

# OPTIMISATION OF KEFIR BIOMASS AND METABOLITE PRODUCTION IN CONJUNCTION WITH SENSORY EVALUATION

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## **DECLARATION**

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

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## ABSTRACT

Developing countries such as South Africa are in dire need of nutritionally adequate dairy food and beverage sources that are ambient stable due to minimal access to refrigeration. One such product is Kefir, a naturally fermented milk beverage that originated in Caucasian China many centuries ago. The microorganisms responsible for fermentation of the milk are held together in a carbohydrate matrix in the form of small grains. These grains are then removed from the beverage prior to consumption, and added to fresh milk for new fermentations. This beverage holds great potential for large scale development due to the self-propagating nature of the grains, the lack of sophisticated equipment and knowledge necessary for production, and the appealing sensory characteristics of this beverage. This study was therefore performed as an initial investigation to determine the optimum fermentation conditions for large-scale grain production and optimal sensory appeal.

Kefir grain production was found to be proportional to incubation temperature in the range studied (18°, 22°, 25° and 30°C), with maximum grain biomass increases of 500% for the Kefir incubated at 30°C over the 10 d trial.

During fermentation of Kefir grains in milk, lactic acid and other metabolites are produced. Lactic acid results in coagulation of the milk, necessary to provide the characteristic texture and flavour of Kefir, as well as exerting a preservative effect. Lactic acid production was found to be strongly proportional to both incubation temperature and inoculum concentration. The samples containing 2% (w/v) Kefir grain inoculum concentration that were incubated at 25°C for 24 h were found to have optimum lactic acid levels for good quality Kefir (pH of 4.4 - 4.6 and TA of 1.0 – 1.15%).

The other metabolites produced during Kefir fermentation are responsible for the specific flavour of Kefir, and include acetaldehyde, diacetyl, ethanol, acetone and 2-butanone. These compounds were studied using headspace gas chromatography over the fermentation period, which yielded good resolution and separation of all these compounds, however, only acetaldehyde, ethanol and acetone were found to be major metabolites in this study. These analytical results were then further compared to sensory results for key identified attributes, as obtained from a trained sensory panel, to enable recommendations for optimum fermentation conditions to

be made. The studied attributes included sourness, sweetness, butteriness, creaminess, yoghurt flavour, cowiness, effervescence, yeastiness, smoothness and overall acceptability. It was apparent from this study that correlations between analytical and sensory data could be drawn, and that panellists were particularly accurate in detecting the attribute sourness resulting from the accumulated lactic acid in the Kefir. Overall acceptability also seemed to be intricately linked to the attribute creaminess, hence the regular literature references to full-cream Kefir as optimum for best sensory appeal.

From this study, it was evident that Kefir with optimal sensory appeal is obtained with incubation for 18 h at moderate temperatures (22° or 25°C) and grain inoculum concentrations (0.8% w/v).

## UITTREKSEL

In ontwikkelende lande soos Suid-Afrika, bestaan daar 'n groot behoefte aan voedsame suiwelprodukte wat stabiel is by kamer temperatuur aangesien 'n groot deel van die bevolking beperkte toegang tot verkoelingsfasiliteite het. Een so 'n produk is Kefir, 'n natuurlike gefermenteerde suiwelproduk wat sy oorsprong eeue gelede in China gehad het. Die mikroörganismes wat verantwoordelik is vir die fermentasie, is saamgebind in 'n koolhidraat matriks in die vorm van klein korrels. Hierdie korrels word verwyder uit die drankie voordat dit gedrink word, en word dan weer by vars melk bygevoeg vir 'n verdere fermentasie. Hierdie gefermenteerde produk het baie potensiaal vir massa-produksie, omdat die korrels natuurlik vermeerder, geen gesofistikeerde toerusting of kennis nodig is nie, en die finale produk hoogs aanvaarbare sensoriese eienskappe het. Die doel van die studie was om 'n inleidende ondersoek uit te voer om die optimum fermentasie toestand vir massakweking van korrels en die mees aanvaarbare sensoriese eienskappe te bepaal.

Uit hierdie studie is gevind dat Kefirkorrel vermeerdering proporsioneel is tot die verhoging in inkubasie temperatuur in die gebied 18°, 22°, 25° en 30°C, met maksimum biomassa toenames van tot 500% vir Kefir wat vir 10 dae by 30°C geïnkubeer was.

Gedurende fermentasie van Kefirkorrels in melk, word melksuur en ander metaboliete gevorm. Melksuur lei tot die verlaging van die pH van die melk, en veroorsaak stolling, wat noodsaaklik is vir die kenmerkende tekstuur en geur van Kefir, maar dien ook as 'n preserveermiddel. Daar is ook gevind dat melksuur produksie 'n direkte verband het met die inkubasie temperatuur en inokulum konsentrasie. Die monsters met Kefirkorrel inokulum konsentrasie van 2% (m/v) wat vir 24 h by 25°C geïnkubeer is, het die optimale melksuur konsentrasies vir goeie kwaliteit Kefir bevat (pH van 4.4 – 4.6 en TA van 1.0 – 1.15%).

Ander metaboliete wat belangrike geurkomponente van Kefir is, is aetaldehyd, diasetiel, etanol, asetoon en 2-butanon. Hierdie metaboliete is bepaal en geëvalueer met bodamp gaschromatografiese tegnieke gedurende die fermentasie, wat 'n goeie resolusie en skeiding gelever het. In hierdie studie is slegs

asetaldehyd, etanol en asetoon as hoof Kefir metaboliete gevind. Die analitiese data is verder vergelyk met die sensoriese data van die hoof sensoriese komponente, soos bepaal deur 'n opgeleide sensoriese paneel, om die mees gunstige fermentasie parameters te bepaal. Die geëvalueerde eienskappe was suurheid, soetheid, botterigheid, romerigheid, joghurt geur, koeismaak, gas inhoud, gisagtigheid, gladheid en algehele aanvaarbaarheid. Uit hierdie data is gevind dat daar wel 'n sterk korrelasie bestaan tussen die analitiese en sensoriese resultate, en dat paneellede in staat was om die suurheid, as gevolg van die gevormde melksuur, te bepaal. Algehele aanvaarbaarheid is definitief gekoppel aan romerigheid, daarom word volroommelk Kefir verkies bo die wat met afgeroomde melk berei is.

Die data uit hierdie studie het ook getoon dat Kefir met optimale sensoriese eienskappe verkry is na 'n inkubasietyd van 18 h by "matige temperature" (22° of 25°C) en 'n Kefirkorrel inokulum van 0.8% (m/v).

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The language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of chapters, each of which are individual entities. Some repetition between chapters has thus been unavoidable.

## CHAPTER 1

### INTRODUCTION

Urbanisation is perceived as one of the biggest driving forces of growth in the South African dairy industry. This is due to increased spending power, access to a greater variety of products, increased awareness of product benefits and, in some instances, access to refrigeration (Hughson, 1995). This is unfortunately not always true in that many people leaving the rural areas for the cities cannot be accommodated in these cities with regard to employment. This results in the development of a community with no access to money and therefore no increase in spending power, nor access to refrigeration.

Studies in South Africa on the sale and consumption of dairy products have shown that in 1990 the higher income group (approximately 12% of the population) consumed 64% of the pasteurised milk produced (Joubert & De Lange, 1992) and 58.7% of the total dairy products produced (Martins, 1991). In comparison, the lower income market consumed 22.9% of the total dairy products (Martins, 1991), yet only 13.6% of the pasteurised products produced (Joubert & De Lange, 1992). It appears that long-life products (UHT milk) are more popular with the lower income consumer in that 33.2% of the UHT products produced were bought by this sector versus 48.2% for the higher income consumers (Joubert & De Lange, 1992). UHT milk is more expensive than pasteurised milk and this trend probably emphasises the limited access of poorer consumers to cooling facilities (Joubert & De Lange, 1992).

In South Africa, as in many other developing countries, the tendency has been to adapt the climate of the country to the food (with, for example, refrigeration) rather than adapting the food to the existing climate (Bachmann, 1984). The simplest and most beneficial solution to this problem is to give fermented milk beverages a more important role in our community. The advantages of fermented products are obvious in that the higher acid concentration in these products facilitates a longer shelf-life and alleviates the need for refrigeration of the product (Bachmann, 1984; Marshall, 1984; Joubert & De Lange, 1992; Roginski, 1988). Paired with this, is the fact that fermented dairy products have many nutritional benefits, including improved flavour,

aroma and textural characteristics (Marshall, 1984; Roginski, 1988), inhibitory action to pathogenic microorganisms (Bachmann, 1984) and decreased lactose levels which is of great importance for lactose-intolerant people (Keller & Jordaan, 1990). A comparison of the suitability of pasteurised vs fermented milks for developing countries is given in Table 1 (Bachmann, 1984).

There have been numerous calls over the last decade for new fermented dairy products. This is due to the fact that products such as cheese and yoghurt are traditionally seen as “luxury” products because of their price, yet are so essential for adequate and sufficient nutrition (Hughson, 1995; Keller & Jordaan, 1990). The scope for development is endless, and so many fermented milk products have been produced for centuries with no ill effect (Kosikowski, 1982).

One such product is kefir, a fermented milk beverage prepared with small white grains consisting of various symbiotic microorganisms (Marshall, 1984; Kosikowski, 1982; Roginski, 1988). The complexity of the microbial community in kefir grains ensures the manufacture of a good flavoured product (Keller & Jordaan, 1990). This product has all the benefits of fermented milks in general, as well as added benefits such as low production costs. This is due to the fact that growth of the grains occurs simultaneously with the formation of the desired product characteristics. The mass of grains can then be removed after fermentation with simple equipment and can be used again to inoculate further batches of milk without any loss of integrity (Kosikowski, 1982; Vedamuthu, 1982). Limited skills are needed for production and no laboratory facilities are necessary as fermentation takes place at room temperature (Keller & Jordaan, 1990). Finally, natural fermented milk products, especially kefir, have high nutritional, biological and dietetic value and are widely recommended for healthy people, as well as patients with gastro-intestinal and metabolic diseases, hypertension, heart disease and allergy (Koroleva, 1982).

The aim of this study is to optimise the production of kefir with regard to maximal grain growth and metabolite production. A correlation between flavour and aroma compound concentrations and optimal consumer appeal will also be drawn with the aid of sensory evaluation.

**Table 1.** Comparison of the suitability of fermented and pasteurised milks for developing countries (Bachmann, 1984).

	<b>Fermented milk</b>	<b>Pasteurised milk</b>
<b>Product</b>	Known, traditional	New, little known
<b>Physiological properties</b>	Good	Uncertain (depending on area)
<b>Production</b>		
- Required equipment	Simple, locally manufactured	Intricate, partly imported
- Investment	Small	Considerable
- Retail packaging	Not required	Usually imported
- Technical know-how	Considerable, local knowledge	Considerable, imported
<b>Energy</b>		
- Required quantity	Moderate	Large
- Type	Heat	Heat and cooling
- Alternate	Possible for entire process	Possible for part of process
<b>Organisation</b>	Simple	Demanding
<b>Marketing</b>	In bulk, without refrigeration	In packages, refrigeration necessary

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## CHAPTER 2

### LITERATURE REVIEW

#### Background

Fermentation is the metabolic process where polysaccharides and related compounds are oxidised with the subsequent release of energy in the absence of any external electron acceptors (Jay, 1992). The final electron acceptors are organic compounds produced directly during the breakdown of the carbohydrates. This results in the partial oxidation of the original compound, and only a small amount of energy is released during the process (Jay, 1992). According to the definition proposed by the International Dairy Federation (IDF, 1988), fermented milk products are “products prepared from milk, skimmed or not, concentrated or not, with specific cultures; the microflora is kept alive until sale to the consumer and may not contain any pathogenic germ”. The fact that lactic conservation takes place without the food having to be put in the refrigerator is of considerable importance for developing countries. Lactic fermentation thus enables foodstuffs to be conserved without the production of artificial cooling which is expensive and in many cases a hazardous operation (Bachmann, 1984).

Lactic acid fermentations are nature’s gift to man. They make it possible to conserve many animal or vegetable derived foodstuffs in an ideal physiological form, where no toxic metabolites are produced. More importantly, there are no losses due to the formation of volatile substances. Lactic acid is responsible for this ideal conversion, for the following reasons:

- i) lactic acid decreases the pH of the product and therefore adversely affects the environmental conditions for the development of many pathogenic organisms
- ii) lactic acid has bacteriostatic and even bactericidal properties and
- iii) lactic acid has excellent physiological properties (Bachmann, 1984).

Other advantages are that the fermented milk can be produced in small dairies with relatively simple equipment. The fermented milk product can also be sold in bulk without retail packaging. This excludes the many hygienic risks present in the sale of fresh milk (Bachmann, 1984).

In the last 30 years, fermented milks have become relatively widespread in South Africa and other countries. Products like maas, yoghurt and fruit yoghurt have become very popular and many of our fresh milk dairy plants produce these products. There is however a need in the market to introduce new or different types of fermented milks which may appeal to the South African market (Keller & Jordaan, 1990) due to the improved nutritional value, the extended shelf life and the cheaper storage requirements of these products. Kefir may be just that product!

Kefir is a self-carbonated, fermented dairy beverage containing about 0.8-1.0% alcohol that is produced by small grains consisting of various microorganisms in a symbiotic relationship. It is made from whole or skim milk, or a mixture, and has a light, refreshing taste (Liu & Moon, 1983). Kefir has been produced in Eastern and Central Europe since the second part of the nineteenth century (countries such as Russia, Poland, Germany, Rumania, Hungary, the old Czechoslovakia and the Scandinavian countries). Russia is the biggest producer of Kefir in the world (Kurmann *et al.*, 1992). The starter cultures employed and the production methods differ dramatically which results in different product qualities and properties (Libudzisz & Piatkiewicz, 1990). These Kefir grains can be found in South Africa where they are known as “jogurt-plantjie” (yoghurt plant). Certain families still have some of these grains they inherited from their forefathers and make their Kefir (or “joghurt”) daily (Keller & Jordaan, 1990).

## History

Kefir is a very old fermented milk product. There is no record of the date or time when it was first made, however one claim is that it originated in the 1300's in the Tibetan Himalayas (Kroger, 1993). A more commonly accepted belief is that it originated in the northern slopes of Caucasian China where two tribes, the Ossetians and Karbadinians, are specifically reputed to be the first Kefir manufacturers

(Duitschaeffer, 1989; Kurmann *et al.*, 1992). These people lived in the village of Karatschajeff (2500 m above sea level) at the foot of Elbur (Duitschaeffer, 1989).

The population of Karatschajeff learnt to make this refreshing beverage from cow and goat milk, the starter for which had a special form called "Kefir grains". Nobody knows where or how these grains first appeared, but legend has it that the grains were given to the orthodox people by Mohammed who also told them how they were to be used (Koroleva, 1988a; Duitschaeffer, 1989). The passing on of grains or Kefir manufacturing secrets was strictly forbidden by Mohammed who claimed that the grains would lose their "magic" strength if these rules were not obeyed (Koroleva, 1988a). There are, however, other explanations for the occurrence of Kefir grains. It has been suggested that during the continuous use of the same containers to make Kefir, their walls became covered with colonies of microorganisms that resemble boiled rice which have subsequently been called Kefir grains (Roginski, 1988). The function and value of these "accidentally formed" grains was apparently realised and from that time onwards Kefir production occurred continuously in many households (Kurmann *et al.*, 1992).

The product was originally prepared in clay pots, wooden buckets (Roginski, 1988), or, more frequently, in leather goat or sheep skin sacks. These were hung in the house during winter and outside during summer, where the milk was fermented by the natural microbial population through a continuous uncontrolled fermentation. This form of temperature control was enforced on a daily basis through the placing of the bags in the sunlight during the day and inside at night, where they were hung near the door so that anyone who exited or entered had to push the sack with their foot in order to mix the liquid (Koroleva, 1988a). Whenever some of the fermented milk was removed from the bags a new batch of fresh milk was always added (Mann, 1985; Koroleva, 1988a; Duitschaeffer, 1989; Kurmann *et al.*, 1992).

Russian medical doctors reported at the turn of the century that Kefir had beneficial properties for the treatment of intestinal and stomach diseases. The first scientific information about Kefir was published at the end of the last century leading to the widespread interest in the industrial scale production of Kefir. The owner of the Moscow Dairy, a man named Blandov (Koroleva, 1988a), needed Kefir grains in order to do just that. In 1908, he sent a beautiful young dairy worker along with a dairy manager from one of his other dairies, to the regional mountains to try to obtain



grains from the local prince, Prince Bek-Mirzey. The Prince was friendly to them, but was not prepared to part with his grains. Whilst the two dairy workers were returning to Moscow through the mountains, their carriage was attacked by mountain people who kidnapped the woman. She was taken back to the Prince where it was explained to her that the Prince liked her, and the mountain custom was to steal a bride. In the meantime, the manager who had accompanied her, went to fetch gendarmes who set her free. In court, the ruling was made that to compensate for the insult to the woman, the Prince had to give her ten pounds of Kefir grains. This is the history attached to the most popular cultured drink manufactured by the Russian dairies at present (Koroleva, 1988a).

## **Nutritional and health aspects**

### a) Natural Kefir

The presence of millions of microorganisms in milk has a powerful and lasting effect, as they change it into fermented dairy products such as Kefir. This can be ascribed to the fact that the concentrations of various categories of microbial nutrients decrease due to their use as substrate for various microbial enzymatic reactions. This decrease is paired with an increase in other metabolites which are produced by the microorganisms present in the milk. An added dimension to this change in the nutritional and organoleptic properties of the milk that must be taken into consideration is the microbial biomass present, although this represents only a small part of the final product (Blanc, 1984). There is a dramatic increase in cell population representing both viable and dormant cells in fermented milk products. The increase continues during storage, although at a slower rate (Alm, 1982).

Another important aspect to be taken into consideration with regard to the fermentation of milk is the decrease in lactose level which allows people regarded as "lactose intolerant" (lactase-deficient) to drink greater quantities of fermented milks than they would be able to raw milk in the same period of time, without ill effect (Blanc, 1984; Kroger, 1993; Roginski, 1988). This is due to the fact that about one quarter of the 5% lactose in milk is converted into lactic acid through the fermentation process, as well as the fact that the bacteria contained in Kefir provide the enzyme

lactase which is necessary for lactose digestion and is lacking in lactose intolerant individuals (Kroger, 1993).

In general, the microbial population of the human gut of healthy adult individuals is fairly stable and is not generally strongly affected by variations in diet. However, the antagonistic relationship between lactic acid bacteria and organisms causing intestinal infections, such as *Escherichia coli*, *Salmonella* spp, *Shigella* spp, *Staphylococcus aureus* and others, has been well documented *in vitro*. This forms the basis of the belief that lactic acid bacteria inhibit the growth of pathogenic organisms also *in vivo* (Koroleva, 1988b; Roginski, 1988). This high degree of anti-biological or probiotic activity towards extraneous intestinal microorganisms is not found in other fermented milk products such as yoghurt manufactured with traditional yoghurt cultures (Libudzisz & Piatkiewicz, 1990).

Improved peristalsis is also noted in patients that drink Kefir on a daily basis, and the lactic acid, acetic acid and anti-bacterial substances present in Kefir inhibit the decomposition processes in the small intestine (Libudzisz & Piatkiewicz, 1990).

Fermented milks have also been compared with milks for their non-protein nitrogen and free amino nitrogen contents and found to give higher values both before and after pepsin digestion *in vitro*. The results of rat feeding trials confirmed the favourable protein utilisation and body mass increments were greater on fermented milk diets than on raw milk diets. This was attributed to the better digestibility of proteins in these products (Mann, 1985). Besides this enhancement of digestion, proteolysis and the reabsorption of proteins is also stimulated by fermented milk products (Blanc, 1984).

There are many other beneficial nutritional and health effects that have been claimed for fermented milks in general (Blanc, 1984; Roginski, 1988). These include:

- i) An improvement in the digestibility and absorption of fats
- ii) Stimulation of digestive secretions such as saliva, bile, gastric and pancreatic juices
- iii) Increases in phosphorus, calcium and iron retention (compared to milk) and
- iv) The alleviation of allergic reactions to milk proteins.

Many of these claims still require convincing experimental proof for a product such as Kefir (Roginski, 1988).

An important question concerning B-vitamins in fermented milk products is their bioavailability. These vitamins function as integrated parts of proteins and may therefore occur both in free form and as cell-bound constituents in fermented milk. It is not known whether lysis of lactobacilli occurs during the digestion process and whether cell-bound vitamins are made available to the host. It may be speculated that one way in which lactobacilli influence the metabolic activities of the normal host microorganisms is by supplying them with B-vitamins (Alm, 1982). The B-vitamins are components of co-enzymes and are extremely important in the metabolism and energy utilisation of the cell, and are therefore essential for starter cultures used in the fermentation of milk. These starters show various needs for vitamins and differing abilities to synthesise B-vitamins in excess (Alm, 1982). A study conducted in Sweden on the effect of fermentation on the B-vitamin content of milk found that fermented milk products showed an increase in folic acid content (for Kefir a 40% increase within the first 24 hours, with a 25% reduction after storage for 11 days) and a slight decrease in the concentration of vitamin B<sub>6</sub>, B<sub>12</sub> and biotin. The concentration of orotic acid showed a more substantial decrease (17.7% in Kefir). Other vitamin concentrations were affected only slightly by the fermentation process (Alm, 1982).

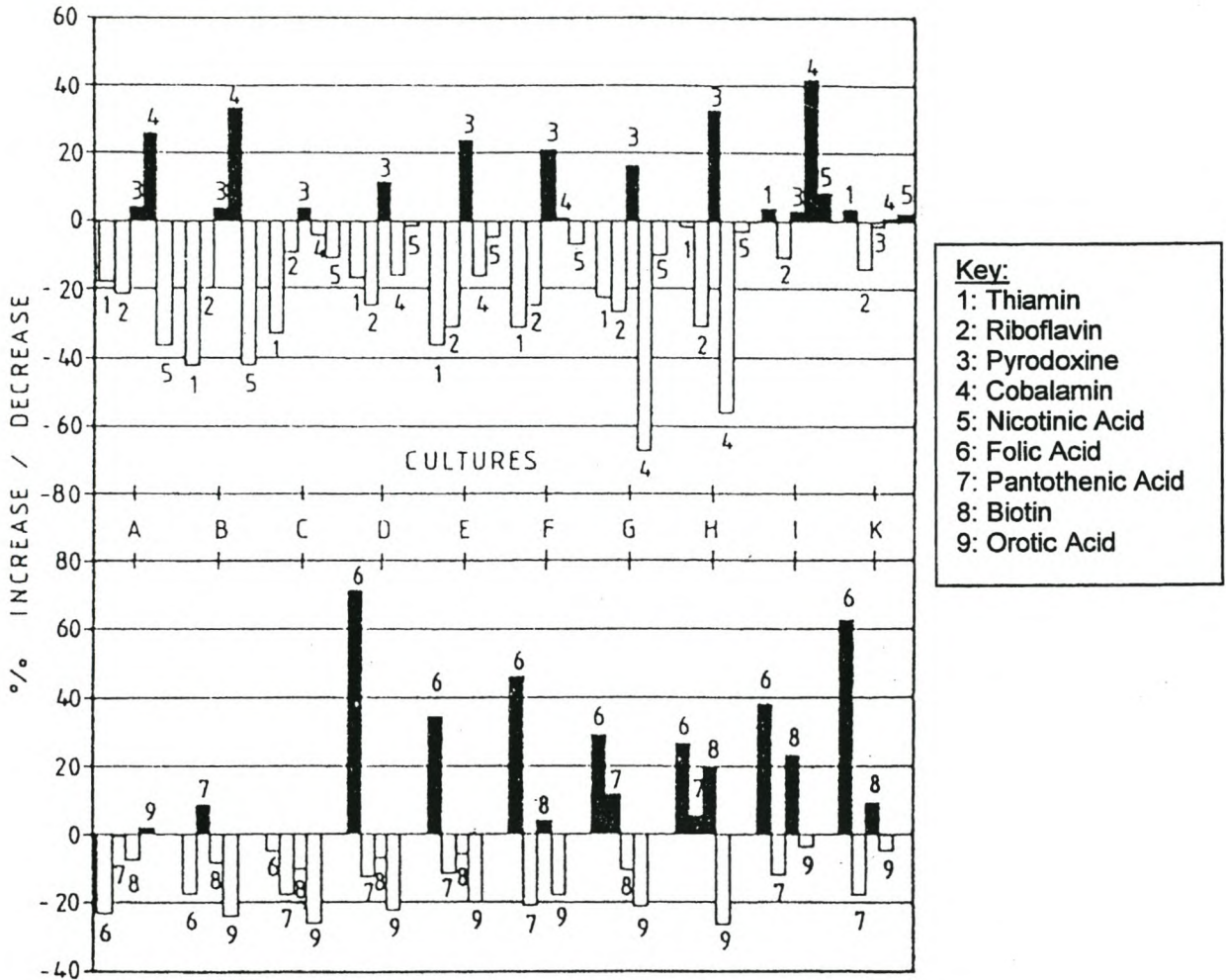
Another interesting aspect of Kefir production and the influence that manufacturing methods have on vitamin production, is the choice of base milk and the actual Kefir starter culture employed. Actively growing Kefir grains are not static, which means their constituent microorganisms vary from culture to culture, depending on source as well as growth conditions. This variation in microbial population is also observed in the same Kefir culture at varying times during the same fermentation process (Duitschaever, 1989; Duitschaever *et al.*, 1988a; Koroleva, 1982; Koroleva, 1988a; Kroger, 1993; Kurmann *et al.*, 1992; Mann, 1983; Mann, 1985; Marshall, 1987; Neve, 1992). In 1991, a study was carried out to determine the influence of the type of milk and the starter culture used in Kefir production, and the effect on the final vitamin properties of the Kefir (Kneifel & Mayer, 1991). It was found that appreciable (> 20%) increases in the relative vitamin content in reconstituted cow milk were only found for pyridoxine in three, cobalamin in three, folic acid in seven and biotin in two of the 10 cultures examined. Two of the cultures did however show increases of greater than 50% for biotin. Losses exceeding 20% were more frequent for thiamine (six cultures), riboflavin (seven cultures), and orotic

acid (six cultures), but also occurred for cobalamin (two cultures), nicotinic acid (two cultures), folic acid (one culture) and pantothenic acid (one culture). Losses of more than 50% were found for cobalamin in two cultures. In Figure 1, a profile of the percentage change in vitamin content in Kefir samples produced with the ten different Kefir grain cultures in reconstituted cow milk, is presented (Kneifel and Mayer, 1991). Three of the 10 cultures examined were then tested on ewe, goat and mare milks. In Figure 2 the percentage change in vitamin content with selected Kefir cultures, in different base milks is illustrated (Kneifel and Mayer, 1991). It was found that all three of these cultures showed increased thiamine, pyridoxine and folic acid concentrations in ewe milk, whereas only one culture showed an increase in folic acid in cow milk. Goat milk was similar to cow milk except for cobalamin, which decreased in one culture, and folic acid which increased in two cultures (as with ewe milk). Mare milk showed small increases in pyridoxine and large losses of biotin with all three cultures. The relevance of these changes must be assessed in terms of the absolute amounts of the vitamins in the milks and Kefirs (Table 1), and the importance of their contribution to the required daily intake of man (Kneifel and Mayer, 1991). In most cases, the losses during Kefir production are slight and probably of little nutritional significance, although those Kefirs which produce increased levels of vitamins for which milk is an important source (riboflavin, cobalamin, pantothenic acid and biotin) may well be significant to certain human populations on restricted diets (Kneifel & Mayer, 1991).

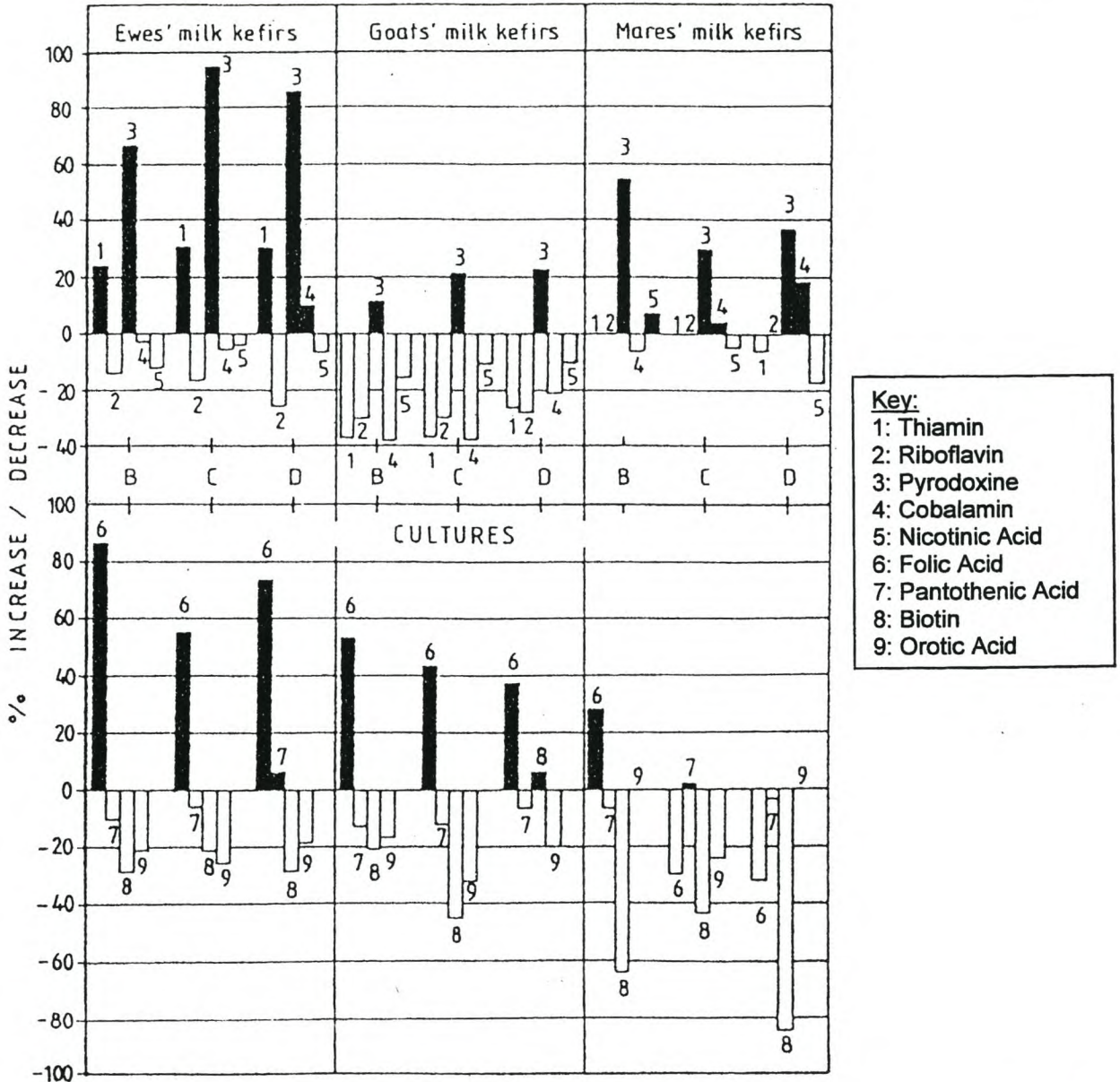
#### b) Biologically Enriched Kefir

The ability of propionic acid bacteria to synthesise vitamin B<sub>12</sub> has been used in research for the enrichment of fermented milk beverages for several years. The fermented milk beverages are enriched either by the combination of the microbial population of the corresponding starter with the production strains of propionic acid bacteria, or by the addition of their dried biomass. "*Propionibacterium shermanii*", which utilises lactose, represents the most widely used strain of this group (Cerna & Hrabova, 1977).

As was discussed in the previous section, lactic acid fermentation of milk usually results in a loss of the B-vitamins. The fermentation of milk by mixed species of lactic acid bacteria and yeasts to a beverage, and subsequent fermentation of this



**Figure 1.** Percentage change in vitamin content in Kefir samples produced with Kefir grain cultures A – K, with reference to base reconstituted cow milk (Kneifel & Mayer, 1991).



**Figure 2.** Percentage change in vitamin content in Kefir samples produced from selected Kefir grain cultures B, C, D, with reference to base reconstituted milk from different species (Kneifel & Mayer, 1991).

**Table 1.** Vitamin content of the reconstituted base milks of different species and their corresponding Kefirs; percentage change during fermentation in parentheses (Kneifel & Mayer, 1991).

Vitamin	Cows' milk		Ewes' milk		Goats' milk		Mares' milk	
	Milk	Kefir	Milk	Kefir	Milk	Kefir	Milk	Kefir
Thiamin (mg kg <sup>-1</sup> )	0.25	0.19 ± 0.03 (-24%)	0.46	0.57 ± 0.03 (+24%)	0.68	0.44 ± 0.04 (-35%)	0.45	0.43 ± 0.02 (-4%)
Riboflavin (mg kg <sup>-1</sup> )	1.74	1.51 ± 0.18 (-13%)	1.58	1.28 ± 0.09 (-19%)	0.83	0.59 ± 0.07 (-29%)	Non-detectable —	
Pyridoxine (mg kg <sup>-1</sup> )	0.20	0.21 ± 0.02 (+5%)	0.16	0.30 ± 0.04 (+89%)	0.55	0.66 ± 0.03 (+20%)	0.09	0.13 ± 0.01 (+40%)
Cobalamin (µg kg <sup>-1</sup> )	3.3	2.7 ± 0.3 (-18%)	4.6	4.6 ± 0.3 —	2.5	1.7 ± 0.3 (-32%)	2.6	2.7 ± 0.3 (+4%)
Nicotinic acid (mg kg <sup>-1</sup> )	0.96	0.90 ± 0.09 (-6%)	2.82	2.61 ± 0.11 (-7%)	2.48	2.18 ± 0.08 (-12%)	1.92	1.82 ± 0.24 (-5%)
Folic acid (µg kg <sup>-1</sup> )	32.4	39.6 ± 8.9 (+22%)	25.2	43.0 ± 4.0 (+71%)	25.6	36.9 ± 2.1 (+44%)	25.1	22.3 ± 8.4 (-11%)
Pantothenic acid (mg kg <sup>-1</sup> )	4.64	4.49 ± 0.52 (-3%)	3.28	3.16 ± 0.29 (-4%)	4.08	3.64 ± 0.13 (-11%)	4.63	4.50 ± 0.20 (-3%)
Biotin (µg kg <sup>-1</sup> )	15.4	13.5 ± 3.2 (-12%)	8.2	5.9 ± 0.4 (-28%)	15.4	12.2 ± 3.9 (-21%)	6.9	1.9 ± 0.8 (-72%)
Orotic acid (mg kg <sup>-1</sup> )	71.5	59.7 ± 7.3 (-17%)	12.1	9.4 ± 0.5 (-22%)	16.8	12.9 ± 1.4 (-23%)	0.8	0.7 ± 0.1 (-13%)

beverage to a product high in vitamin B<sub>12</sub>, is an example of the sequential production of lactate and fermentation of lactate by "*P. shermanii*". The addition of propionibacteria to Kefir results in increases, or in some cases only small losses, of vitamin B<sub>12</sub> and the product thus obtained has a high nutritional value and is rich in terms of proteins and vitamins (Liu & Moon, 1983).

Cerna and Hrabova (1977) investigated the biological enrichment of Kefir with vitamin B<sub>12</sub> and folic acid. Their results indicated that the enrichment degree is strictly proportional to the type of fermented beverage microbial population. Other investigators have also come to this conclusion based on the fact that Kefir microorganisms do not synthesise vitamin B<sub>12</sub>, yet stimulate the production of this vitamin to a large degree when the vitamin B<sub>12</sub>-synthesising organisms are present (Roczniakowa *et al.*, 1974). Coenzyme A might be the key factor in this case since it is essential for both the biosynthesis of vitamin B<sub>12</sub> and for the metabolism of lactic acid bacteria. In mutual symbiosis, the coenzyme A is not available to the *Propionibacterium* to the same degree as it would be if the two organisms were cultivated separately in milk, and the biosynthesis of vitamin B<sub>12</sub> is consequently retarded. The opposite scenario occurs if a yeast is present, as is the case in Kefir. The yeast produces coenzyme A, which means that the biosynthesis of vitamin B<sub>12</sub> is not interfered with, and the same values for vitamin B<sub>12</sub> are obtained as is the case for milk. This fact is illustrated in Table 2 where the various types of beverages and the corresponding degrees of vitamin B<sub>12</sub> enrichment by "*P. shermanii*" are given (Cerna & Hrabova, 1977).

Cerna and Hrabova (1977) also reported that the biosynthesis of vitamin B<sub>12</sub> occurred simultaneously with that of folic acid. In Table 3 the changes in the content of free and total folic acid in fermented milk beverages brought about by the addition of "*P. shermanii*" are shown. As was the case for vitamin B<sub>12</sub>, the biosynthesis of folic acid occurred to the greatest extent in Kefir milk, however, in this case no analogy of the inhibitory effect due to the microbial population of the fermented beverages could be drawn due to the fact that the starter cultures stimulated folic acid production in all the studied cases. An important aspect to consider in enrichment studies is the fact that the addition of propionic acid bacteria did not affect



**Table 2.** Vitamin B<sub>12</sub> biosynthesis in fermented milk beverages by the addition of "*P. shermanii*" (Cerna & Hrabova, 1977).

Beverage	Micro-organisms	Ripening Time (h)	Ripening Temp. (°C)	Vitamin B <sub>12</sub> Content		Enrichment Degree
				- <i>P. sherm.</i>	+ <i>P. sherm.</i>	
Whole milk	N/a	16	30	0.27	14.60	54x
Kefir	Rods, cocci, yeasts	24	18	0.51	14.30	28x
Yoghurt	Diplococci, rods	16	30	0.47	1.70	3.6x
Acidophilus milk	Rods	16	37	0.40	1.34	3.3x
Bifidus milk	Rods	16	37	0.42	1.30	3.1x

**Table 3.** Folic acid biosynthesis in fermented milk beverages by the addition of "*P. shermanii*" (Cerna & Hrabova, 1977).

Beverage	Folic Acid µg/100 ml	
	Free	Total
Reconstituted whole dry milk	2.0	2.0
Reconstituted whole dry milk (inoculum) + <i>P. shermanii</i>	2.2	3.8
Kefir milk	2.5	5.0
Kefir milk + <i>P. shermanii</i>	15.3	27.4
Streptococci cultured milk	2.8	3.8
Streptococci cultured milk + <i>P. shermanii</i>	6.1	6.6
Acidophilus milk	3.9	4.2
Acidophilus milk + <i>P. shermanii</i>	5.7	6.5

the organoleptic properties of any of the investigated beverages unfavourably (Cerna & Hrabova, 1977).

### **Starter Cultures**

Kefir is one of the few fermented milk products which is industrially manufactured without using pure microbial cultures, but rather a natural symbiotic starter, the “Kefir microorganisms” (Koroleva & Bavina, 1970). Typically, there are three types of starters employed for Kefir production, the first, the “Kefir grains” themselves, the second, a “starter” obtained by growing Kefir grains in milk, and the third, a “single-use culture” that is made by grinding Kefir grains in neutralised Kefir and subsequently mixing with a yeast preparation (Koroleva & Bavina, 1970; Koroleva, 1988b).

There are various general criteria for starter cultures. According to Buckenhuskes (1993), these are:

- i) Safety - Starter cultures must not be in possession of any pathogenic or toxic activity and their preparation must be of a high hygiene standard
- ii) Technological effectiveness - The starter organisms must dominate over the spontaneous microbial population and must perform the required metabolic activity without the incorporation of any technologically precarious infections or substances and
- iii) Economical aspects - The propagation must be feasible from an economical point of view. The starter culture must be able to be preserved by freezing or freeze-drying with little practical loss of activity, and be stable under defined storage conditions for several months. It is extremely important that the handling of the starter culture be as easy as possible.

In more general terms:

- i) Starters must continuously provide good quality products at high efficient manufacturing rates
- ii) They must ensure technological process stability (resistance of the starter microbial population to bacteriophage, seasonal variations in milk quality, etc.)

- iii) They must provide products with certain properties attractive to the consumer (flavour, aroma, consistency) and
- iv) They must help to produce products that possess some beneficial and therapeutic values (Semenichina, 1984).

#### a) Kefir Grains

Kefir grains are a natural starter that consist of yeasts and lactic acid bacteria in a strong and specific symbiotic relationship (Koroleva, 1988b). These grains do not appear to be influenced by bacteriophage. They have a low sensitivity to the inhibitors present in milk, and only a slight seasonal change in activity. They have therapeutic properties, as previously discussed, and the Kefir drink is enjoyed by many people around the world. Kefir grains, as a starter for a fermented product, thus satisfy the prerequisites for starter cultures, as recommended by Semenichina (1984).

The natural Kefir grains resemble cauliflower florets and have an irregular form, with folded or uneven surfaces (Marshall, 1984a). The grains are insoluble in water and common solvents, but when added to milk, they swell (Liu and Moon, 1983). They are gelatinous, have a white or slightly yellow colour and have a diameter of 1-2 to 3-6 mm, or more. Duitschaever *et al.* (1988a) cites the average diameter of grains from their country as 20 to 30 mm. The total solids content of the Kefir grains is about 10%, being composed of 3.5% fat, 32.6% protein, 6% ash and 56.2% carbohydrates, of which 24% are water soluble (Kosikowski, 1982), on a dry matter basis (Mann, 1985). Active Kefir grains float on the milk surface. They always have a definite structure and conduct themselves as biologically viable organisms. They grow and impart their properties to next generations, as do the new grains. In practice, new grains are propagated through splitting the grains (Koroleva, 1988b; Roginski, 1988). In spite of several attempts, no-one has been able to obtain Kefir grains from the mixed culture of the microorganisms present in the original grains (Liu & Moon, 1983). The main reason for this inability to reconstitute Kefir grains from pure or crude cultures is probably due to the composition and structure of the Kefir grain and its importance to the grain organisms (Liu & Moon, 1983). Only Kefir prepared with the grain starter has the typical taste and aroma of Kefir, and it is therefore advisable to manufacture Kefir using only grain starter, even on an

industrial scale (Koroleva, 1988b). Kefir that is obtained through fermentation of fresh milk with Kefir grains is often called “**starter 1**” (Koroleva, 1988b).

#### b) Kefir Culture

Marshall (1984a) reports that although organisms are continuously shed from the grains into the milk, Kefir milk cannot be used as the starter for the next batch of milk. Any attempts to replace Kefir grains by a starter composed of a mixture of pure microorganism cultures is not effective because the pre-established ratio between microorganisms is altered in the process of transfer and cultivation (Koroleva, 1988b). This fact is illustrated by the loss of balance of the microorganisms after subculturing as shown in Table 4 (Marshall, 1984a). Other investigators have however reported that Kefir milk obtained with Kefir grains can be used to ferment a further batch of milk without too much loss of integrity of the Kefir drink.

Sometimes, due to the lack of the equipment necessary for separating Kefir grains from the milk, a bulk starter is used. This starter is prepared by fermenting pasteurised milk with Kefir milk obtained from the fermentation with the grains (**starter 1**) (Koroleva, 1988b). This starter is called “**starter 2**”. The drink obtained in this fashion does not however possess the typical Kefir characteristics, and it is therefore not desirable to prepare Kefir using this method (Koroleva, 1988b).

Kefir culture starter (**starter 2**) prepared by fermentation with Kefir grains (**starter 1**) possesses properties which are very important for Kefir manufacture due to the fact that the conditions for symbiotic growth of the microorganisms are optimal. This starter is not affected by seasonal fluctuations in milk quality, nor by the presence of normally detected quantities of antibiotics or other inhibitors in the milk (Koroleva, 1988b). The starter is also resistant to phages, and once again, the product obtained is both desirable and therapeutic. Kefir culture starter thus satisfies the starter prerequisites as the Kefir grains themselves did, although, as previously mentioned, any further sub-culturing results in an undesirable product (Koroleva, 1988b; Semenichina, 1984).

**Table 4.** The effect of subculturing on Kefir microorganisms and metabolites (Marshall, 1984a).

	<b>Yeasts (cfu/ml)</b>	<b>Lactobacilli (cfu/ml)</b>	<b>Ethanol (%)</b>	<b>Acetaldehyde (µg/ml)</b>
Kefir milk	$2 \times 10^5$	$8 \times 10^7$	1.00	12.0
1 <sup>st</sup> subculture	$1 \times 10^4$	$5 \times 10^8$	0.09	1.0
2 <sup>nd</sup> subculture	$5 \times 10^2$	$1 \times 10^9$	0.04	0.0
3 <sup>rd</sup> subculture	$2 \times 10^2$	$1 \times 10^9$	0.01	0.0
4 <sup>th</sup> subculture	30	$1 \times 10^9$	< 0.01	0.0
5 <sup>th</sup> subculture	< 10	$9 \times 10^8$	< 0.01	0.0

### c) Single-Use Kefir Starters

Studies have been undertaken into the development of fast single-use Kefir cultures that can be used for the production of Kefir starter and Kefir that does not differ in flavour, properties and biochemical characteristics from the product obtained by traditional methods. One successful starter is a lyophilised culture prepared from Kefir grains, which is standardised to 10% of the whole microbial population by the addition of yeasts isolated from these Kefir grains (Libudzisz & Piatkiewicz, 1990). In other studies, two stages have been found to be necessary in the starter production in order to simulate the bacterial and yeast reactions that occur naturally in milk fermented by Kefir grains. Kramkowska *et al.* (1982) prepared a culture by first mixing Kefir grains with Kefir that had been neutralised twice during cultivation. They then suspended yeasts (obtained from Kefir grains) in a sucrose medium which was then mixed with starch and dried at 40°C. This was followed by mixing the two dry samples and placing the mixture in fresh milk and then incubating at 23°C. The Kefir so produced had the typical flavour and consistency of traditional Kefir.

Secondary and two-stage fermentations for Kefir-like products have also been developed which modify the final aroma. These second fermentations involve a buttermilk or yoghurt culture to enhance diacetyl and acetaldehyde flavour respectively in the final products (Marshall, 1987). Although relatively good results can be obtained for Kefir manufactured in this way, this single-use starter culture method is rather complicated, and the obtained product cannot compete with conventional Kefir (Koroleva, 1988b).

### **Microbiological Aspects**

Kefir made with or without Kefir grains has been classified as a mixed lactic-acid and ethanol fermentation. This classification is based on the system proposed by Kurmann (1984) in which fermented milks are classified according to the organisms used for their manufacture. The microorganisms found in Kefir grains and those found in Kefir milk are similar, however, there are variations, and many conflicting reports are found in the literature (Kandler & Kunath, 1983).

### a) Kefir Grains

Kefir grains usually consist of many different types of organisms. The macroscopic, as well as microscopic, appearance of the grains has shown little variation over the years. In spite of many investigations, no definite agreement has been reached on a systematic classification of bacteria associated with the Kefir grain. Identification of the real microorganisms associated with the Kefir grain is difficult because of the diverse population present. Microbial interactions caused by unfavourable cultivation conditions further hamper the identification process (Liu & Moon, 1983), as does a lack of aseptic handling of the grains (Marshall *et al.*, 1984b). It is known that the organisms in the same Kefir culture can even differ at different times during the same fermentation process (Libudzisz & Piatkiewicz, 1990).

The basic microbial community of Kefir grains has been described as the heterofermentative and homofermentative lactic acid bacteria, acetic acid bacteria and lactose fermenting and non-fermenting yeasts (Koroleva, 1988b; Marshall *et al.*, 1984b), although in a study of Portuguese Kefir grains no acetic acid bacteria were observed (Pintado *et al.*, 1996). Heterofermentative lactic acid streptococci are also often isolated from Kefir grains (Neve, 1992). All these microorganisms are mesophilic (Koroleva and Bavina, 1970; Molska *et al.*, 1982) or thermophilic, depending on the manufacturing conditions applied (Koroleva & Bavina, 1970). These organisms can be divided into the following groups:

- i) The lactobacilli which appear to dominate the microbial population of the grain (Pintado *et al.*, 1996). Of these lactobacilli *Lactobacillus kefir* (previously known as *Lactobacillus brevis*) is most frequently found (Kandler & Kunath, 1983; Marshall, 1987; Neve, 1992; Pintado *et al.*, 1996; Roginski, 1988). These lactobacilli are encapsulated with a capsular polysaccharide (Pintado *et al.*, 1996). Another species often isolated is *Lactobacillus kefiranofaciens* (Fujisawa *et al.*, 1988; Neve, 1992). In a Polish study of the lactobacilli present in Kefir grains, all the examined strains (12 in total) were classified as one species of *Lactobacillus casei* (Molska *et al.*, 1982). Other lactobacilli often reported in Kefir grains are strains of *Lactobacillus acidophilus* (Marshall, 1987). Two new species of lactobacilli have also

recently been isolated from Kefir grains. These are named *Lactobacillus kefirgranum* and *Lactobacillus parakefir* (Takizawa *et al.*, 1994).

- ii) The lactic acid streptococci which include strains of lactococci and leuconostocs have been reported but are rarely found in Kefir grains (Koroleva & Bavina, 1970; Neve, 1992). *Lactococcus lactis* subsp. *lactis* has been isolated in Portuguese Kefir grains (Pintado *et al.*, 1996).
- iii) Acetic acid bacteria of the genera *Acetobacter* and *Gluconobacter* are also frequently present in Kefir grains. These microorganisms are considered important for the maintenance of the symbiosis between the Kefir grain microorganisms (Koroleva, 1988b). They are Gram-negative, catalase-positive obligate aerobes that oxidise ethanol to acetic acid. They also oxidise ethanol, acetate and lactate to CO<sub>2</sub> and H<sub>2</sub>O. Of the four species of *Acetobacter*, only *A. aceti* has been reported to be present in Kefir grains (Roginski, 1988).
- iv) Yeasts, which form remarkably stable associations with lactic acid bacteria, are also part of the dominant members of the Kefir consortium. These include strains of *Kluyveromyces marxianus* subsp. *marxianus* (formerly known as either *Saccharomyces kefir*, *Saccharomyces fragilis* or *Kluyveromyces fragilis*), *Candida kefir* (formerly either *Saccharomyces kefir*, *Torulopsis kefir* or *Candida pseudotropicalis* var. *lactosa*) and *Saccharomyces cerevisiae* (Marshall, 1987; Neve, 1992; Roginski, 1988). Another important yeast associated with Kefir grains is *Torulopsis holmii* (Iwasawa *et al.*, 1982). Apart from these, an investigation into the yeast composition of commercial Kefir and Kefir cultures distributed in Switzerland included strains of *Torulopsis delbrueckii*, *Debaryomyces polymorphus*, *D. hansenii*, *Candida inconspicua*, *C. tropicalis* and *C. versatilis* (Geiges and Spillmann, 1994; Neve, 1992). The yeast species isolated in Portuguese Kefir grains was *Saccharomyces unisporus* or, synonymously, *Saccharomyces delbrueckii* (Pintado *et al.*, 1996). Yeasts play an important role by promoting symbiosis among the microorganisms, as do the previously mentioned acetic acid bacteria (Koroleva, 1988b).

Generally, lactobacilli (homo- or hetero-fermentative) constitute about 65-80% of the microbial content of Kefir grains, with the remaining 20% consisting of streptococci



(souring and aroma forming) and different species of lactose fermenting and non-fermenting yeasts. These yeasts usually make up approximately 5% of the microbial content (Libudzisz & Piatkiewicz, 1990). The concentration of the various microorganisms in the Kefir grains should be  $1 \times 10^8$  to  $1 \times 10^9$  lactic acid streptococci and lactobacilli and  $1 \times 10^8$  yeasts cells per gram (Libudzisz & Piatkiewicz, 1990).

Sometimes the surfaces of the Kefir grains are covered with a white mould (the asexual stage in the life-cycle of a yeast), *Geotrichum candidum*. This mould does not appear to affect the grains' performance, and fermentation continues in the usual manner (Bottazzi & Bianchi, 1980).

Scanning electron microscopy (SEM) of Kefir grains show closely interwoven threads that form the basic structure of the grains in which the microorganisms are entrapped (Koroleva, 1988b; Mann, 1985), as well as a "cobweb-like network" that holds the microorganisms loosely together at the centre of the grains. This less prominent, "cobweb-like network" is thought to be produced by a yeast (Mann, 1985). The insoluble threads of the main structural material are thought to be composed of a branched hexasaccharide repeating unit consisting of glucose and galactose residues that has subsequently been named Kefiran (Neve, 1992; Mukai *et al.*, 1990). Hydrolysis of Kefiran yields various D-glucose to D-galactose ratios, depending on the relative concentrations of the various microorganisms present in the grains (Toba *et al.*, 1987). Neve (1992) found in his studies that the glucose and galactose residues are present in equal concentrations in hydrolysed Kefir grains. Kefiran can form weak gels in conditions of low water activity, which has potential practical applications (Pintado *et al.*, 1996). This polymer makes up at least 25% of the dry mass of the Kefir grains (Neve, 1992). The insoluble nature of the polymer may be expected to prevent diffusion of nutrients and end metabolites so that organisms embedded within it may eventually die, remain enmeshed and become part of the grain structure (Marshall, 1984a). It appeared at first that Kefiran was a capsular material formed by *Lactobacillus kefir* (Pintado *et al.*, 1996; Roginski, 1988). This is consistent with the findings of Pintado *et al.* (1996) that *Lactobacillus kefir* is encapsulated by a polysaccharide. However, in some Kefir grains, *Lactobacillus acidophilus* has been shown as the organism responsible for its formation (Liu and Moon, 1983). Neve (1992), in contrast, reported that *Lactobacillus kefiranofaciens* is the main producer of kefiran. Investigators do not seem to be in accord as to which

organism is responsible, except that the organism responsible is a homofermentative lactobacillus species (Toba *et al.*, 1987). Kefir grains also appear to contain a protein/lipid complex that is slimy, but extremely resilient. These various embedding materials are of great survival value for the microorganisms due to the structural support and protection they offer (Liu & Moon, 1983). This densely packed fibrillar, amorphous support material is the embedding material for the short and elongated bacterial rods (bacilli) (Duitschaever *et al.*, 1988a).

Closer examination of the Kefir grains reveal that they occur in different forms. Some of the smaller structures can be unfolded into sheet-like forms (Marshall *et al.*, 1984a). These show asymmetry; one side is smooth and shiny and the other rough and convoluted. The smooth side is populated by small, short lactobacilli such as *Lactobacillus kefir*, and the obverse side is composed of a mixed population of lactobacilli and yeasts (Marshall, 1984a).

If a cross-section is made of a Kefir grain, it is evident that the different microorganisms occur at different depths in the Kefir grain (Bottazzi & Bianchi, 1980). The most richly colonised part of the grain is the part nearest to the exterior of the grain; this part is composed almost exclusively of short, rod-shaped bacteria and a few yeasts (Bottazzi & Bianchi, 1980). The yeast concentration increases towards the middle of the grain where few bacteria are present. This increase in yeast concentration continues until the yeasts achieve dominance at the centre of the granule (Bottazzi & Bianchi, 1980). This is consistent with the findings that the yeasts found in Kefir grains are the least affected by environmental conditions of all the microorganisms present (Pintado *et al.*, 1996).

The Kefir grain is unique in its ability to self-regulate its microbial population. This is demonstrated by the fact that different grain starter samples cultivated under the same conditions have similar populations (Koroleva, 1988a). This in turn means that there are many variables linked to the microorganisms found in Kefir grains, and hence those found in the Kefir drink (Koroleva, 1988a).

## b) Kefir Culture

As discussed previously, Kefir culture (**starter 2**) is the product obtained when milk is fermented by Kefir grains (Koroleva & Bavina, 1970; Koroleva, 1988b). For the routine manufacture of Kefir of standard quality, a Kefir starter of constant and desired microbial population and biochemical characteristics is necessary. Appropriate treatments of the Kefir grains and culture during the fermentation process allow, in principle, Kefir with any specified microbiological composition to be obtained (Koroleva & Bavina, 1970). This explains why it is so difficult to obtain a Kefir drink with optimal and constant composition. Any deviation from the established environmental conditions for the cultivation of Kefir grains will lead to changes in the microbial composition of the Kefir grains and consequently to changes in the character and duration of the fermentation, as well as the quality of the product (Koroleva, 1988b).

As with Kefir grains, the microbial community of Kefir culture consists of several functionally different groups of microorganisms:

- i) Mesophilic homofermentative lactic acid streptococci. These microorganisms include species of *Streptococcus lactis* and *Streptococcus lactis* subsp. *cremoris* and make up the largest and most active part of Kefir culture, in contrast to the dominant microorganisms in the Kefir grains themselves (Koroleva, 1988b). This fact suggests that a microbiological shift occurs during fermentation of milk with Kefir grains (Neve, 1992). These microorganisms provide rapid acid development during the first hours of fermentation, although they are inhibited at high acidity levels (Koroleva, 1988b).
- ii) Lactobacilli such as *Lactobacillus kefir*, *L. casei* subsp. *rhamnosus*, *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus*. The number of these mesophilic bacteria does not exceed  $10^2$ - $10^3$  per ml of Kefir culture, and subsequently these organisms are not the main determinant of quality in the final product (Koroleva, 1988b).
- iii) Mesophilic heterofermentative lactic acid streptococci. These include members of species such as *Leuconostoc mesenteroides*, *L. mesenteroides* subsp. *dextranicum* and *L. dextranicum*. *Leuconostoc dextranicum* is

activated at elevated temperatures and in the presence of large numbers of yeasts. *Leuconostoc mesenteroides* and *L. mesenteroides* subsp. *dextranicum* participate in the formation of the specific taste and aroma of Kefir, however, if growth is excessive, excessive gas formation is observed which results in packaging problems of the Kefir beverage (Koroleva, 1988b).

- iv) Yeasts. These can include *Kluveromyces marxianus* subsp. *marxianus*, *Torulaspora delbrueckii*, *Saccharomyces cerevisiae* and *Candida kefir*. The heterogeneity of the species found can be explained by the different techniques employed by dairy workers in the Kefir grains' cultivation. The yeasts are responsible for CO<sub>2</sub> formation and the development of characteristic tastes and aromas in the Kefir beverage. Excessive growth of these organisms can cause high levels of gas formation in the product and consequently packaging problems (Koroleva, 1988b).
- v) Acetic acid bacteria of the species *Acetobacter aceti* and *A. rasens*. The presence of these bacteria result in an improved consistency of the Kefir by increasing its viscosity. This is accomplished by the lowering of the pH of the milk and the resulting coagulation of the milk. Acidification activity is increased when streptococci are cultivated together with acetic acid bacteria due to the stimulating effect of the streptococci on the acetic acid bacteria (Koroleva, 1988b).

Due to the fact that the microbial population of Kefir drink depends on the method of preparation and the starter culture employed, investigations have mainly been based on light microscopical techniques (Duitschaever *et al.*, 1988a). Photographs of Kefir made with pure cultures of lactobacilli, streptococci and yeasts show a population structure consisting of only these three groups of microorganisms (Duitschaever *et al.*, 1988a). In contrast, the microorganisms found in Kefir prepared with Kefir grains consisted mainly of cocci and a few yeast cells. The presence of these yeast cells may explain the buttermilk character and slight effervescence of this Kefir (Duitschaever *et al.*, 1988a). The organisms present in Kefir made from grain-free starters consisted exclusively of cocci (Duitschaever *et al.*, 1988a). It is therefore apparent that rod-shaped bacteria and the many yeast strains that are associated with the Kefir grain either do not detach themselves from the Kefir grain, or are lost in the Kefir production process (Duitschaever *et al.*, 1988a). This loss of yeasts results

in a product with less effervescence and a decrease in organoleptic quality due to a decrease in the concentration of the flavour and aroma compounds usually manufactured by the yeasts (Duitschaeffer *et al.*, 1988a).

Occasionally, contaminating yeasts of the *Mycoderma* type appear which impair the taste of the Kefir. Another yeast that impairs the taste and quality of the Kefir is *Geotrichum candidum* which appears on the surface of Kefir that has been kept for a prolonged period (Koroleva, 1988b).

It is generally recommended that a properly prepared Kefir beverage should have  $1 \times 10^8$  to  $1 \times 10^9$  homofermentative mesophilic lactic acid streptococci,  $1 \times 10^5$  thermophilic lactobacilli,  $1 \times 10^7$  to  $1 \times 10^8$  heterofermentative lactic acid streptococci,  $1 \times 10^5$  to  $1 \times 10^6$  yeasts and  $1 \times 10^5$  to  $1 \times 10^6$  acetic acid bacteria per ml (Koroleva, 1988b).

### c) Single-Use Kefir Starter

There have been a number of patents for these single-use starters which generally consist of two starters. In one developed by Klupsch (1984), as cited by Marshall, 1987, the first starter contains *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *diacetylactis* and *Lactococcus lactis* subsp. *cremoris*, as well as *Lactobacillus acidophilus*, *Lactobacillus kefir* and *Lactobacillus brevis*. This starter is added at a concentration of approximately 2% per volume to fresh milk. The milk is then incubated at 24°-27°C until the pH of the milk reaches 4.4. This mixture is then cooled and a second starter consisting of a strain of *Candida kefir* and of *Lactobacillus brevis* is added. No further incubation takes place, which results in a product with little ethanol content or gassiness. Another patent, developed by Pettersson in 1984, as cited by Marshall, 1987, involves a lyophilised starter. In this case, selected strains of homofermentative lactic acid streptococci, lactobacilli and *Candida kefir* are cultivated separately at a constant pH. Each strain is then concentrated, lyophilised and finally mixed together to give a final content of 75% *Lactococcus lactis*, 24% *Lactococcus lactis* biovar *diacetylactis*, 0.5% lactobacilli and 0.1% yeast. This starter produces a consistent product with a good aroma and flavour in terms of more diacetyl, and less of a yeasty flavour than that found in traditional Kefir. The low numbers of yeasts result in a product with less ethanol and CO<sub>2</sub> than found in traditional Kefir (Marshall, 1987). Kefir starters obtained from

lyophilised cultures should contain approximately 80% streptococci, 15% lactobacilli and 5% yeasts (Libudzisz & Piatkiewicz, 1990).

A disadvantage of lyophilisation of Kefir grains is the loss of the intrinsic inhibitory power of the grains with respect to pathogenic bacteria (*Saccharomyces cerevisiae*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*). The percentage loss of inhibition is a function of the lyophilisation substrate; a total loss is observed for water (Brialy *et al.*, 1995).

## **Manufacturing Procedures**

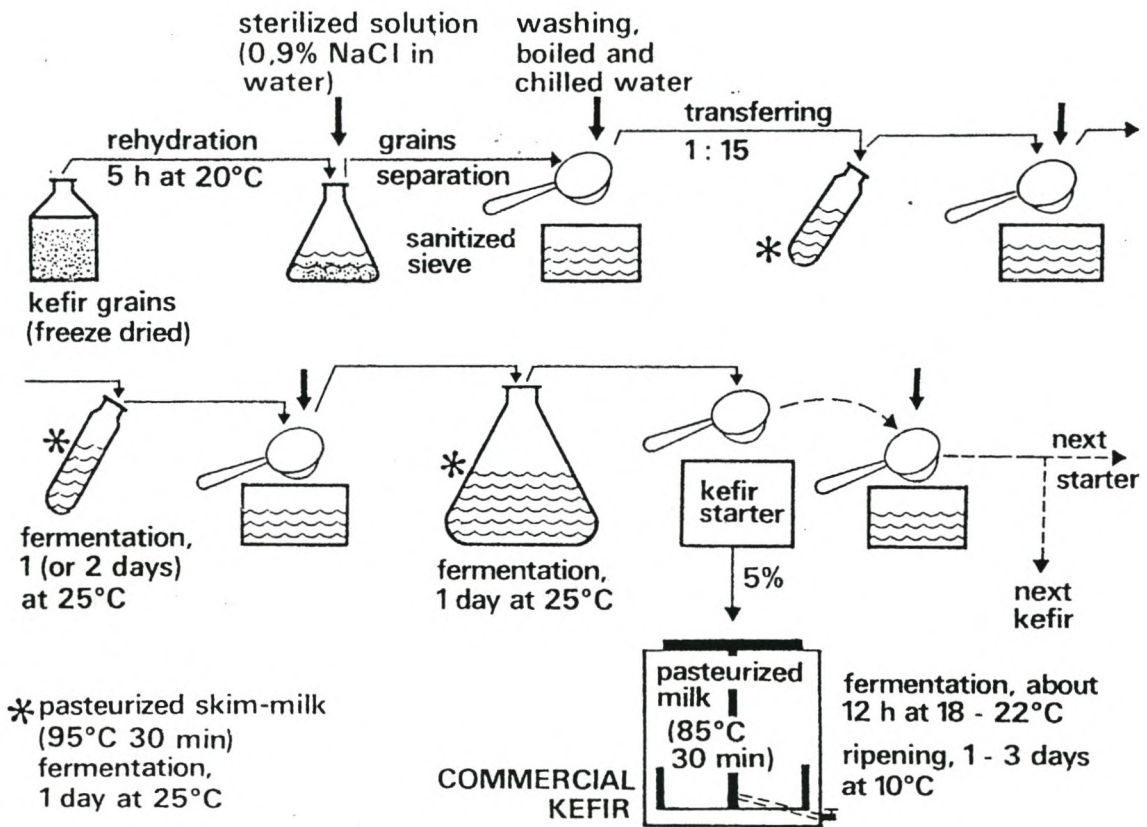
Just as there are contradicting reports regarding the microbiological aspects of Kefir, there are many different beliefs as to which production method provides the most desirable product. Kefir can be made from whole, low fat or skim milk, although with a lowered fat content, the body and mouthfeel of the final product may be lacking. This problem can be overcome by adding 1-4% non-fat milk solids, such as skim milk powder to the substrate (Kroger, 1993). The total solids content of the milk should never be lower than 8% (Koroleva, 1988a). Whatever the type of milk used, the milk must be of a high chemical and microbiological quality and should be subjected to strict microbiological, organoleptic and physico-chemical control before use as a substrate (Koroleva, 1988a).

The production process employed obviously depends to a large extent on the starter used, however, regardless of the type of starter, the first step in the production process is the homogenisation of the milk at 12.5-17.5 MPa (Koroleva, 1988a). This is followed by the pasteurisation of the milk (Koroleva, 1988a; Kroger, 1993) at 85°-87°C for 5-10 minutes or 90°-92°C for 2-3 minutes (Koroleva, 1982). The milk can alternatively be homogenised during the pasteurisation process (Koroleva, 1982). This pasteurisation step is necessary in order to ensure that no unwanted microorganisms are included in the Kefir beverage. An added benefit of the pasteurisation process is that an extreme heat treatment such as 95°C for 10-15 minutes, or even for as long as 30 minutes (Libudzisz & Piatkiewicz, 1990) denatures the whey proteins which results in a subsequent stabilising of the Kefir and a better mouthfeel in the final product (Kroger, 1993). The heat-treated milk must then be cooled to an inoculation temperature of 18-22°C (Kroger, 1993).

The three main categories for Kefir manufacture are manufacturing using Kefir grains (traditional Kefir), using a Kefir culture, and Kefir manufacture using pure or "single-use" starter cultures. The relationship between Kefir grains and Kefir culture is illustrated in Figure 3 which is a schematic representation of the preparation of traditional Kefir and Kefir culture (Kosikowski, 1982).

#### a) Kefir Grains

After the heat treatment of the milk and the subsequent cooling, Kefir grains should be added to the milk at a concentration of 2-5% (Kroger, 1993), or 0.5-10% (Roginski, 1988). Libudzisz and Piatkiewicz (1990) recommend that the ratio of grains to milk should be maintained at 1:10. The milk containing the grains should then be incubated at 18° to 23°C for approximately 12-24 hours (Kroger, 1993; Libudzisz & Piatkiewicz, 1990; Roginski, 1988) in order to stimulate a lactic fermentation (Roginski, 1988). During this fermentation period, two brief intermittent stirrings of the milk containing the grains should be carried out (Kroger, 1993). The Kefir grains can then be sieved from the milk using a metal sieve (Kosikowski, 1982; Kroger, 1993; Libudzisz & Piatkiewicz, 1990; Roginski, 1988). At this point, the grains can be inoculated into a fresh milk sample that has been treated as previously described. Other authors claim that the grains should first be washed with cold tap water (Kroger, 1993), or sterile water (Libudzisz & Piatkiewicz, 1990). This washing process is not recommended as it tends to disturb the microbial ratio in the grains and many essential microorganisms are lost in the process which leads to a lower quality product (Koroleva, 1988b; Koroleva & Bavina, 1970). Roginski (1988) includes an extra step in Kefir production in order to ensure alcoholic fermentation, with the resultant saturation of Kefir with carbon dioxide - the coagulum should be bottled and then stored at 10°-15°C for 1-3 days. This Kefir must then be cooled to approximately 8°C before consumption (Koroleva, 1988a; Kroger, 1993), although refrigeration temperatures also result in satisfactory Kefir quality (Roginski, 1988).



**Figure 3.** Schematic outline for producing Kefir and transferring Kefir grains from lyophilised culture (Kosikowski, 1982).



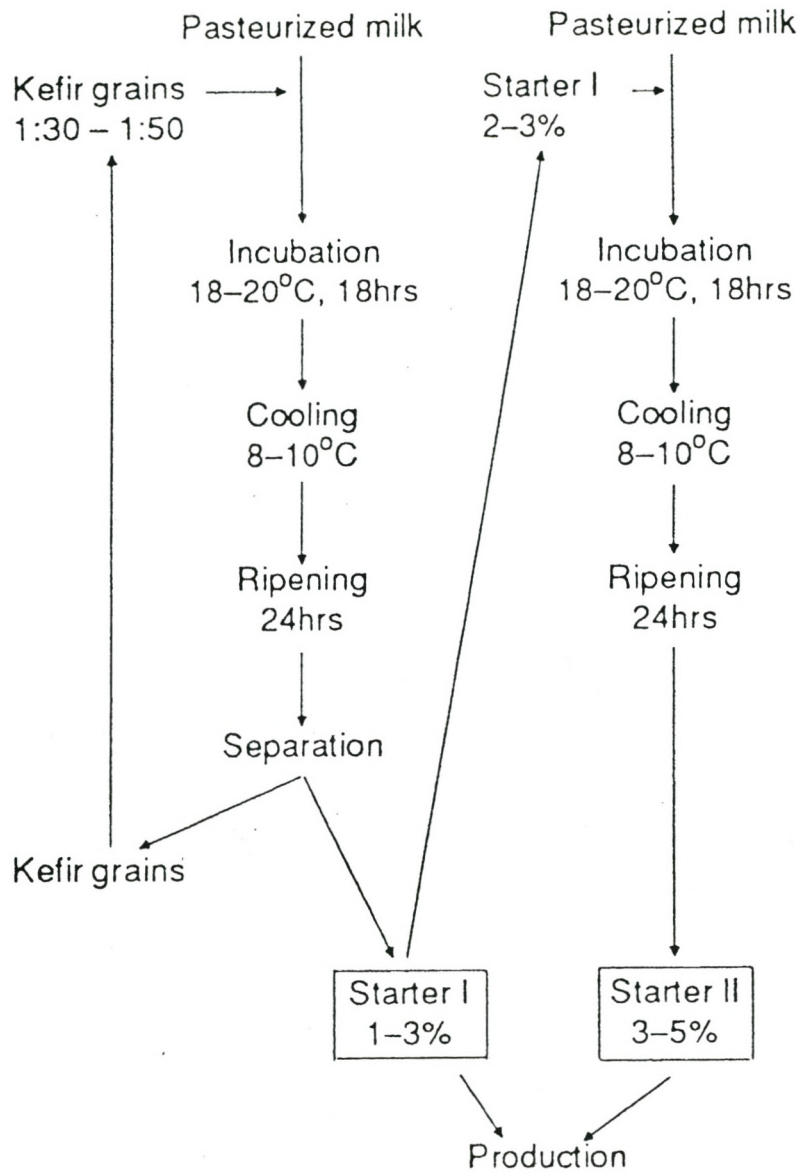
There are disadvantages associated with the use of Kefir grains for industrial scale Kefir production in comparison to the use of single-use starter cultures, namely;

- i) The cultivation of Kefir grains requires time, appropriate laboratory conditions and a skilled staff and
- ii) The composition of the microbial population associated with the Kefir grains not only varies from source to source, but also fluctuates constantly during the propagation of the grains, as previously discussed (Duitschaeffer, 1989).

#### b) Kefir Culture

In this case, a mother culture is prepared by adding Kefir grains to severely heated milk (95°C for 10-15 minutes) at a concentration of 100-200 grams per litre (Duitschaeffer, 1989), or a ratio of 1:30 to 1:50, and incubating this milk at 18°-22°C for 18-24 hours (Kurmann *et al.*, 1992; Rasic, 1987). The fermenting product must be agitated twice, once after 15-16 hours of fermentation and again after 22 hours (Kurmann *et al.*, 1992; Rasic, 1987). The grains are then removed and used for further mother culture preparation (Duitschaeffer, 1989; Kurmann *et al.*, 1992). This prepared culture of Kefir is inoculated into fresh milk that has been subjected to a pasteurisation process of 95°C for 10-15 minutes (Kurmann *et al.*, 1992) at a 2-3% concentration (Koroleva, 1988a; Kurmann *et al.*, 1992; Rasic, 1987), and the milk is then incubated at 20°-22°C (Kurmann *et al.*, 1992; Rasic, 1987) for 8-12 hours. This is followed by ripening in order to develop the specific taste and aroma associated with a good quality Kefir (Koroleva, 1988b). In this ripening process, the fermented milk is agitated and slowly cooled during 10-12 hours to 8°-10°C (Koroleva, 1988a; Kurmann *et al.*, 1992).

If a large quantity of Kefir is to be prepared, a mother culture (**starter 1**) is used for the preparation of a bulk culture (**starter 2**). This bulk culture is prepared by the addition of 3-5% of mother culture to pasteurised and cooled milk (Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990). This mixture is then incubated at approximately 20°C for 20 hours (Libudzisz & Piatkiewicz, 1990). Figure 4 is a schematic diagram of the preparation of the various starters for Kefir production and their utilisation in production. Each of these starters is in itself a Kefir beverage that



**Figure 4.** Preparation and utilisation of starters in Kefir production (Koroleva, 1988b).

can be consumed once cooled. The production of Kefir starters is thus analogous to the use of Kefir grains for Kefir production (Koroleva, 1988b).

Another popular method described in the literature for the cultivation of Kefir using starter cultures is a method called the stirred method. This method employs a vessel with both a heating element and a cooling sleeve, as well as a stirrer. In this method, a continuous process for Kefir manufacture is thus employed. Milk is pasteurised with homogenisation as for traditional Kefir manufacture (Koroleva, 1982), and then cooled to between 18° and 22 °C. The milk is then inoculated with 2-3% of **starter 1** and is carefully agitated. The fermentation process usually takes approximately 10-12 hours, and is followed by ripening in the course of slow cooling to 8°C over a 12-24 hour period (Koroleva, 1982; Koroleva, 1988b).

There are three specific methods for industrial scale Kefir production in Poland which all use a bulk culture (Libudzisz & Piatkiewicz, 1990). These are:

- i) Kefir production using the in-final-package method. For this production method skim or standardised milk of fat content 1.5, 2.0 or 3.1% is pasteurised at 85°-87°C by a continuous method with a holding time of 10 minutes. It is then cooled to a temperature of 19°-23°C (depending on the season). The culture is then added at a volume of 2-7% (also depending on the season), after which the milk is carefully mixed for 3-5 minutes. This mixture is then poured into returnable glasses, capped and incubated at 19°-23°C for 12-14 hours. The drink is then ripened at 8°-10°C for 12 hours (Libudzisz & Piatkiewicz, 1990).
- ii) Kefir production using the fermentation-in-tank method. Bulk starter is added to milk prepared as in method (i) at a volume of 2-7%. This milk-containing starter is then carefully mixed and left to ferment in the tank at a temperature between 19° and 23°C, depending on the season, for 6-8 hours. The curd thus obtained is carefully mixed, cooled to 14°C and poured into polyethylene foil packs covered with aluminium foil lids. This in-pack Kefir is then ripened at 8°-10°C for 12-14 hours (Libudzisz & Piatkiewicz, 1990).
- iii) Production of luxury Kefir. Milk of 2% fat content and dry mass content of 13% is standardised by the addition of skim milk powder (97% of dry mass). This milk is then homogenised at 65°-70°C and a pressure of 15 MPa.

Pasteurisation of this milk at 93°-97°C for fifteen minutes is the following step, after which the milk is cooled to 22°-25°C. The bulk culture is then added at a concentration of 3-5% and the fermentation is carried out at a temperature of 22°C for about 20 hours. The curd thus obtained is then cooled to 5°C and poured into polyethylene foil packs covered with aluminium foil and ripened at 5°-8°C for between 15 and 20 hours (Libudzisz & Piatkiewicz, 1990).

All these finished products should be stored at a temperature of up to 10°C. In this temperature range, these products have a shelf life of approximately three days (Libudzisz & Piatkiewicz, 1990).

As discussed previously, the disadvantage of using Kefir culture for Kefir manufacture is that the culture from which the grains are removed does not contain the same microorganisms as the grains themselves. The lactobacilli, and to a lesser extent, the yeasts, seem to remain embedded in the grains and do not migrate into the surrounding milk during incubation. This explains why the products obtained from a "grain-free" starter are so different from those obtained using Kefir grains (Duitschaever, 1989).

### c) Single-Use Kefir Culture

The easiest of the methods, in this case one gram of a lyophilised culture is added to three litres of pasteurised and cooled milk. The milk containing the culture is mixed well and incubated at approximately 20°C for 20-24 hours (Libudzisz & Piatkiewicz, 1990). If lyophilised cultures are to be used for the preparation of bulk starters, a concentration of 1% per volume of milk must be used, with a fermentation temperature of 22°C for 18-22 hours (Rasic, 1987).

A procedure for the preparation of Kefir using pure cultures has been described by Duitschaever (1989). This procedure involves a two-stage fermentation process, the first using the lactic acid bacteria, and the second utilising a yeast fermentation in order to produce the traditional ethanol and gassiness in the final product.

Another process for preparing Kefir that involves the use of two starter cultures has been described by Mann (1985). The end-product obtained using this method is said to possess the typical flavour and alcohol content of Kefir, as well as the correct

microbial population. Following severe pasteurisation, the milk is incubated at 24°-27°C with 2% of a mixed culture consisting of "*Streptococcus*" *lactis*, *Streptococcus diacetylactis*, *Streptococcus thermophilus*, *Streptococcus cremoris*, *Lactobacillus acidophilus*, *Lactobacillus brevis* and *Lactobacillus lactis* in a lactobacilli:streptococci ratio of 1:20 until the pH is reduced to 4.40 (Mann, 1985). After cooling, a culture of *Candida Kefir* and *Lactobacillus brevis* is added at a rate of 0.5-5.0 litres per 1000 litres.

There is even a method to produce Kefir that uses three stages, a pure lactic acid fermentation, a combined yeast/lactic fermentation and the addition of a mixture of a combined starter (Kefir lactobacilli and Kefir yeasts). These fermentations are followed, after packaging, by storage at 5°C for 24 hours with concurrent milk fermentation characterised by ethanol production and a small increase in carbon dioxide concentration. The end-product contains  $1 \times 10^7$  to  $1 \times 10^8$  yeasts per millilitre and 0.03% ethanol, and has a balanced, slightly yeasty and aromatic flavour (Hlavata, 1987).

Kefir tablets have also been produced in Poland for Kefir preparation in the home. One or two of these tablets are dissolved in a glass of milk that is then incubated at a temperature of 25°-30°C for 18-26 hours until a curd is formed. This curd can then be used as a starter for the production of Kefir; four to five tablespoons are added to one litre of milk. This mixture is incubated at 20°-22°C for 14-18 hours until coagulation occurs, after which it is cooled and kept in the refrigerator until consumption. The tablets are quick and easy to use, allowing the preparation of a drink under home conditions which meets the needs and preferences of the consumer (Libudzisz & Piatkiewicz, 1990).

### **Product Characteristics**

The product has a sour, cream-like consistency and specific flavour (Marshall, 1982; Rasic, 1987). This flavour can be described as mildly alcoholic, yeast-sour with a tangy effervescence (Vedamuthu, 1982) which is often characterised by a specific "biting" taste (Koroleva, 1982; Koroleva, 1988a). Other descriptions of the flavour of Kefir range from rather cheesy to slightly rancid (Marshall, 1984a).

The fat content of the final product depends on the fat content of the milk used for production, and there are whole milk Kefirs, low-fat milk Kefirs, skim milk Kefirs, and even Kefirs containing six or more percent fat (Kurmann *et al.*, 1992; Rasic, 1987). The major end-products of the fermentation of Kefir are lactic acid, ethanol, diacetyl, acetoin, and carbon dioxide (Kramkowska *et al.*, 1982; Rea *et al.*, 1996), and its typical flavour is due mainly to an optimum ratio of diacetyl to acetaldehyde and complex alcohols of three to one. Acetoin appears to play a minor role in the flavour of Kefir (Kosikowski, 1982; Vedamuthu, 1982). Another metabolite found in Kefir produced from Irish “Kefir-like” grains is acetate (Rea *et al.*, 1996). Kefir, before ripening at low temperatures, has the aroma of freshly baked bread (Marshall, 1984b). A good quality Kefir foams and fizzes like beer when agitated, has a soft curd, and is pourable (Kosikowski, 1982; Marshall, 1982; Vedamuthu, 1982).

The end metabolites of the associative growth of the Kefir microorganisms result in a product with a titratable acidity, or lactic acid concentration of 0.8% (pH 4.3-4.4) (Marshall, 1982; Marshall, 1984a; Marshall, 1984b; Koroleva & Bavina, 1970; Kosikowski, 1982; Vedamuthu, 1982) to 1% (Koroleva & Bavina, 1970; Kurmann *et al.*, 1992; Rasic, 1987); an acetaldehyde concentration of 1-2ppm; a diacetyl concentration of 3ppm; and an ethanol concentration that is very variable (Marshall, 1982; Marshall, 1984b). There are reports of it being as high as 1% in terms of ethyl alcohol (Koroleva & Bavina, 1970; Kosikowski, 1982; Vedamuthu, 1982), although the alcohol content of Kefir produced by modern manufacturing methods does not usually exceed 0.1% (Koroleva, 1982; Koroleva, 1988a; Rasic, 1987), and is even often as low as 0.01% (Koroleva, 1982; Kurmann *et al.*, 1992). The carbon dioxide content is also usually very low (Koroleva, 1988a; Rasic, 1987), as is the amount of carbonic acid (Koroleva, 1982). Generally, the higher the alcohol content, the more yeasty the flavour (Marshall 1984b).

In an investigation into the chemical changes that occur during fermentation and ripening of Kefir (Gawel & Gromadka, 1978), it was found that the main chemical changes in Kefir occur during the first day of fermentation. In this period, there is a considerable increase in the volatile acidity, simple nitrogen compounds, acetoin and carbon dioxide. The ethanol content is low, and the concentrations of orotic acid and citric acid decreased. The orotic acid is probably used for growth of the microorganisms and the resulting synthesis of nucleotides, while the citric acid is

probably converted to diacetyl and acetoin. There is great variation in the content of acetaldehyde and diacetyl. The organoleptic properties of the Kefir were related to the chemical composition of the Kefir, and these chemical properties were dependent on the activity of the Kefir grains and starter (Gawel & Gromadka, 1978). In another investigation into the effect of fermentation on the volatile acid and ethanol content of Swedish dairy products, it was found that propionic and butyric acid content is low in Kefir, however ethanol content is very high in comparison with other fermented products at approximately 1.2%. As this value is lower than the value cited in other reports, it is mentioned that the mode of Kefir manufacture in Sweden is adjusted for the production of little ethanol and carbon dioxide. The level of acetic acid in Kefir is intermediate when compared to other fermented products, and this concentration of acetic acid is not influenced by storage (Alm, 1981).

In an investigation into the properties of Kefir prepared using different manufacturing methods (Duitschaever *et al.*, 1988b), it was found that Kefir prepared using pure cultures and a sequential fermentation had a higher acidity and viscosity than Kefirs prepared using Kefir culture and those using a single fermentation with pure cultures. The panellists gave the highest organoleptic score to the Kefir prepared using this sequential fermentation (Duitschaever *et al.*, 1988b). These results are however inconclusive, as Kefir prepared using Kefir grains was not included in the experiment, yet most of the other authors show a preference for traditionally prepared Kefir.

### **Factors Affecting Kefir Quality**

The quality of the Kefir beverage is strongly affected by many parameters, especially changes in environmental conditions of either the grains or the Kefir culture. Environmental and handling conditions have a direct influence on the microbial population of the Kefir grains and the Kefir culture, which has a drastic influence on the quality of the final product.

When Kefir grains are to be cultivated in order to obtain enough grains of a standard and desirable quality for production processes, many aspects regarding the cultivation techniques and environment need to be considered. The most important is probably the sustenance of the grains' microbial population activity when they are

not being actively used for fermentation processes. When Kefir grains are not removed from the fermented product, excessive acid production will gradually damage the viable organisms. With refrigeration (4°C), acid production is inhibited, but the organisms still lose their activity after about 10 days (Kroger, 1993). Grains are active for 8-10 days if stored in clean water at a temperature of 4°C (Kosikowski, 1982; Vedamuthu, 1982). Pintado *et al.* (1996) found that Kefir grains stored in sterile water at refrigeration temperatures could be stored for three months without much loss of microorganism activity. This is in contrast to Garrote *et al.* (1997) who showed that Kefir grains stored at 4°C do not increase their weight and the product obtained after re-inoculation does not have the acidity or viscosity of the standard product. Several successive daily transfers (usually about three) may renew the vitality of the Kefir grains and re-establish a desirable mix of the microorganism species (Kroger, 1993; Vedamuthu, 1982). A long-term storage method which allows the grains to remain active for well over a year is to wash the grains with clean cold water and to dry these grains on cheesecloth or paper for two days at room temperature (Kosikowski, 1982; Kroger, 1993), or in a warm oven (Vedamuthu, 1982). These grains should then be stored in aluminium foil or in a paper envelope in a cool, dry place in order to prolong the storage period without detriment to the grains (Kroger, 1993; Vedamuthu, 1982). Grains that have been dried have a lag-phase of approximately three days upon re-introduction to milk where slow growth is observed (Pintado *et al.*, 1996). This is consistent with the commonly accepted belief that microorganisms in a dried form take a considerable time to readapt their metabolic system to biomass production (Pintado *et al.*, 1996). In a more recent study, the effect of freezing on the grains was investigated. Kefir grains stored at -20°C and -80°C were found to maintain their microflora and increase their weights at a rate comparable to that found with nonstored grains. The Kefir prepared from these grains showed the same microbial populations, rheological behaviour, acidity and carbon dioxide content as is usually obtained with unstored grains. This implies that storage of Kefir grains at household freezer temperatures is a good method to preserve their necessary metabolic activity (Garrote *et al.*, 1997). Another similarly benign way to store the grains on a long term basis is to freeze-dry them (Kroger, 1993).



The various factors that are important in the production of Kefir drink of a constant and desirable composition are:

a) Effect of the starter used

As discussed previously, the starters used in the manufacture of Kefir are either the Kefir grains themselves or the Kefir obtained by the cultivation of the grains. When the grains are used for Kefir production, the content of the homofermentative lactic acid streptococci and yeasts in the resulting Kefir is higher than in Kefir prepared using a Kefir culture, however, the number of thermophilic lactobacilli is higher in Kefir obtained using Kefir culture (Koroleva, 1988a; Koroleva *et al.*, 1978a). An excess of thermophilic lactobacilli leads to high acidity in the Kefir, and so their numbers should not be too high. This is another reason why it is preferable to use Kefir grains for Kefir production (Koroleva, 1988a; Koroleva *et al.*, 1978a). The starter used should contain sufficient acetic acid bacteria, although not more than  $1 \times 10^6$  per ml, as these microorganisms improve the consistency of Kefir (Koroleva, 1988a; Koroleva *et al.*, 1978a). Due to the fact that acetic acid bacteria do not seem to be influenced by the choice of starter, the consistency of Kefir prepared with grains or culture shows minimal variation (Koroleva, 1988a; Koroleva *et al.*, 1978a).

b) Effect of the concentration of starter used

If Kefir is to be made using Kefir culture, a decrease in starter concentration from 5% to 1% makes very little difference to the final composition of the product. The only influence is a slightly prolonged fermentation time (Koroleva *et al.*, 1978a). It must however be noted that the maximum quantity of homofermentative mesophilic lactic acid streptococci have been detected in Kefir made from milk inoculated with 1-2% of Kefir starter. This can probably be explained by the fact that smaller quantities of inoculum create more favourable conditions for the growth of this group of organisms. Similar results have been obtained for the heterofermentative lactic acid streptococci. The content of the other microorganisms in Kefir, especially for the thermophilic lactobacilli and the acetic acid bacteria are not influenced by the quantity of inoculated starter (Koroleva, 1988a).

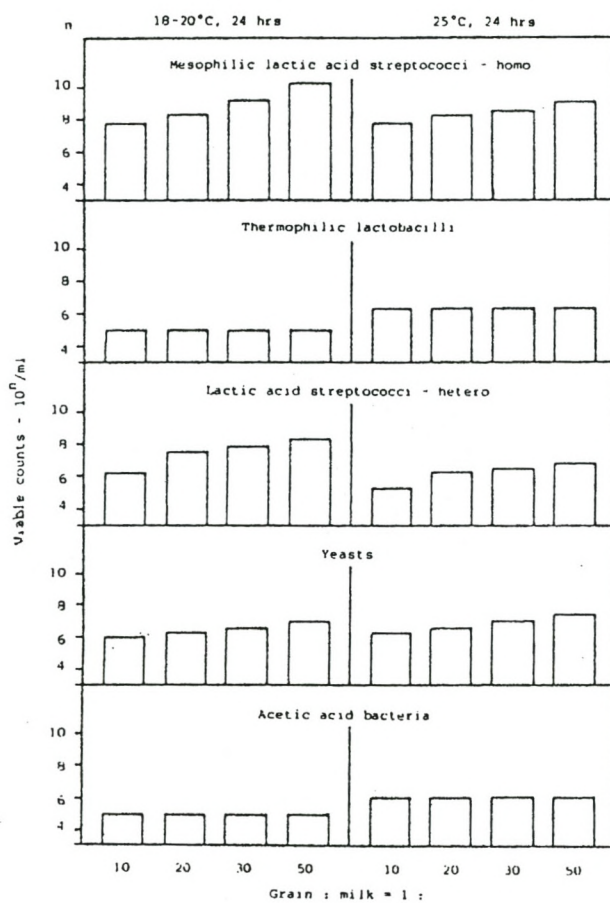
### c) Effect of the Kefir grains to milk ratio

The larger the ratio of grains to milk, the shorter the fermentation process. This results in a minimum concentration of homofermentative and heterofermentative lactic acid streptococci and yeasts due to the rapid accumulation of lactic acid, hence the shortened fermentation (Koroleva, 1988b). A decrease in the amount of Kefir grains added to milk (1-2%) results in an increased content of some groups of Kefir culture microorganisms, higher biochemical indices and better taste of the finished product due to a slightly prolonged fermentation time in comparison to higher grain ratios (3-5%) (Koroleva *et al.*, 1978a). Thermophilic lactobacilli and acetic acid bacteria do not respond to starter to milk ratio changes. A low pH (3.6-3.8) is obtained in Kefir that has been prepared with a high grains to milk ratio (in the order of 1:10), while higher pH values (4.4-4.6) are obtained for lower grains to milk ratios (in the order of 1:30 to 1:50) (Koroleva, 1988b). For the cultivation of good quality grains, the ratio of grains to milk should be kept in the range 1:30 to 1:50 (Koroleva, 1988b).

By reducing the quantity of Kefir grains inoculated into the milk, the number of the major groups of microorganisms tends to increase, with a grains to milk ratio of 1:50 resulting in the most active development of all the groups of microorganisms. This ratio also results in the highest enrichment of Kefir with volatile fatty acids and CO<sub>2</sub> which is probably connected to a more intensive growth of yeasts and heterofermentative lactic acid streptococci. This intensive growth is observed in the grains to milk ratio range of between 1:30 and 1:50 (Koroleva, 1988b).

By varying the ratio between the Kefir grains and milk from 1:10 to 1:50, the numbers of aroma producing streptococci, yeasts, mesophilic lactic acid streptococci and acetic acid bacteria can be regulated from, respectively,  $1 \times 10^7$ - $1 \times 10^8$ ,  $1 \times 10^5$  -  $1 \times 10^6$ ,  $1 \times 10^7$ - $1 \times 10^{10}$  and  $1 \times 10^4$ - $1 \times 10^6$  per ml (Koroleva & Bavina, 1970).

The correlation between grains to milk ratio and the main groups of microorganisms encountered in Kefir is illustrated in Figure 5 (Koroleva, 1988b).



**Figure 5.** Correlation between main groups of microorganisms in Kefir and Kefir grain and milk ratio (Koroleva, 1988b).

#### d) Effect of the fermentation temperature

It has been found that the pH change of milk is directly proportional to the incubation temperature of the milk (Korovkina *et al.*, 1978). When the cultivation of Kefir is carried out at elevated temperatures (25°-27°C), the growth of thermophilic lactic acid bacteria (streptococci and lactobacilli) is more intensive, which results in a drastic pH drop to levels that are inhibitory to homofermentative and heterofermentative lactic acid streptococci and yeasts. This level of acidity is reached within 6-8 hours of fermentation, and has as a result a decrease in these organisms' concentrations in the Kefir, hence an atypical Kefir taste. More acid-resilient groups of microorganisms, such as the acetic acid bacteria, favour higher incubation temperatures. At 20°-22°C the fermentation time is 10-12 hours. If the coagulum is then cooled to 8°-10°C, the heterofermentative lactic acid streptococci and yeasts still do not develop, also resulting in an atypical Kefir taste (Koroleva, 1988a; Koroleva 1988b).

In another report, it is stated that an increase in the incubation temperature of Kefir has as a result an increase in CO<sub>2</sub> production, viscosity and acidity, with a temperature of 25°C regarded as optimal for the maximum production of ethanol and volatile fatty acids, as well as a good specific flavour and consistency (Korovkina *et al.*, 1978).

To accumulate all the principle microbial groups in the Kefir, it is necessary to hold the coagulated Kefir which still contains the grains at the fermentation temperature for another 5-6 hours. This is then often followed by incubation of the Kefir and grains at a low temperature (7°-10°C), although this further incubation does not appear to change the character and quantitative composition of the microbial population. Thus, the total cycle of Kefir manufacture is 20-24 hours, which includes fermentation at 20°-22°C for 10-12 hours and ripening, with slow cooling to 8°-10°C over the next 10-12 hours (Koroleva, 1988a).

#### e) Effect of agitation during fermentation

Agitation during the cultivation of grains in milk results in a ten-fold increase in the content of homofermentative lactic acid streptococci and yeasts. This agitation has no effect on the content of the heterofermentative lactic acid streptococci,

thermophilic lactobacilli and acetic acid bacteria or on the concentration of volatile fatty acids produced in the Kefir. The effect of agitation on the microorganism counts in Kefir is illustrated in Figure 6 (Koroleva, 1988b). For the cultivation of Kefir grains of a high quality, the milk containing the grains should be agitated three times during the fermentation process in order to bring all the milk into contact with the grains (Koroleva, 1988b).

Kefir viscosity depends strongly on the pH of the fermented milk at the time of stirring. It has been found that agitation at a pH of 4.4-4.5 produces a Kefir of typical consistency and stable body, without whey separation (Korovkina *et al.*, 1976, as cited by Mann, 1979). The additional stirring prevents the growth of moulds on the Kefir surface and promotes a more even distribution of the microbial metabolites in the milk (Koroleva, 1988b).

#### f) Effect of washing the Kefir grains

Weekly washing of Kefir grains, which is sometimes practised in the dairy industry, leads to a sharp decrease in the numbers of all microorganisms on the surface of the Kefir grains. As yeasts are found deeper in the Kefir grain, their numbers are not decreased by washing the grains, and in some instances washing of the grains actually stimulates yeast growth. This decrease in the surface microbial count results in a decrease in the proportion of acid-forming microorganisms which decreases the activity of the grains. This has the effect of increasing the fermentation time and decreasing the lactic acid and volatile acid content, thereby compromising the taste and consistency of the Kefir (Koroleva, 1988b; Koroleva & Bavina, 1970). This decrease in the microorganisms in Kefir is illustrated in Figure 7 (Koroleva, 1988b). The composition of the normal microbial population is restored after further cultivation of the grains in the usual manner for 3-5 days after the washing process (Koroleva, 1988b).

#### g) The quality and type of milk used for Kefir production

The milk used should be subjected to strict microbiological, organoleptic and physio-chemical control and should be of a consistently high quality (Koroleva, 1988a). For the production of Kefir, milk of any fat content desired can be used, although lower fat products are lacking slightly in mouthfeel (Kroger, 1993), and

therefore often receive lower organoleptic scores in sensory evaluations (Piechocka *et al.*, 1977, as cited by Mann, 1979). For grain cultivation, skim milk is preferable (Koroleva, 1988b).

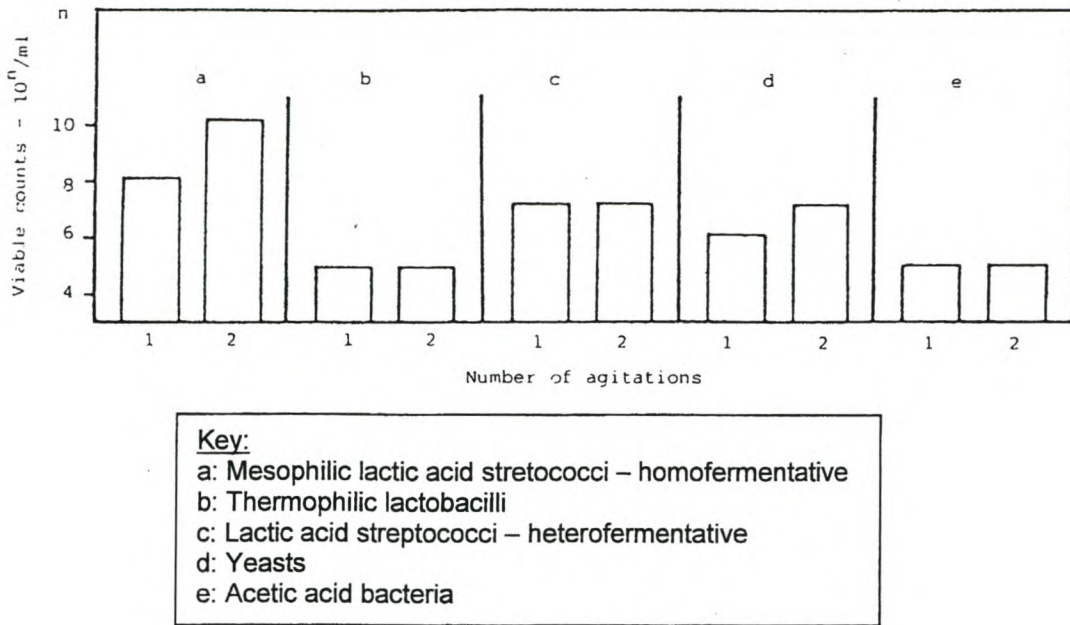
A study in Poland revealed that Kefir made from homogenised mixtures of skim milk concentrated by ultrafiltration, whey protein concentrate prepared by the ultrafiltration of whey and cream has a better structure and better organoleptic properties than traditionally made Kefir. Furthermore, this product does not undergo whey separation upon standing (Chopnowski *et al.*, 1978, as cited by Mann, 1979).

#### h) The solids-not-fat content of the milk

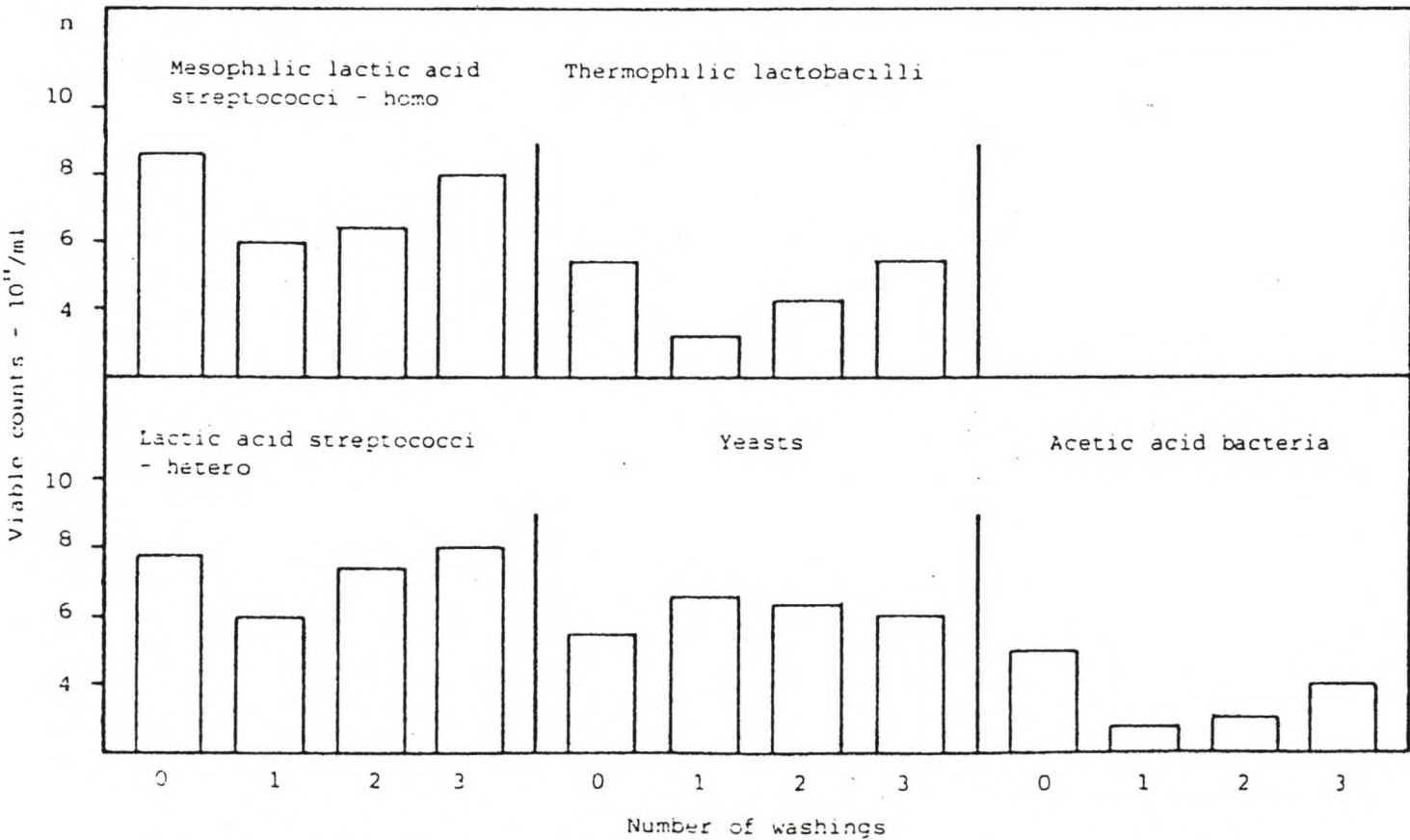
The solids-not-fat content of the milk should be at least 8% in order to ensure a product with a good consistency (Koroleva, 1988a).

#### i) Treatment of the milk prior to inoculation

The milk should be homogenised before fermentation or the consistency of the final product will be adversely affected (Koroleva, 1988a). The consistency and mouthfeel of cultured milk beverages also depends to a large degree on the pasteurisation temperature and holding time of the raw milk (Berzhinskas *et al.*, 1978). For this reason, the milk should be subjected to an extreme heat pasteurisation process such as 85°-87°C for 5-10 minutes or 90°-95°C for 2-3 minutes in order to denature the whey proteins so that they can stabilise the coagulum (Koroleva, 1988a; Koroleva, 1988b). In a study of the effects of heat treatment on the quality of Kefir, four different heat treatments of Kefir milk were compared, namely 85°-87°C for 5-10minutes; 92°-95°C for 20-30 minutes; 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 minutes (Bondarev, 1977, as cited by Mann, 1979). In this study, the consistency of Kefir improved with the severity of heating. The optimal heating of the milk was found to be 92°-95°C for 20-30 minutes. Other investigators have found that pasteurisation of milk at 87°C, followed by a temperature fall of 10°C, and a further rise in temperature to 87°C increases the level of denaturation of the whey proteins and thereby increases the dispersion of the casein molecules. This results in an increase in the firmness,



**Figure 6.** Influence of agitation during the 24 h incubation of Kefir on the main microorganism groups (Koroleva, 1988b).



**Figure 7.** Effect of washing of Kefir grains on the main microorganism groups in Kefir (Koroleva, 1988b).

elasticity and viscosity of the curd, and decreases syneresis (Berzhinskias, *et al.* 1978).

j) The composition of the coagulum

An effort should be made to ensure that no whey is included in the coagulum, or a compromise in the consistency of the Kefir will be the result (Koroleva, 1988a).

k) The fermentation time

For the cultivation of top quality grains, the milk must be renewed at the same time daily. This is due to the fact that Kefir grain microorganisms are not static and there is a constant microbial population shift during the fermentation period (Koroleva, 1988b).

l) The storage conditions of the product

If Kefir is stored at either 2°-4°C or 4°-6°C, the changes in the normal Kefir microbial population take place slowly, and Kefir of reasonable qualitative composition is still obtained after seven days of storage (Koroleva *et al.*, 1978b). At these storage temperatures, the number of mesophilic lactic acid streptococci and yeasts begins to decrease after the fourth day, with the numbers in comparison to fresh Kefir nearly halved by the seventh day. The number of thermophilic lactic acid bacilli appear to remain constant during storage at these temperatures, as do the numbers of acetic acid bacteria. This is not however the case for Kefir stored at room temperature, where the number of mesophilic lactic acid streptococci and aroma producing bacteria are halved after only one day of storage. Marked changes in the yeast and acetic acid content of the Kefir are also observed by the second day of the storage of Kefir at room temperature (Koroleva *et al.*, 1978b). The prepared Kefir should therefore preferably be stored at 8°C for a maximum of 36 hours in order to ensure a good quality final product, although, if the Kefir is stored in glass bottles at 3°-4°C, the shelf life can be increased to 8-10 days (Koroleva, 1988a).



#### m) The handling of the coagulum

Prepared Kefir should not be subjected to rough mechanical treatment during filling as this adversely affects the consistency of the product. For this reason, it is recommended that the distance between the processing vessel and filling machine be as short as possible and that suitable pumps for the transportation of the product are installed in Kefir manufacturing plants (Koroleva, 1988a).

#### n) The shelf life of the product

The prepared Kefir should not be stored for too long as it has been determined that long-term holding of the Kefir at low temperatures after coagulation results in a reduction in flavour due to the intensive development of *Mycoderma*-type yeasts and white milk mould, as discussed in the section on the microbiological aspects of Kefir. This decrease in flavour is not compensated for by any appreciable increases in the number of aroma producing yeasts and bacteria that improve the flavour and aroma of Kefir. This long term storage also results in a more acidic product (Koroleva & Bavina, 1970).

### **Regulatory Issues**

There are no well-known standards for the composition of Kefir (Kroger, 1993). Standards were issued in California in the U.S.A. in the 1991 Food and Agriculture Code, however the description is clearly not based on traditional Kefir which involves yeast cells, a dual lactic and alcoholic fermentation, and the presence of yeast cells in the final product. These details are contradicted in item 38871 of the above-mentioned standard. This is probably the reason for Californian Kefir being called "Kefir milk". The sections dealing with Kefir (not including fruit Kefir) are cited from Kroger (1993), and appear as follows:

Article 36. Kefir Milk - A Cultured Milk

**38871.** Market milk or market milk combined with non-fat milk from market milk, with or without added milk solids, flavouring, or seasoning, which is certified raw milk or has been pasteurised and afterwards fermented by *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Lactobacillus*

*caucasicus* may be sold as Kefir, low-fat Kefir, or non-fat Kefir, or such names as may be characteristic for the product and approved by the director. Such product may contain harmless edible stabiliser not to be exceeded six-tenths of one percent. It shall contain no more than 10 coliform bacteria per gram and shall be free of moulds, yeasts, and other fungi, and other objectionable bacteria which may impair the quality of the product.

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

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

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

There is another standard, drawn up since the Californian Standard, namely the General Standard of Identity for Fermented Milks as found in the International Dairy Federation (IDF) Standard **163** of **1992**. As with most standards, this begins with definitions. The first is the definition of fermented milks, followed by definitions and scopes for raw materials, cultures, ingredients and additives. There follows a classification system consisting of four categories for fermented milks, under which Kefir is classed in the lactic acid and alcoholic fermentation category. The section dealing with the quality requirements for some typical fermented milks devotes the following two paragraphs to Kefir (IDF Standard 163, 1992):

### Culture:

Starter prepared from Kefir grains, whose microbial population is constituted by yeasts both lactose fermenting (*Kluyveromyces marxianus*) and non lactose fermenting (*Saccharomyces unisporus*, *Saccharomyces cerevisiae* and *Saccharomyces exiguus*), *Lactobacillus Kefir*, species of the genera *Leuconostoc*, *Lactococcus* and *Acetobacter* growing in a strong, specific relationship.

### Composition:

Acidity not lower than 0.60% per weight expressed as lactic acid. Minimum counts of specific microorganisms at the time of sale: lactic acid bacteria  $10^7$  cfu/g and yeast  $10^4$  cfu/g.

The standard then has references to other standards for the general labelling of fermented milks (with the proviso that the absolute fat content of the product is expressed as %/g/100g in the total product), and various other referrals to other standards for the methods of sampling and analyses for fermented milks in general. Attached to the standards is an appendix containing the new names of the various lactic acid bacteria as they have appeared in the International Journal of Systematic Bacteriology since 1991 (IDF Standard 163, 1992).

## **Conclusion**

As can be seen throughout this literature review, there is much research still needed on the sacred "Mohammed Grains", as well as all the possibilities for the future manufacture of Kefir. There is no doubt that fermentation is of great importance to man, especially when the product obtained through the fermentation process has a better nutritional value than the substrate, is easier and cheaper to store, has an enjoyable flavour and is easy to prepare. In the manufacture of Kefir, the fermentation conditions are easily attained and the fermentation is easy to control. Kefir as a nutritious and tasty beverage offers endless possibilities to the millions of starving people in Third World Countries such as ours. It most certainly is "a gift of the Gods" as the people from previous centuries called it (Kosikowski, 1982), and it would be a pity to waste such a useful and enjoyable gift!

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## CHAPTER 3

### INFLUENCE OF INCUBATION TEMPERATURE AND INOCULUM CONCENTRATION ON KEFIR GRAIN AND ACID PRODUCTION

#### Summary

Kefir is a fermented milk beverage that has its origin in Caucasian China. This beverage is prepared from Kefir grains consisting of various microorganisms in a symbiotic relationship. Kefir grains are sensitive to growth parameters including incubation temperature and starter grain concentration. In terms of biomass increase, fermentation at 22°, 25° or 30°C appears to yield similar results (up to 500% increase in 10 days) which are preferable to results obtained with incubation at 18°C (approximately 200% increase over the same 10 day period). As these grains grow, they produce metabolites including lactic acid which lowers the pH of the milk from approximately 7.0 to as low as 4.5, depending on the incubation temperature. This lactic acid has a preservative effect on the Kefir. Concurrent with this pH drop is a titratable acidity (TA) increase from approximately 0.18% to as high as 1.35%, also dependent on incubation temperature. For optimum acid production (pH 4.4 - 4.6; TA 1.0 - 1.15%) an incubation temperature of 25°C and inoculum concentration of 2% appears to be preferable to higher (30°C) or lower (18° and 22°C) incubation temperatures and lower starter grain concentration combinations (0.8%).

#### Introduction

Kefir is a fermented dairy beverage that is produced by the incubation of Kefir grains in fresh milk. As these grains grow, they produce various metabolites with the major end-products being lactic acid (0.8 - 1.5%), which serves to lower the pH of the resultant Kefir, and ethanol ( $\pm$  1.0%), which together with CO<sub>2</sub> (0.025 - 0.073%) (Korovkina *et al.*, 1978) gives Kefir its characteristic flavour and "sharp bite" (Koroleva, 1982; Koroleva 1988). The lowered pH of the product lengthens its shelf-life and ensures that the milk coagulates to form a smooth, creamy product (Kneifel & Mayer, 1991; Rasic, 1987).

Kefir grains are small grains consisting of various yeast and bacterial species in a symbiotic relationship that are sensitive to growth parameters such as incubation temperature and grain inoculum concentration (Kosikowski, 1982; Marshall, 1984a; Roginski, 1988). Very little information is available on Kefir grains biomass increase, although the modern production of grains is based on continuous cultivation in milk, resulting in an increase in biomass of 5 - 7% per day (Libudzisz & Piatkiewicz, 1990).

In terms of the optimum inoculum concentration for good quality Kefir, the literature shows no agreement or even correlation, and no standard production method is available. Some authors believe that the Kefir grain to milk ratio should be maintained at 1:10, and then incubated at  $20^{\circ} \pm 1^{\circ}\text{C}$  for 24 h (Libudzisz & Piatkiewicz, 1990). Other authors cite optimum concentrations of inoculated grains to milk of up to 5% (w/v) with an incubation temperature of  $22^{\circ} - 25^{\circ}\text{C}$  for 18 - 24 h (Kneifel & Mayer, 1991; Merin & Rosenthal, 1986), or even for as short a period as 8 - 12 h (Mann, 1989). Roginski (1988) maintains that for traditional Kefir manufacture, a 0.5 - 10% inoculum concentration of Kefir grains must be added to heat-treated milk and incubated at  $20^{\circ} - 23^{\circ}\text{C}$  for 1 - 3 d. Marshall (1984b) in contrast, recommends 200 g (wet weight) Kefir grains per litre of milk as the necessary inoculation concentration, although concentrations of 1:20 to 1:50 grains per volume of milk incubated at  $18^{\circ} - 22^{\circ}\text{C}$  also appear to prove satisfactory in terms of Kefir quality (Rasic, 1987).

A report from Russia on the effect of milk fermentation temperature on the biochemical properties and consistency of Kefir showed that  $\text{CO}_2$  production, viscosity and acidity all increased with incubation temperature in the range  $19^{\circ} - 28^{\circ}\text{C}$  with an inoculum concentration of 5% (Korovkina *et al.*, 1978). In a study using UHT milk, a sharp decrease in pH (paired with a sharp increase in titratable acidity) was noted for the first nine hours of incubation (at  $23^{\circ}\text{C}$ ), followed by a gradual decrease in pH to a steady state. In this study, 1 and 3% milk fat samples were compared with regard to acid production for up to 48 h. The acid production was found to be almost identical in both instances (Merin & Rosenthal, 1986).

With all these discrepancies and large inoculum concentration ranges, as well as the fact that Kefir is not usually manufactured in an aseptic environment, it is not surprising that Kefir quality differs so widely and is assumed to be relatively unstable. Any change in fermentation conditions, as well as the possible contamination of Kefir

grains with extraneous microorganisms, will result in an altered microbiological consortium with a resultant shift in the metabolic pathways, and thus a change in the type of, as well as concentration of the characteristic Kefir metabolites (Duitschaeffer, 1989; Koroleva, 1982; Kroger, 1993; Mann, 1979; Marshall, 1984a; Rasic, 1987).

This study was undertaken to investigate the influence of incubation temperature and period and initial Kefir grain inoculum concentration on biomass and acidity increase over time in order to determine the optimal conditions for Kefir grain growth and acid production.

## **Material and methods**

### *Kefir grain activation*

Frozen Kefir grains were obtained from the ARC-Animal Nutrition and Animal Products Institute, Irene, Pretoria, South Africa (Dr J.F. Mostert, 1996, Personal Communication). These grains were originally from various South African households, and were without specifications of characteristics or composition. Prior to aseptic inoculation into fresh pasteurised milk, these grains were defrosted at ambient temperature and the container was then placed in a temperature-controlled waterbath (22°C) for 24 h. The grains were retrieved using a stainless steel sieve sterilised in a *Milton* solution [1.25% (v/v)] for 30 min and then rinsed using sterile distilled water. These grains were then re-inoculated into locally purchased pasteurised skim milk that was re-pasteurised at 80°C for 10 min in order to ensure a good and consistent microbiological quality. This process was repeated three times over a period of three days in order to activate the grains fully.

### *Analyses*

TA was determined using the method described by Dixon (1973). In this method, a 9 ml bulb pipet is used to remove the coagulated sample and the contents allowed to drain into an Erlenmeyer flask. The pipet is then kept in the flask as the phenolphthalein indicator is added. The contents of the flask are then titrated against NaOH [0.1 M] until the first noticeable pink colour is detected. The pink liquid in the Erlenmeyer is then re-introduced into the pipette and left to drain under gravity in order to remove all traces of the initial coagulum. The colour of the contents of the

Erlenmeyer flask then return to colourless, and further titration is necessary to again reach the titration end point. The pH was determined using a *Knick Calimatic Portamess 75*.

### *Experimental design*

The study was undertaken in four major sections and the experimental design has been summarised in Table 1. The first Experimental Study (I) was an investigation into biomass and titratable acidity (TA) increases and pH decreases over the entire fermentation process as obtained at the end of each fermentation period at different incubation temperatures. Active Kefir grains (2.0 g) were aseptically inoculated into each of eight 500 ml bottles containing 250 ml freshly pasteurised skim milk. Uninoculated duplicate pasteurised milk samples were used as controls. Duplicate containers were placed in each of four constant temperature waterbaths (18°, 22°, 25° or 30°C) and the fermentation was allowed to proceed for 24 h. The average pH and TA values for each incubation temperature were then determined and the grains retrieved and thoroughly rinsed with sterile distilled water. This water was allowed to drain from the grains for 30 min, after which time the grains were weighed and an average weight for each incubation temperature was obtained from the duplicate samples. The weighed Kefir grains were quantitatively re-inoculated into fresh pasteurised milk, and the process repeated for 10 consecutive days.

In the second Experimental Study (II), the pH decreases and titratable acidity increases during each fermentation process at different incubation temperatures were investigated. This study was undertaken to provide a more detailed profile of the entire Kefir fermentation process. For this, six 500 ml containers, each with 500 ml pasteurised skim milk, were inoculated with 4.0 g, and a further six inoculated with 10.0 g grains. Two containers of milk with each inoculum concentration were incubated at each of 22°, 25° and 30°C, in order to obtain duplicate pH and TA values for each temperature. The pH and TA were measured at two-hourly intervals for a duration of 24 h, with uninoculated pasteurised milk serving as the 0 h control. In this study, fresh pasteurised milk (18 ml) was added to each fermentation vessel after each two-hourly TA measurement. This was necessary to replace the volume

**Table 1.** Summary of Experimental Design.

Study	Inoculum Concentration	Incubation Temperature	Parameters Investigated	Monitoring Intervals & Duration	Additional Comments
I	2 g / 500 ml skim milk	18, 22, 25 & 30°C	Biomass pH TA	24 h for 10 d	Grains quantitatively re-inoculated into fresh milk daily
II	4 g & 10 g / 500 ml skim milk	22, 25 & 30°C	pH TA	2 h for 24 h	Fresh milk supplemented for removed Kefir (18 ml / day)
III	8 g & 20 g / 1 l skim milk	22, 25 & 30°C	pH TA	0, 10, 18, 24, 28, 32 & 42 h	No fresh milk addition
IV	20 g & 140 g / 1 l skim milk	25°C	pH TA	0, 10, 18, 24, 28, 32 & 42 h	No fresh milk addition
	8 g & 20 g / 1 l skim milk	30°C			

of substrate (Kefir) removed in order to maintain the original volume and thus inoculum concentration of each vessel.

Experimental Study III was undertaken to obtain a more accurate profile of the fermentation process at different incubation temperatures. For this, an extended fermentation period was necessary to ensure that the fermentation was complete for all the investigated samples. Larger original substrate volumes were also used and fewer analyses performed during the fermentation period in order to minimise substrate losses and thus eliminate the need for fresh milk supplementation. In this section, the volume of substrate was increased from 500 ml to 1 l with the relative inoculum concentrations maintained (six samples with 8.0 g and six with 20.0 g of active Kefir grains). Duplicate samples of skim milk containing each of the three Kefir grain inoculum concentrations were incubated at the same temperatures as before (22°, 25° and 30°C). Uninoculated pasteurised skim milk again served as the 0 h standard. In this study pH and TA were measured at 0 h (uninoculated pasteurised milk), 10, 18, 24, 28, 32 and 42 h of incubation.

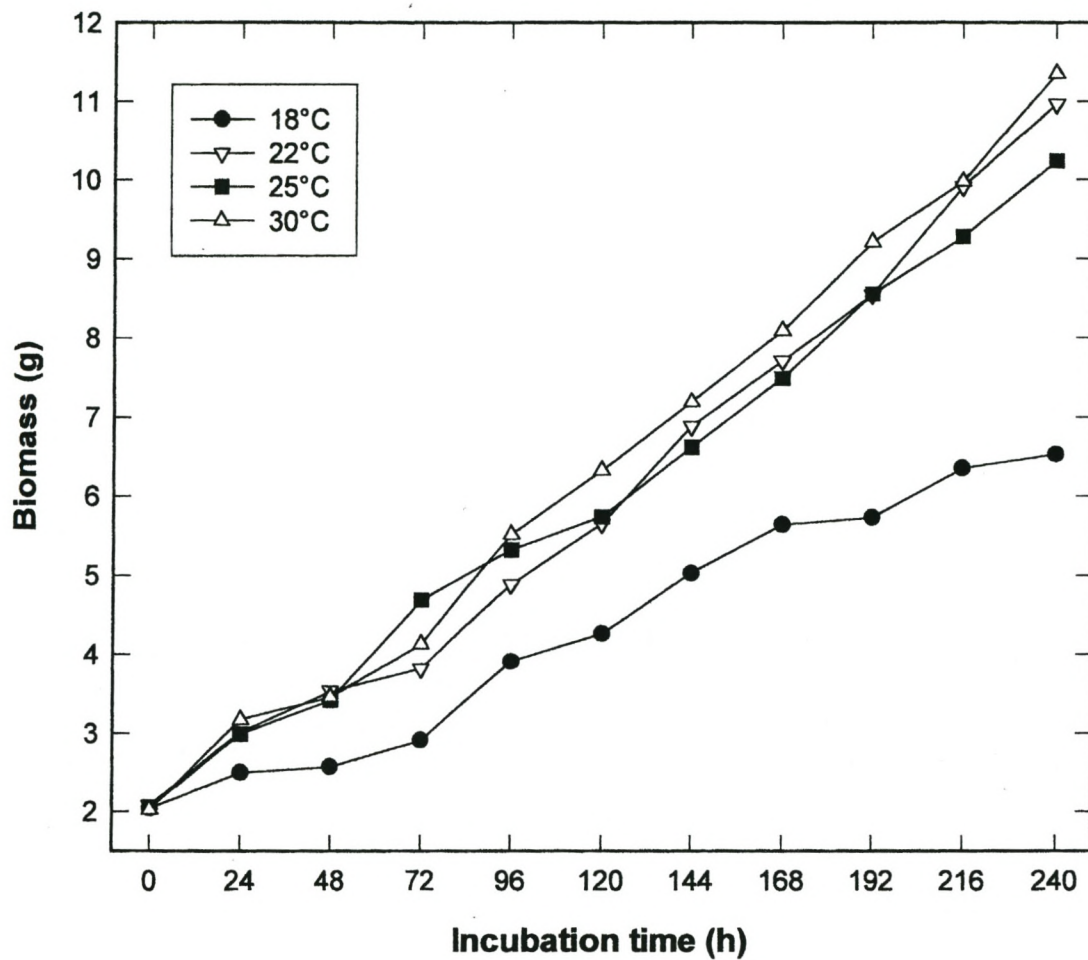
In the fourth study (IV), an investigation into the effect of very large inoculum concentrations on growth profiles was undertaken. This was necessary to investigate the validity of literature references citing optimum inoculum concentrations of 100 - 200 g Kefir grains per litre of milk (Marshall, 1984b; Roginski, 1988). Due to the fact that an excessive amount of Kefir grains would be necessary as inoculum if duplicate fermentations at each temperature (22°, 25° and 30°C) were to be carried out, it was decided to investigate this elevated inoculum concentration at only one incubation temperature. For this study, duplicate samples containing both a large (140 g) and smaller (20 g) grain inoculum concentration per litre of skim milk were incubated at 25°C. Alongside these samples were another two sets of duplicate samples with either 8 g or 20 g of Kefir grains that were incubated at 30°C. These samples containing lower inoculum concentrations were necessary to act as reference points for the new data. The measurements and measurement intervals used were the same as for study III.

## Results and discussion

### *Experimental study I*

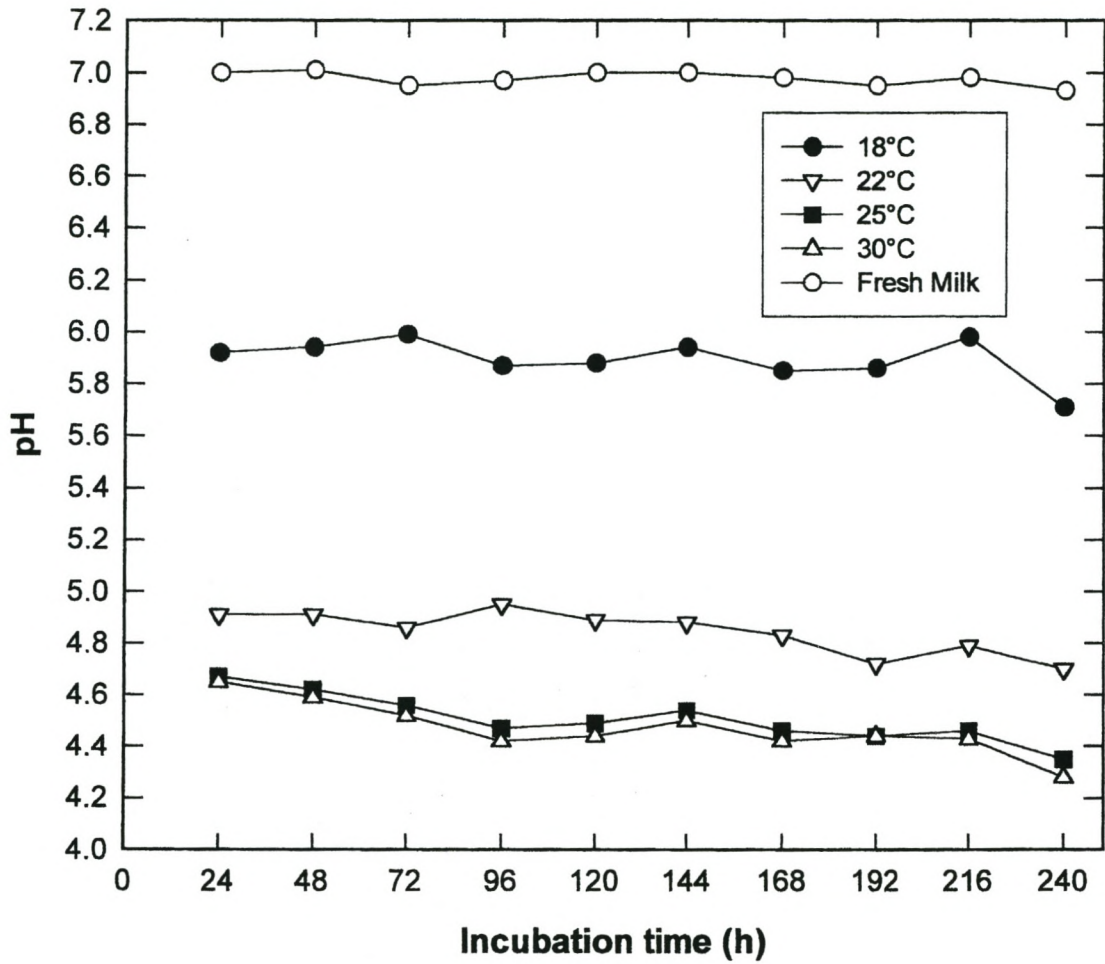
The increases in Kefir biomass over the 10 d period at four different incubation temperatures in skim milk are illustrated in Fig. 1. It is evident that the Kefir grain mass increased significantly for all the incubation temperatures, however it is clear that incubation at 18°C resulted in a much slower biomass increase. This is consistent with the observation that metabolic processes, and thus biomass increases, normally occur faster at elevated temperatures up to a specific maximum temperature (Jay, 1992). Incubation of the Kefir grains at 22°, 25° and 30°C yielded fairly similar biomass increases, with increases of up to 9.5 g in 10 d, which equates to a 500% increase in mass from day one to day 10. This is similar to reported values of biomass increases of 5 - 7% per day in a continuous grain cultivation system (Libudzisz & Piatkiewicz, 1990). These biomass increases could possibly have been larger if the grains were not rinsed daily, as rinsing of grains may also limit biomass increases and Kefir activation levels slightly due to the loss of surface bacteria (Koroleva & Bavina, 1970). For the purposes of this study, the grains were washed so that weight of grains was not affected by Kefir coagulum attached to the grains.

The changes in the pH of skim milk inoculated with the Kefir grains and incubated at 18°, 22°, 25° and 30°C for the 10 d period are illustrated in Fig. 2. The pH of the fresh pasteurised milk (control at  $t_0$ ) was found to be constant at a value of approximately 7.0 over the 10 d period. The plots of pH for each incubation temperature show a fairly similar consistency in final pH values, although a slight daily lowering of pH values was noted for each incubation temperature over the 10 d period. This was probably due to the fact that with each successive inoculation, the inoculum concentration was increased ( $\pm 5\%$  per day) in comparison to the previous day due to the daily biomass increase, as well as the fact that the grains became more active through repeated propagation (Kroger, 1993). The data also shows that the rate of acidification (pH decrease) was strongly influenced by incubation temperature. Incubation at 18°C for each 24 h period yielded a product with a final pH value in the region of of 5.9 which was not sufficient to conform to the prescribed description of Kefir, which should have a final pH value of between 4.0 and 4.6



**Figure 1.** Influence of incubation temperature on kefir grain biomass increase in skimmed milk.



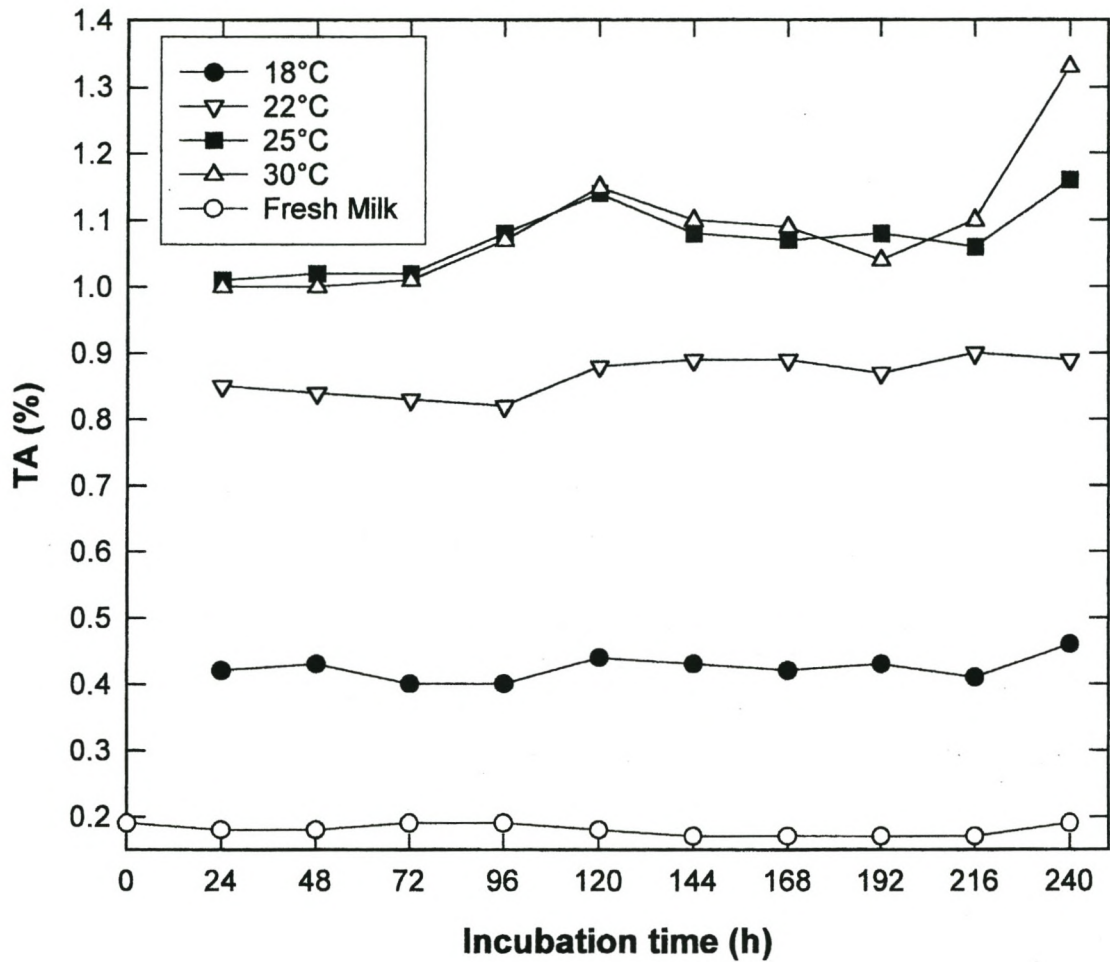


**Figure 2.** The influence of incubation temperature on pH in skimmed milk.

(Duitschaeffer, 1989; Marshall, 1982). It was also noted that the Kefir did not coagulate in all instances when incubated at 18°C (coagulation occurs in the pH range 5.4 - 5.5). In the case of incubation at 22°C, the final pH values were sufficient to allow coagulation (4.8 - 5.0), although the pH values were again too high for true Kefir (Duitschaeffer, 1989; Marshall, 1982). The pH values obtained after incubation at 25° and 30°C yielded similar results (values below pH 4.6), which conform to the requirement that the final pH of Kefir should be in the range of 4.0 - 4.6 (Duitschaeffer, 1989; Marshall, 1982).

The change in titratable acidity (TA) of Kefir over the same 10 d period for the four incubation temperatures (18°, 22°, 25° and 30°C) and for fresh pasteurised milk (control) is illustrated in Fig. 3. As was found in the pH studies (Fig. 2), the TA of the control remained constant (between 0.15 - 0.2%) during the study. According to the literature, desirable TA values for good quality Kefir are between 0.8 and 1.0% (Kosikowski, 1982; Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983; Vedamuthu, 1982), and acidity levels may not be lower than 0.6% per weight expressed as lactic acid (IDF, 1992). As per the findings for pH, it is evident that incubation at 18°C did not result in sufficient acidification of the Kefir (TA level of 0.4 - 0.5%) to produce a high quality product, nor to promote coagulation (coagulation to occurs in the TA range 0.52 - 0.55%). The final TA values for incubation at 22°C were in the desired range resulting in adequate coagulation, while values for incubation at 25° and 30°C yielded very similar results (as was observed with the final pH values). These TA values were all in the range 1.00 – 1.15 %, except the final TA value (240 h) for incubation at 30°C which reached a level of approximately 1.35%. These TA values exceed the desirable values slightly, especially towards the end of the study where increased inoculum concentration (due to biomass increase), as well as the higher level of Kefir grain activation (due to prolonged propagation) had an effect (Kroger, 1993). It is therefore apparent that inoculum concentration also has a direct influence on TA increase, which is in accordance with the findings for pH (Fig. 2).

Due to the lowered activity of Kefir grains and the inferior quality of the product obtained with incubation at 18°C, it was decided to omit incubation at 18°C in the subsequent studies.



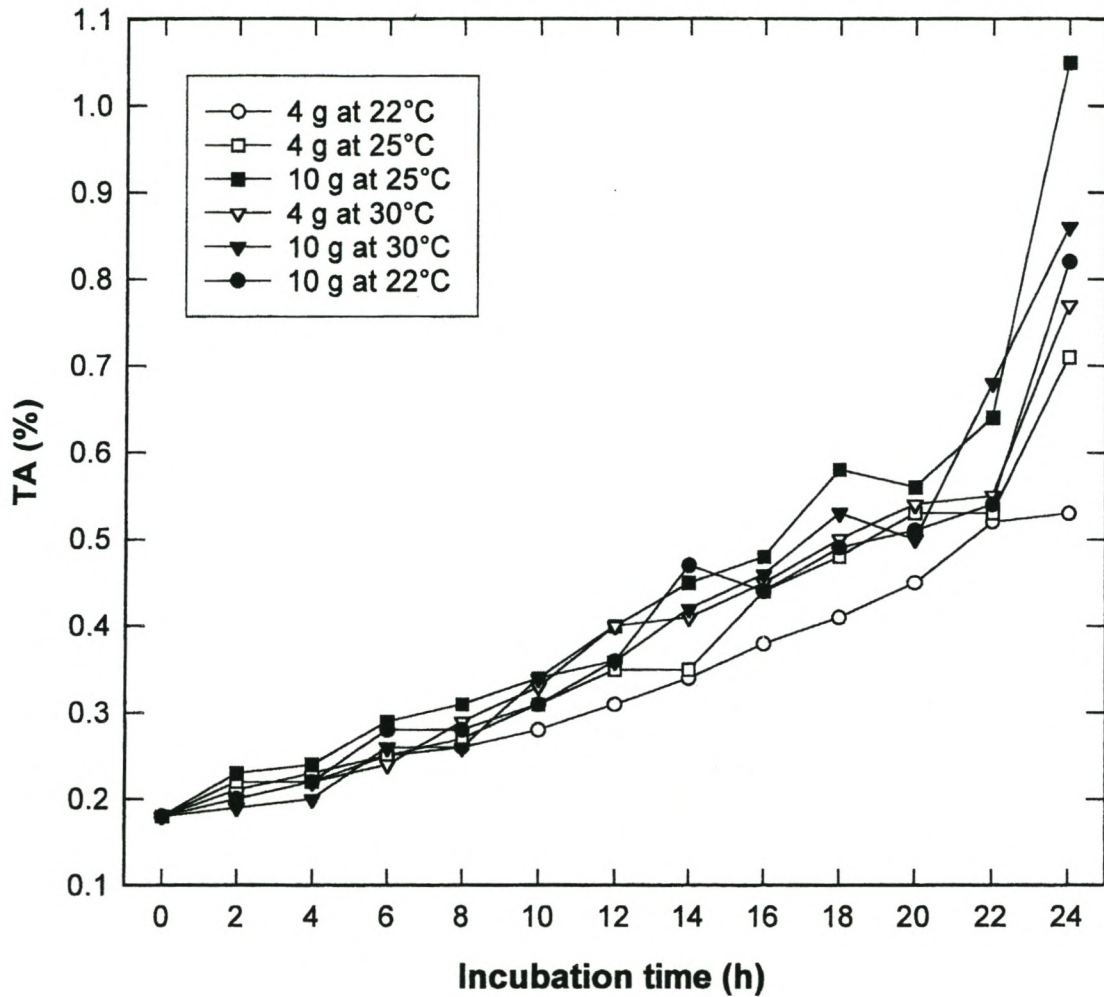
**Figure 3.** Influence of incubation temperature on titratable acidity in skimmed milk.

### *Experimental study II*

The average of the duplicate TA values over the 24 h incubation period at 22°, 25° and 30°C for both the 4 g and 10 g Kefir grain inoculum concentrations are shown in Fig. 4. The TA profiles for each sample were similar for the first 12 h of incubation, with the 4 g inoculum usually slightly lower. After the first 12 h, the samples containing the higher inoculum concentrations (10 g grains) showed an increased acid production in comparison to those with the lower grain inoculum concentrations. Incubation of 10 g of Kefir grains at 25°C resulted in the fastest and highest acid production, followed by the sample containing 10 g grains incubated at 30°C and then the 10 g grain sample incubated at 22°C. TA increases were lowest for the 4 g grain inoculum incubated at the lowest temperature (22°C). It therefore appears that, as before, acid production is proportional to inoculum concentration and to a lesser extent incubation temperature, with incubation of 10 g of Kefir grains at 25°C yielding the highest acid production.

Most of the samples in this study did, however, conform to the requirements of good quality Kefir (TA of 0.8 - 1.0%) (Kosikowski, 1982; Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983; Vedamuthu, 1982), with the exception of the sample containing 4 g grains incubated at 22°C which had a final TA of about 0.5%. This TA value is not high enough to permit the name Kefir for the product (IDF, 1992). The highest final TA value ( $\pm 1.06\%$ ), obtained for the sample with 10 g Kefir grains incubated at 25°C, is slightly higher than the cited optimum of 1.0%, but is still considered acceptable (Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983).

The data also shows that the largest increase in acidity occurs after 22 h of incubation. The final TA profiles are quite similar to the TA profiles after 24 h in the first experimental study (I), suggesting that the highest TA was reached within 24 h and maintained at that value. One difference is that the TA values obtained in this study (II) after 24 h of fermentation are lower than those observed in Experimental Study I, although the original grain inoculum concentrations were higher. This is probably due to the repeated two-hourly removal of the fermentation vessels from the constant-temperature incubators for sampling purposes, paired with the replacement of an equal volume of sampled Kefir with fresh, unfermented milk. This is an aspect that must be taken into consideration when continuous cultivation of Kefir grains is undertaken in future studies.



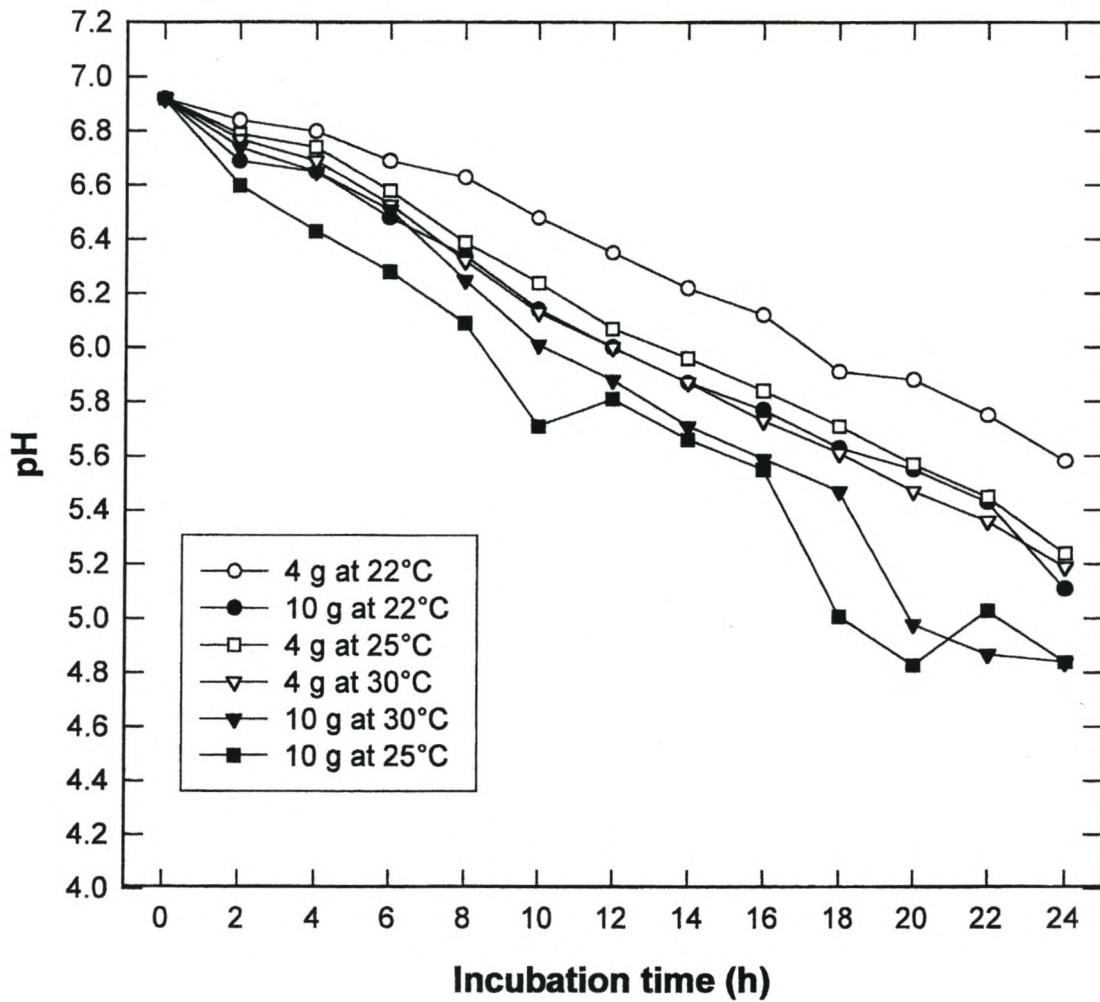
**Figure 4.** Influence of kefir grain inoculum concentration and incubation temperature on titratable acidity in 500 ml skimmed milk.

In this study, a similar trend is noted for pH changes for the same samples over the same incubation period (Fig. 5). The lowest pH values are obtained for the samples with the higher inoculum concentrations (10 g) that were incubated at the higher temperatures (25° and 30°C). As with the TA values, acidification was fastest in the sample containing 10 g grains per 500 ml milk incubated at 25°C, with the 10 g sample incubated at 30°C yielding very similar results. The slowest acidification was again found in the sample containing 4 g Kefir grains incubated at 22°C. The pH profiles over the 24 h period are all very similar for the remaining samples. This emphasises the fact that acid production is strongly influenced by the initial inoculum concentration as well as incubation temperature.

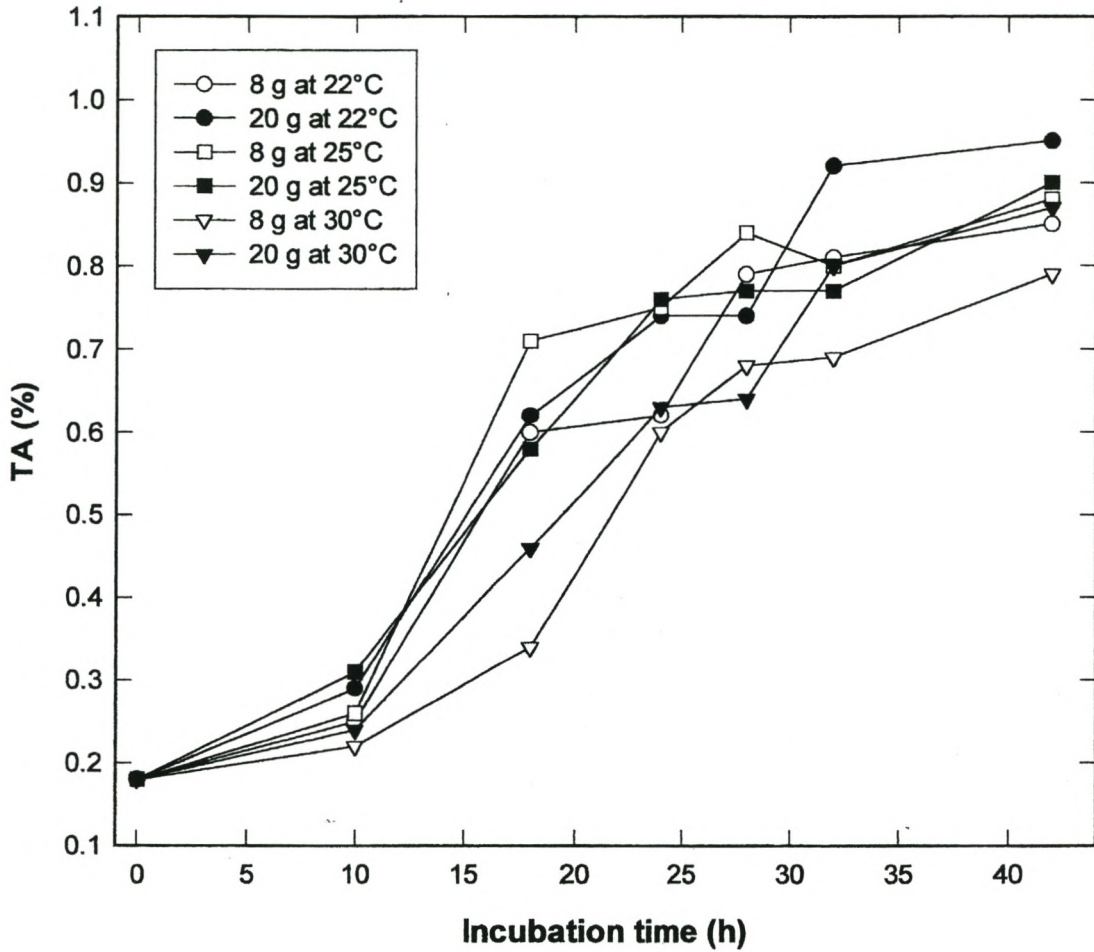
The final pH's of each sample in this study were sufficiently low to effect coagulation in all but the low temperature, low inoculum concentration sample (4 g grains incubated at 22°C), however, none of the samples had a final pH that was low (4.0 - 4.6) enough to provide a good quality product, as recommended by Duitschaever (1989) and Marshall (1982). These slightly elevated final pH values in comparison to those obtained in Experimental Study I can once again be attributed to the interrupted fermentation due to continual removal of the fermenting samples and the addition of fresh milk. It is also possible that the continuous presence of the grains at a low pH led to a slower acid production.

### *Experimental Study III*

In this section the milk and Kefir grain volumes were increased and the fermentation period extended. The relative grain to milk concentrations were kept the same as in Experimental Study II, thus 8 g and 20 g Kefir grains were inoculated into 1 l skim milk and incubated at the same temperatures as before: 22°, 25° and 30°C. In this study there was no substitution of fresh milk for removed Kefir. The averages of the duplicate TA values obtained over the 42 h incubation period are shown in Fig. 6. From the data, it is apparent that even after a prolonged fermentation (42 h) with a larger milk volume, the TA of Kefir did not exceed 1.0 %. All the samples, with the exception of one (8 g grains incubated at 30°C), did, however, reach the minimum recommended TA level of 0.8 % for quality Kefir during the 42 h incubation period (Kosikowski, 1982; Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983; Vedamuthu, 1982). Most of the samples reached the acceptable lower



**Figure 5.** Influence of inoculum concentration and incubation temperature on pH in 500 ml skimmed milk.



**Figure 6.** Influence of inoculum concentration and incubation temperature on titratable acidity in 1 litre skimmed milk.

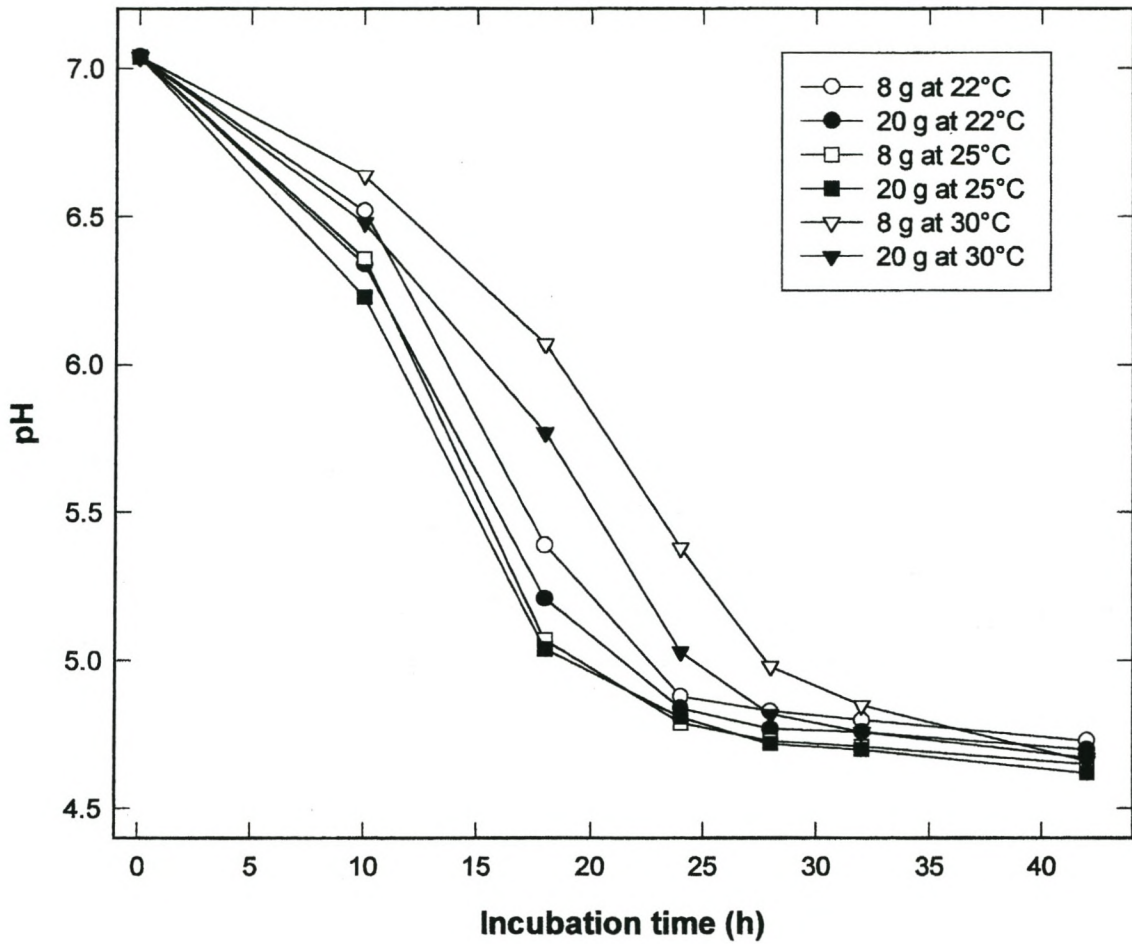


TA level in a far shorter period, with the 8 g/25°C sample reaching this level soonest (after approximately 26 h). All the samples, with the one exception, had fairly similar TA increase profiles until 30 h of incubation. After 30 h of fermentation, the sample containing 20 g of Kefir grains incubated at 22°C had a TA value that was higher than the values of the other samples and remained at this level for the remainder of the fermentation period. This highlights the fact that the profiles reaching the highest TA levels soonest also levelled off soonest whilst the others continued climbing steadily. This resulted in samples with final TA values that had a relatively small variation between them. It is also apparent that the slowest fermenting sample (8 g/30°C) had a TA profile that had not yet levelled off. It was thus concluded that acid production was still occurring in this sample at a reasonable rate at the 42 h period.

It was concluded from this study that higher inoculum concentrations (20 g) resulted in higher final acidity levels after a prolonged fermentation (42 h), with the highest acid production favoured in the 22° - 25°C region. It therefore appears that acid production is inhibited to some extent at very high temperatures (30°C) and low inoculum concentrations. The relationship between inoculation concentration and TA increase is in accordance with the findings of Experimental Study IIa, however, in that study, TA increases were also proportional to incubation temperatures up to 30°C, whereas in this study inhibition appears to occur at this elevated temperature.

The fact that all the samples with the exception of the 8 g/30°C sample, reached an acceptable TA level for good quality Kefir is in agreement with the conclusion that excessive handling of the fermentation vessels and repeated removal from the incubator resulted in inadequate acid production and thus inadequate coagulation in Experimental Study II.

The corresponding graph showing the pH changes over time for the same period is shown in Fig. 7. In this instance, all the samples have similar pH profiles and it appears that all the incubated samples have final pH values in a very narrow range, between 4.60 and 4.72. As the prescribed pH maximum for good quality Kefir is 4.6 (Kosikowski, 1982; Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983; Vedamuthu, 1982), only one sample (20 g/25°C) conforms to the true definition of good quality Kefir. This sample also had the fastest acid production. In accordance with the findings for TA (Fig. 6), the slowest acid production as measured by pH was observed in the 8 g/30°C sample.



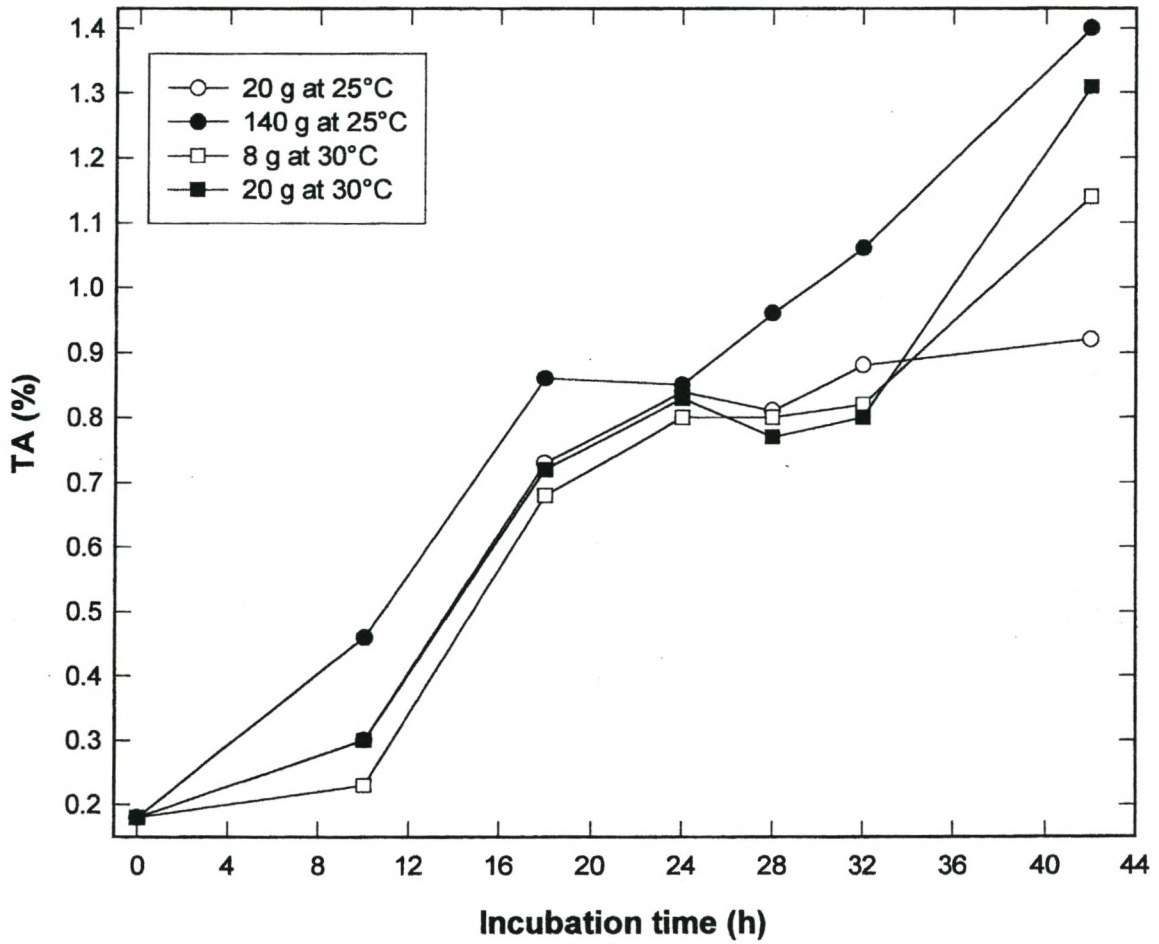
**Figure 7.** Influence of inoculum concentration and incubation temperature on pH in 1 litre skimmed milk.

The data from this study shows that acid production is more related to incubation temperature than inoculum concentration, with a temperature of 25°C being optimal (fastest acid production), while incubation at 30°C resulted in the slowest acid production. The possible inhibitive effect at the elevated temperature (30°C) may be due to the presence of predominantly mesophilic organisms in the dynamic microbial consortium of the grains. Incubation of both inoculum concentrations of grains at 22°C resulted in pH curves between these two extremes. Incubation temperature thus plays the primary role in determining the speed of acid production, whilst inoculum concentration has a secondary role. This is evident in that for each temperature, the sample containing the lower inoculum concentration (8 g Kefir grains) has a slower acid production than the sample containing the higher inoculum concentration (20 g Kefir grains). This relationship between inoculum concentration and acid production is in accordance with the findings for TA values in as illustrated in Fig. 6.

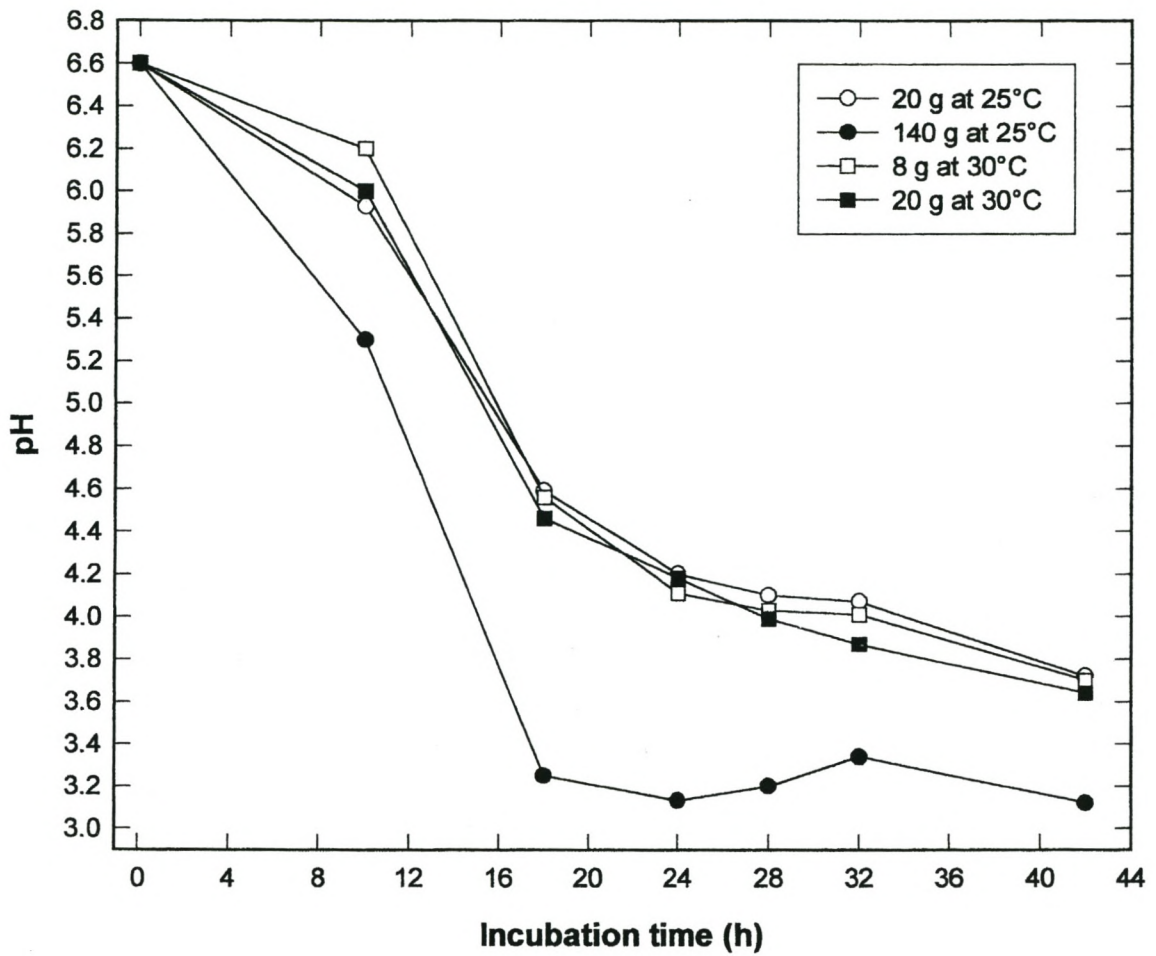
#### Experimental Study IV

In this study, the effect of very large inoculum concentrations (140 g grains per litre milk) was studied. In Fig. 8 the TA values for the investigated samples over the 42 h incubation period are shown. In this instance, all the samples reached an acceptable TA level of at least 0.8% within the 24 h incubation period (Kosikowski, 1982; Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983; Vedamuthu, 1982), with the sample 140 g/25°C having the fastest acid production throughout the 42 h period. Also, all the samples investigated in this study were found to coagulate within 10 hours of incubation, irrespective of grain concentration or incubation temperature. The samples containing 20 g Kefir grains that were incubated at either 25°C or 30°C and the 140 g sample had TA profiles that were fairly similar in TA increase up until 24 hours of incubation.

The corresponding curves for pH changes over the incubation period can be seen in Fig. 9. In this case there is a clear distinction between the pH decrease for the sample containing 140 g Kefir grains and the other samples which all have pH values and curves in a very similar range at each measured time interval. The larger inoculum concentration (140 g) resulted in a very noticeable faster and lower pH drop over the period with a final value of 3.1 after 42 h. In all instances pH lowering was



**Figure 8.** Influence of inoculum concentration and incubation temperature on titratable acidity in 1 litre skimmed milk.



**Figure 9.** Influence of inoculum concentration and incubation temperature on pH in 1 litre skimmed milk.

accelerated until about 18 h of incubation, followed by a stabilisation phase as shown by the levelling-off of the pH curves. This levelling off is largest for the sample containing 140 g Kefir grains where further fermentation resulted in minimal pH decreases.

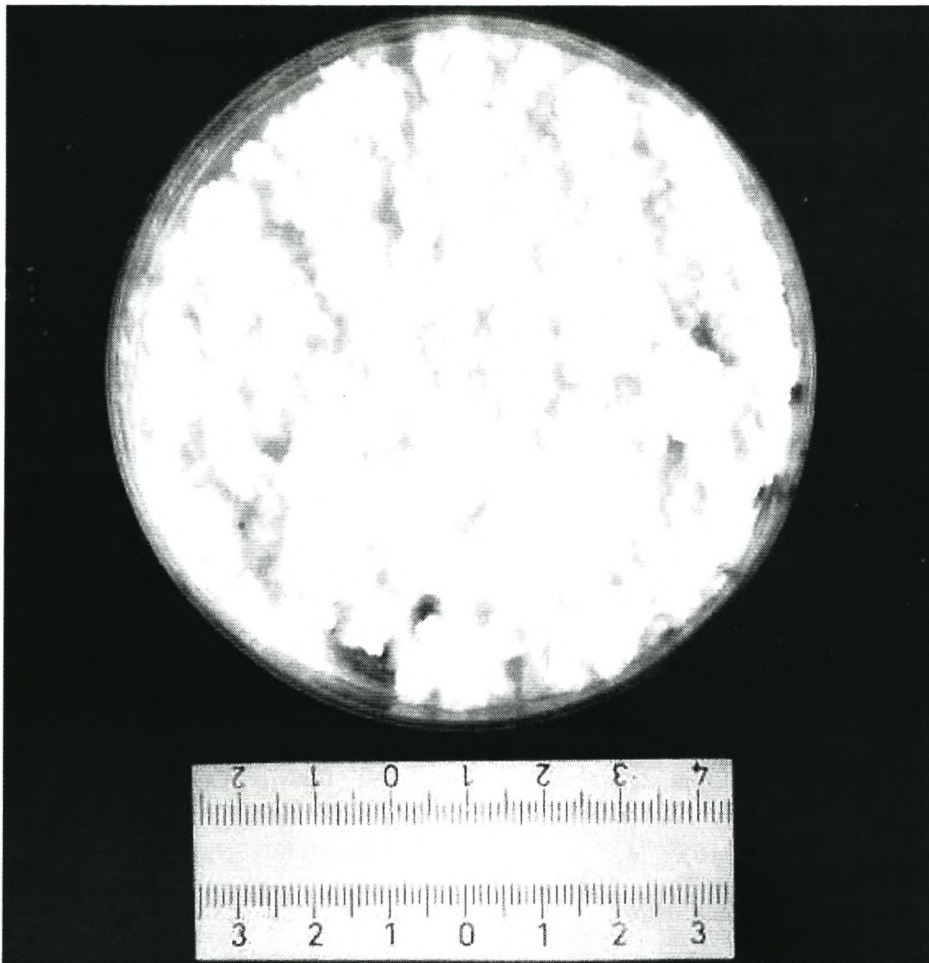
As regards the TA (Fig. 8), acid production in all the samples appeared to slow down after approximately 18 h of fermentation and resume again between 24 (in the 140 g/25°C) and 28 h of incubation (the remaining samples). One possible explanation for this sudden apparent change in acid production is that the repeated handling of the samples resulted in separation of the curds and whey. This separation would have influenced the TA results obtained, as different fractions of curds and whey would have been removed for measurement. This seems especially plausible if the TA curves are compared to the corresponding pH curves (no removal of substrate for measurements), which are far smoother, demonstrating the typical profile of an accelerated growth phase followed by a levelling off period. Another possibility is that this separation of the coagulum into curds and whey resulted in a shift in growth conditions for the grain microbial population, which resulted in slower metabolic processes and thus slower acid production. After the grains' population had acclimatised to the new substrate environment, they resumed active growth and acid production, resulting in the ensuing second accelerated TA increase phase. A further possible explanation for the second accelerated growth phase is that the less acid tolerant microbial population in Kefir grains are inhibited by the high acid content in the Kefir, allowing more acid tolerant microorganisms to become dominant. These acid tolerant microorganisms continue with their metabolic processes and thus further increase the acid concentration. This second accelerated growth phase appears to have affected the TA more than the pH, possibly due to the effect of buffers on pH measurement, although a slight dip in pH decrease is also evident over the corresponding time period (18 – 32 h). This also seems plausible in that the more favourable growth conditions (higher inoculum concentrations and higher incubation temperatures) result in a less severe lag phase with the ability of the grains to adapt easier and to grow faster than those with less favourable conditions. All these acid production lag phases are followed by an accelerated growth phase, except in the 20 g/25°C sample. In this instance, the TA profile remained levelled off for the rest of the incubation period. The curves for the samples that entered a

second accelerated growth phase had not yet levelled off for the second time after 44 h of incubation (even at TA levels of up to 1.4%).

As TA values greater than 1.0% are not conducive to good quality Kefir (Kosikowski, 1982; Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983; Vedamuthu, 1982), it does not appear beneficial to extend Kefir fermentation periods to more than 30 h with large inoculum concentrations, or 38 h for incubation of samples at 30°C. The 20 g/25°C sample had a TA in the acceptable quality range of 0.8 – 1.0% from approximately 24 h of incubation until the end of the trial (with a final TA of 0.92%). The prescribed pH range for good quality Kefir of 4.0 - 4.6 (Kosikowski, 1982; Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983; Vedamuthu, 1982) was reached by all the samples within a 20 h incubation period, with the sample containing 140 g of grains reaching this desired range within 14 h. Extended fermentation resulted in the pH falling below the desirable range after 28 h of incubation for all the samples except the one containing 140 g of grains that reached this level after only 16 h of incubation. It therefore appears that very high inoculum concentrations and lengthened incubation periods for fully activated Kefir grains result in a poor quality product.

The fact that these curves illustrate more active acid production than in the previous experiments is probably due to the fact that this study was conducted after all the other studies on growth were completed. Kefir grain activation was conducted using methods obtained from personal correspondences and from the available literature which cited 3 subsequent propagations as being adequate to fully activate the grains (Kroger, 1993; Vedamuthu, 1982). Re-activation was thus considered complete after repeated inoculation of the grains into fresh milk as explained earlier in the Methods section. This assumption may in fact not have been correct, with the grains becoming more and more active during all the trials with full activation only being realised at this stage of the trial.

Fig. 10 is a photograph of activated Kefir grains in a petri dish with a scale alongside illustrating the grain dimensions.



**Figure 10.** A photograph of active Kefir grains.



## Conclusions

Kefir grains can be activated and cultivated by repeated inoculation into fresh milk. Activation is an ongoing process, and from the data obtained in this study and from the literature, grains become more active with each subsequent fermentation process they undergo. The biomass increases that were obtained using this method of continuous grain cultivation are in the region of 5% per day, however this can be faster at higher temperatures (up to 30°C), or slower at lower temperatures (18°C). Rinsing of grains should be avoided in grain cultivation as this practise may limit biomass increases and Kefir activation levels slightly, as surface bacteria are lost through this process (Koroleva & Bavina, 1970).

Due to insufficient acid formation at an incubation temperature of 18°C, it was found that the resultant Kefir did not coagulate properly and did not have the desirable pH or TA values necessary for good quality Kefir. Also, biomass increase at this temperature was very low in comparison to higher temperatures, up to 30°C. Kefir grains should therefore not be incubated at 18°C or lower during Kefir grain cultivation, nor for actual Kefir production. At the higher end of the temperature scale (30°C), Kefir grain growth was found to be faster (Experimental study I), however Kefir quality is compromised due to rapid and excessive acidification (Experimental Studies II and III).

Inoculum concentration was also found to have a direct influence on acid production, with higher concentrations resulting in faster acidification at any specified temperature. At extremely high inoculum concentrations (140 g/litre milk, Experiment IV), the fermentation time for optimal acid production could be reduced to approximately 16 hours. Unfortunately, if extended fermentation is allowed to continue with such high inoculum concentrations, the quality of the resultant Kefir is compromised in that both the pH lower and TA upper maximum limits for good quality Kefir are exceeded. High inoculum concentrations did not however appear to have the inhibitory effect on grain growth and acid production that elevated incubation temperatures did.

It is recommended that care be taken to limit the excessive handling of the fermentation vessels as movement during coagulation may result in separation of Kefir into curds and whey. This separation results in an inferior product and makes

representative sampling difficult, which may impact on the accuracy of quality measurements (Experimental study IV). The removal of the fermentation vessels from the constant temperature incubators also needs to be limited due to the effect that change in environmental temperature can have on the fermentation process and thus the resultant Kefir quality.

The growth curves of Kefir grains were typical in a 24 h period, with an initial accelerated growth phase followed by a lag phase. It appears from Experiment IV that continuing incubation for 42 h results in Kefir grain adaptation to this high acid environment, with a subsequent second accelerated growth phase before inhibition for the second time. The resultant Kefir quality is however compromised due to excessive acid content.

From the overall study it is therefore apparent that the optimal conditions for Kefir grain growth are higher incubation temperatures (up to 30°C) and larger inoculum concentrations (Experimental study I). However, for optimal acidification and Kefir quality, it appears that moderate temperatures (22° and 25° C) and raised inoculum concentrations (at least 20 g per litre of milk) are beneficial. The fermentation period necessary for Kefir production at 25°C can be reduced from 24 – 32 h (20 g grains / litre milk) to 16 – 18 h if very large inoculum concentrations (140 g grains / litre milk) are used. Care must however be taken to ensure that fermentation does not proceed too far, as this will be detrimental to final product quality.

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## CHAPTER 4

### IMPACT OF ENVIRONMENTAL CONDITIONS ON METABOLITE PRODUCTION DURING KEFIR GRAIN FERMENTATION IN SKIM AND FULL-CREAM MILK

#### Summary

Various volatile flavour components including acetaldehyde, acetone, 2-butanone, ethanol and diacetyl are produced during the fermentation of Kefir grains in milk during the production of Kefir. The concentration of each of these compounds was studied in duplicate samples of both skim and full-cream milk containing different inoculum concentrations and incubation temperatures over a 24 h incubation period using headspace gas chromatography. The prepared samples contained either 4 g or 10 g of active Kefir grains that were incubated at either 22°, 25° or 30°C, with sampling occurring every six hours. All these compounds could be isolated, detected and quantified using an optimised headspace gas chromatography method, however, only acetaldehyde, ethanol and acetone were found to be major end-metabolites in the Kefir produced during this study (24 h). Diacetyl, an important flavour compound in Kefir, was produced during the fermentation but was metabolised with extended incubation.

#### Introduction

When Kefir grains are incubated in milk, microorganisms (streptococci, lactobacilli and yeasts) are shed from the grains into the milk where they continue to multiply (Marshall, 1982). The growth and metabolic processes of these microorganisms result in the production of lactic acid, flavour components, other metabolites and secondary structural changes in the milk (Kroger, 1993). The metabolites produced depend primarily on the microbial consortium of the grains and the fermentation conditions employed (Liu & Moon, 1983; Mann, 1979; Korovkina *et al.*, 1978; Kneifel & Mayer, 1991).

Two of the main metabolites formed in Kefir are lactic acid and ethanol. Lactic acid typically occurs at levels of 0.8 - 1.5%, although some authors cite lower maximum values of 0.9 - 1.0% (Marshall, 1982; Semenichina, 1984). Ethanol content is very variable (Marshall, 1984a), with typical levels of between 0.1 and 2.0% (Duitschaever, 1989; Kneifel & Mayer, 1991; Marshall, 1984b; Rasic, 1987), and an optimum level of about 1.0% (Marshall, 1984a; Kosikowski, 1982; Vedamuthu, 1982), however, one study cited typical Kefir ethanol content to be as low as 0.023% (Gawel & Gromadka, 1978). Another metabolite that may be present is acetic acid (Marshall, 1982), however this appears to be the exception rather than the rule.

During Kefir fermentation, other volatile compounds and small amounts of carbon dioxide are also produced (Merin & Rosenthal, 1986; Marshall, 1982; Marshall, 1984a; Rasic, 1987; Semenichina, 1984). Diacetyl is typically present at levels of approximately  $0.48 \text{ mg.l}^{-1}$  (Gawel & Gromadka, 1978; Kramkowska *et al.*, 1982) to  $3 \text{ mg.l}^{-1}$  (Marshall, 1982; Marshall, 1984a; Vedamuthu, 1982). Acetaldehyde has a central role in flavour metabolism, as acetoin, diacetyl and ethanol can all be produced from this compound (Marshall, 1982; Marshall 1984a). It is typically found in Kefir at levels of  $1 - 2 \text{ mg.l}^{-1}$  (Gawel & Gromadka, 1978; Marshall, 1982; Marshall, 1984b; Vedamuthu, 1982). Acetone (Gorner *et al.*, 1972; Marshall, 1984a; Ulberth & Kneifel, 1992; Vedamuthu, 1982) and small amounts of 2-butanone (Gorner *et al.*, 1972; Palo, 1971) are sometimes also present in Kefir, but play a less important role in Kefir quality and flavour (Blanc, 1984). Acetoin can be important in Kefir flavour, however the concentration is very variable and none is usually detected within the first 24 h of incubation (Rea *et al.*, 1996).

The concentrations of end-metabolites formed in Kefir can be regulated by several physical factors and by the proportion of various organisms in the starter culture. Incubation at a temperature of  $15^{\circ} - 22^{\circ}\text{C}$  yields a product high in acid and low in alcohol and carbon dioxide due to lactic acid fermentation and some yeast inhibition, whereas a product high in alcohol and carbon dioxide is obtained with incubation at  $4^{\circ} - 15^{\circ}\text{C}$  (Liu & Moon, 1983). Similarly, in other studies, the largest amount of ethanol was produced with incubation at  $10^{\circ}\text{C}$  (Mann, 1979), and carbon dioxide production, viscosity and acidity all increased with incubation temperature in the temperature range  $19^{\circ} - 28^{\circ}\text{C}$  (Korovkina, *et al.*, 1978). Similarly, inoculum

concentration had a direct influence on ethanol levels (Mann, 1979). Incubation of Kefir at 25°C is recommended to give maximum production of ethanol and volatile acids as well as good specific flavour and consistency (Liu & Moon, 1983; Korovkina *et al.*, 1978).

Numerous studies have been undertaken to determine the concentration of volatile flavour metabolites in Kefir and other dairy products. These have been based on chemical (Rea *et al.*, 1996) and enzymatic analyses (Alm, 1982), gas-liquid (Alm, 1982), direct gas (Palo & Ilkova, 1970), extract gas (Kang *et al.*, 1988) and headspace gas chromatography (GC) (Gorner *et al.*, 1972; Hild, 1980; Kang *et al.*, 1988; Monnet *et al.*, 1994). The direct (Palo & Ilkova, 1970) and headspace GC methods (Gorner *et al.*, 1972; Hild, 1980; Kang *et al.*, 1988; Monnet *et al.*, 1994) are the favoured methods as all the compounds can be evaluated simultaneously and no isolation of the individual compounds is necessary prior to measurement. The direct GC method is, however, not always accurate due to interactions between water and the other components in dairy systems (Monnet *et al.*, 1994). Headspace GC is effectively able to separate all the possible volatiles found in Kefir, including acetaldehyde, propionaldehyde, acetone, ethanol, isopropanol, 2-butanone, diacetyl, isobutanol and isoamyl alcohols, without these disruptive component interactions (Palo, 1971). Thus, of these methods, headspace GC yields the best results for the determination of the concentration of volatile components responsible for flavour in dairy products such as Kefir (Hild, 1980; Kang *et al.*, 1988).

This study was undertaken to investigate the influence of fat content, incubation temperature, length of incubation and initial Kefir grain inoculum concentration on volatile metabolite production during Kefir production.

## **Material and methods**

### *Headspace gas chromatography*

Separation of volatiles was accomplished using a Fisons 8 000 Series Gas Chromatograph (Fisons Instruments S.p.A., Milan, Italy) that was equipped with a flame ionisation detector with a split ratio of 100:1. Separation was effected using a 30 m DB5 capillary column with a bonded methyl-5% phenyl-silicone layer as stationary phase (film thickness 0.25 micron; Quadrex Corporation, Newhaven. C.T.).

Operating parameters were: injector temperature, 200°C; detector temperature, 150°C; helium carrier gas at a flow rate of 2 ml.min<sup>-1</sup>; hydrogen flow rate at 30 ml.min<sup>-1</sup> and air flow rate at 300 ml.min<sup>-1</sup>. The oven heating cycle was programmed at 45°C for 1 min, followed by a temperature increase of 5°C per min for 4 min.

#### *Internal standard and peak identification*

Analytical grade external standard stock solutions (1 000 mg.l<sup>-1</sup>) of all the volatile flavour compounds listed in the literature for Kefir were prepared, and included diacetyl (Sigma-Aldrich), acetaldehyde (Merck), acetoin (Merck), 2-butanone (Merck), ethanol (Univar Saarchem-Holpro Analytical) and acetone (Merck). These standards were used to optimise the chromatograph operational conditions so that each compound could be effectively separated and resolved, and the retention time of each compound obtained. The standard stock solution was prepared by mixing 2 ml of each of the prepared stock solutions together in a hermetic gas chromatography vial, placing the vial in a waterbath at 90°C for 1 h and then injecting 0.2 ml of the headspace into the chromatograph using a gas-tight heated syringe (95°C). The elution was allowed to continue for 60 min (maximum temperature of 220°C). The same 1 000 mg.l<sup>-1</sup> stock solutions were used for peak identification of Kefir volatiles obtained from different Kefir samples. For this identification, 10 ml prepared Kefir was placed in a hermetic gas chromatography vial and incubated in a waterbath (90°C) for 1 h. One ml of the headspace gas was injected into the chromatograph using a hermetic syringe warmed to 95°C, and the relevant peaks obtained. Five 9.5 ml Kefir samples from the same source were then spiked with 0.5 ml of each of the individual 1 000 mg.l<sup>-1</sup> stock solutions, and the same procedure followed as for the unspiked Kefir. The peak that increased in height was assumed to represent the compound that was used to spike the Kefir. Butanol (0.25 ml of a 1 000 mg.l<sup>-1</sup> solution per 9.75 ml Kefir) (Protea Laboratory Services) was chosen as the internal standard as it had a retention time similar to the other investigated volatiles and did not interfere with any of the other peaks. All the standards and the stock solutions were well secured and refrigerated (4°C) for the duration of the trial to ensure that none of the volatile components were lost.

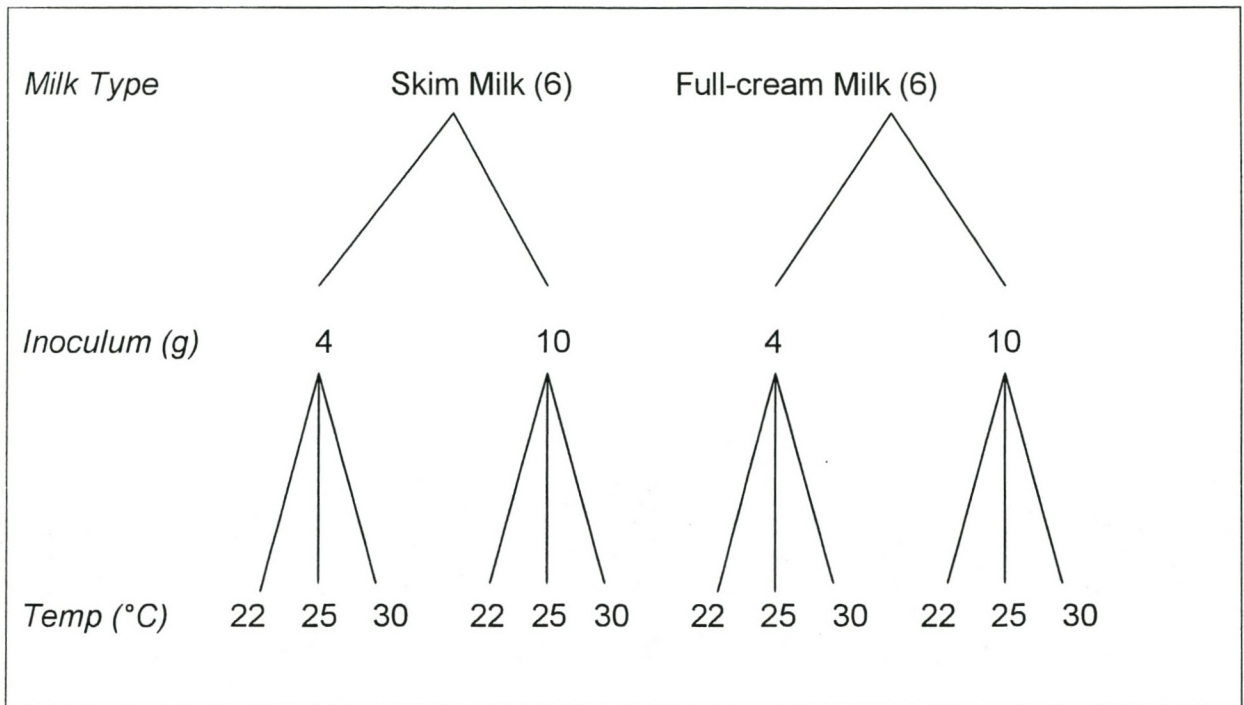


### *Kefir grain activation*

Frozen Kefir grains were obtained from the ARC-Animal Nutrition and Animal Products Institute, Irene, Pretoria, South Africa (Dr J.F. Mostert, 1996, Personal Communication). These grains were originally obtained from various South African households, and were without specifications of characteristics or composition. Prior to aseptic inoculation into fresh pasteurised milk, the grains were defrosted at ambient temperature and then placed in a temperature-controlled waterbath (22°C) for 24 h. The grains were retrieved using a stainless steel sieve sterilised in a *Milton* solution [1.25% (v/v)] for 30 min and then rinsed using sterile distilled water. The grains were then re-inoculated into a fresh skim milk sample using a sterilised stainless steel laboratory spoon-type spatula. This process was repeated three times over a period of three days in order to fully activate the grains. Locally purchased milk (although pasteurised) was re-pasteurised at 80°C for 10 min in order to ensure a good and consistent microbiological quality.

### *Kefir production and sampling*

As discussed in Chapter 3 of this thesis, there is little correlation in the literature regarding optimum inoculum concentrations and incubation temperatures for Kefir production. Commonly cited inoculum concentrations of between 0.5 – 10% and incubation temperatures of 22° - 30°C have been used as guidelines for this study (Kneifel & Mayer, 1991; Marshall, 1984b; Merin & Rosenthal, 1986). A diagrammatical summary of the testing set-up is illustrated in Fig. 1. Three 500 ml glass containers, each with 500 ml pasteurised skim milk, were inoculated with 4.0 g, and a further three with 10.0 g active Kefir grains. This set-up was repeated in a further six 500 ml containers that contained 500 ml pasteurised full-cream milk. A container of each milk type with each grain inoculum concentration was incubated at each of 22°, 25° and 30°C. Each of these containers was removed at six-hourly intervals for the duration of the trial (24 h) and placed in the refrigerator (4°C) for 30 min prior to sampling in order to ensure that no volatile components were lost. Duplicate samples of 9.75 ml of Kefir were aseptically removed from each container and placed in a 20 ml headspace vial containing 0.25 ml of the internal standard (butanol). Each vial was then hermetically sealed with a rubber-aluminium crimped cap and placed in a waterbath at 65°C for 30 min. One ml of headspace was



**Figure 1.** Schematic diagram of testing procedure for Kefir production.

removed from each vial using a heated (70°C) gas-tight syringe and injected into the gas chromatograph. Uninoculated pasteurised skim or full-cream milk served as the relevant zero hour control for the headspace gas chromatography analysis.

## Results and discussion

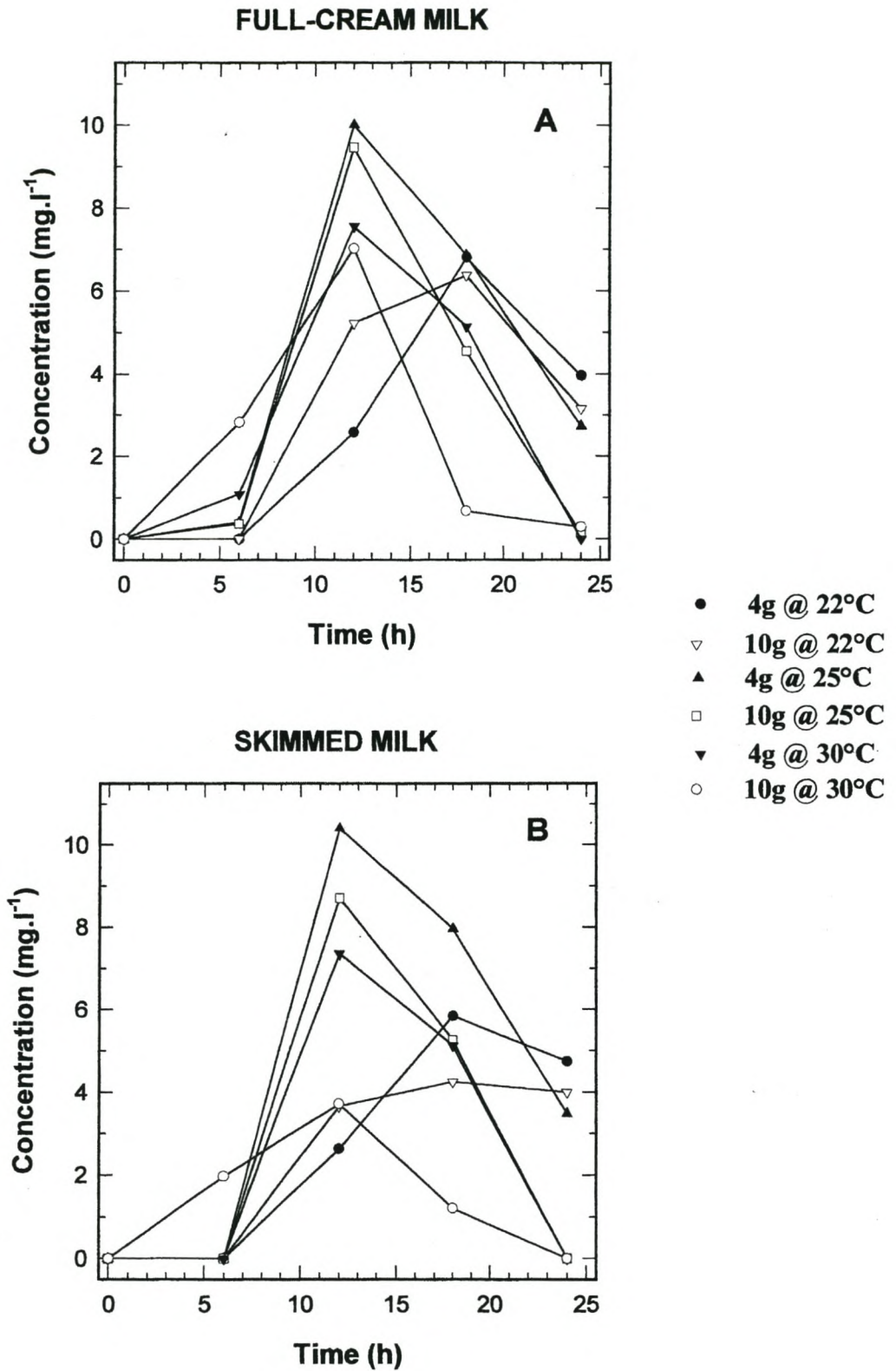
### *Identification of peaks*

The peaks obtained for the compounds tested all had excellent resolution and separation using the operational parameters discussed above. The compounds that were positively identified in the Kefir samples were acetaldehyde (boiling point (b.p.) 20.8°C; retention time (RT) 1.83 min.), acetone (b.p. 56.2°C; RT 1.92 min.), 2-butanone (b.p. 78.5°C; RT 2.00 min.), ethanol (b.p. 78.5°C; RT 2.36 min.) and diacetyl (b.p. 88°C; RT 2.42 min.). There was no elution of acetoin, so it was assumed that for the purposes of this study, acetoin was not an important Kefir metabolite. This has been a common finding by other researchers, with the concentration of acetoin being very variable and none usually detected within the first 24 h of incubation (Rea *et al.*, 1996). The internal standard (butanol; b.p. 117.5°C) had a RT of 2.95 min.

### *Metabolite production - acetaldehyde*

Acetaldehyde is a major flavour and aroma contributor in fermented milks. At low levels, the flavour is pleasant, but at higher levels it can be undesirable as becomes more green and "yoghurt" like (Vedamuth, 1982). It can be produced directly from lactose or from proteins through released amino acids during fermentation (Blanc, 1984). Acetaldehyde has a central role in flavour metabolism, as acetoin, diacetyl and ethanol can all be produced from this compound (Marshall, 1982; Marshall 1984a). It is typically found in Kefir at levels of 1 - 2 mg.l<sup>-1</sup> (Gawel & Gromadka, 1978; Marshall, 1982; Marshall, 1984b; Vedamuthu, 1982).

The acetaldehyde concentrations for the different incubation temperature/inoculum concentration combinations over the 24 h period for Kefir prepared from full-cream milk are illustrated in Fig. 2A, and the corresponding data for skim milk is shown in Fig. 2B. The maximum acetaldehyde concentration obtained for full-cream milk was 10.1 mg.l<sup>-1</sup>, and for skim milk 10.5 mg.l<sup>-1</sup>, both for the samples that contained inoculum concentrations of 4 g of Kefir grains that were



**Figure 2.** Acetaldehyde concentration changes for Kefir prepared with different inoculum concentrations and incubation temperatures.

incubated at 25°C for 12 h. It is apparent from these curves that acetaldehyde is not present in fresh full-cream or skim milk (acetaldehyde concentration at  $t=0$  is  $0 \text{ mg.l}^{-1}$  for both studies), but is produced by the Kefir grains during fermentation. It was noted from the data that the rate and maximum amount of acetaldehyde produced is directly related to the sample incubation temperature for each inoculum concentration. This is evidenced in the fact that the acetaldehyde concentration in both the skim and full-cream milk samples incubated at 25° and 30°C reach a maximum after only 12 h of incubation, whereas the samples incubated at 22°C reach a maximum only after 18 h of incubation. The maximum acetaldehyde concentrations are also higher for the samples incubated at higher temperatures (25° and 30°C) than the samples incubated at 22°C. Incubation at 25°C was the most favourable for acetaldehyde production, followed by incubation at 30°C. This influence of incubation temperature on acetaldehyde production is attributable to the fact that the main microorganisms responsible for acetaldehyde production are in the mesophilic to upper mesophilic range of microorganisms (Blanc, 1984). These organisms consist of *Lactobacillus bulgaricus*, *L. jugurti* and *Streptococcus thermophilus* (Blanc, 1984). Of these, *S. thermophilus* and *L. bulgaricus* are known to produce larger quantities of acetaldehyde, and lack the enzymes necessary for the metabolism of other flavour compounds (Marshall, 1982; Marshall, 1984a).

It was also evident that for each incubation temperature, the lower inoculum concentration (4 g) samples had higher acetaldehyde concentrations than the higher inoculum concentration (10 g) samples (Fig. 2). Acetaldehyde production, therefore, appears to be inversely related to inoculum concentration at a specified incubation temperature.

The acetaldehyde concentration peak maxima (Fig. 2A and 2B) for each incubation temperature/inoculum concentration combination were followed by a rapid decrease in acetaldehyde concentration over time. These decreases were especially noticeable in both the full-cream and skim milk samples that contained 10 g of grains incubated at 25°C, 4 g of grains incubated at 30°C and 10 g of grains incubated at 30°C. In all these samples, the final acetaldehyde concentration after 24 h of incubation, was below  $0.5 \text{ mg.l}^{-1}$ . This is below the cited average values in the literature of 1 - 2  $\text{mg.l}^{-1}$  (Marshall, 1982; Marshall, 1984b; Vedamuthu, 1982). All the other samples had final acetaldehyde concentrations in the range 3 - 5  $\text{mg.l}^{-1}$ , which

is slightly higher than the cited range, however still far lower than the values cited for UHT milk ( $73 \text{ mg.l}^{-1}$ ) that were obtained after an extended fermentation period of 48 h (Merin & Rosenthal, 1986).

The decrease in acetaldehyde concentration with further incubation is probably due to the metabolism of acetaldehyde to acetoin, diacetyl or ethanol (Marshall, 1982) by the numerous yeast species and *L. acidophilus* strain in Kefir grains. The yeast species are known to be able to produce acetaldehyde from pyruvate, however, they also possess an alcohol dehydrogenase that is used to convert acetaldehyde to alcohol (Marshall, 1984a). *Lactobacillus acidophilus* is known to convert pyruvate mainly to lactate (Marshall, 1982), however, this species is also capable of producing acetaldehyde from pyruvate (Marshall, 1984b). The acetaldehyde produced by *L. acidophilus* is, however, not secreted into the milk as strains of this species also possess alcohol dehydrogenase that can metabolise any produced acetaldehyde to ethanol. This results in an elevation of the ethanol level rather than the acetaldehyde level in the fermented milk product (Marshall, 1984b).

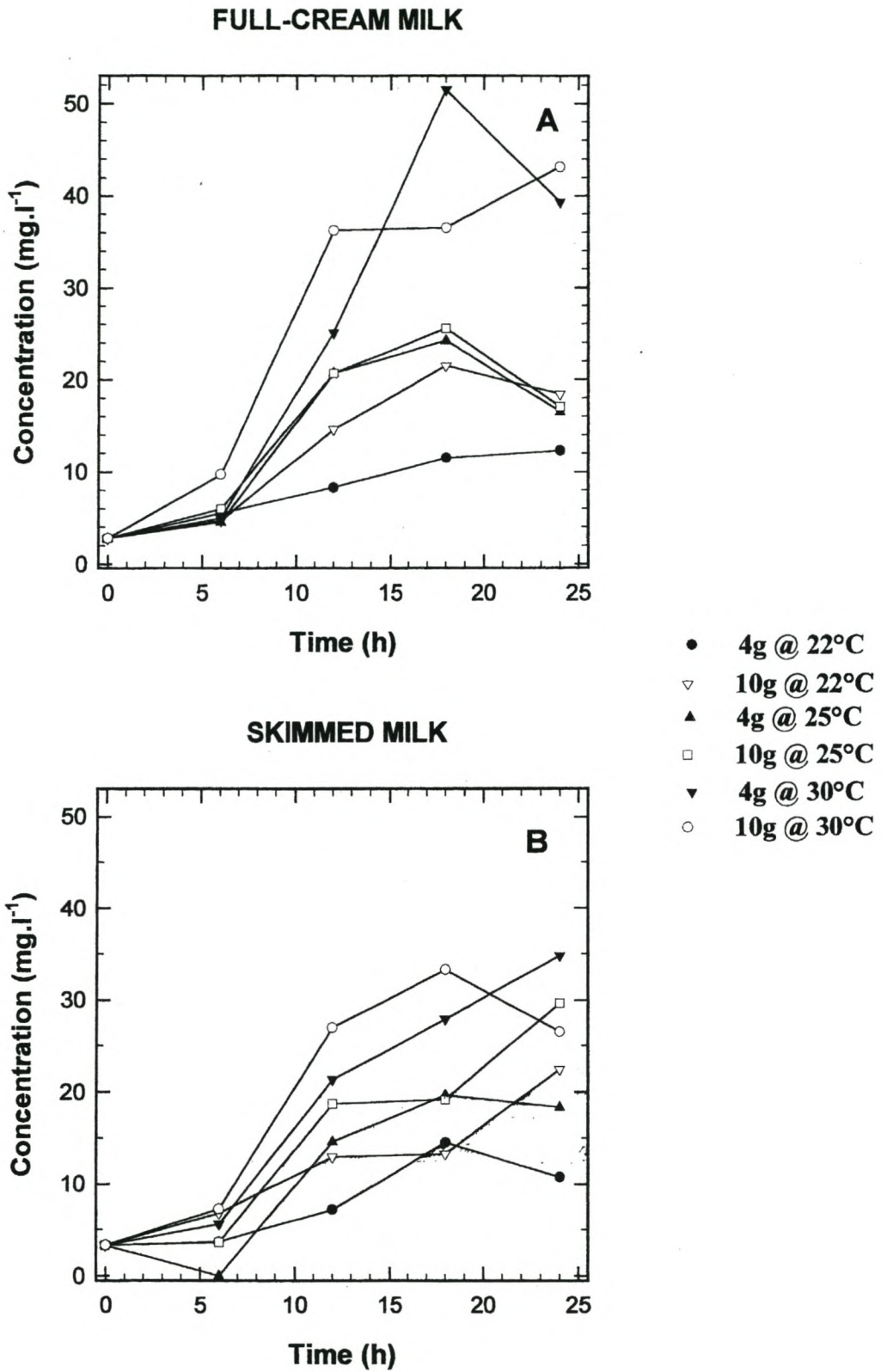
The acetaldehyde concentration data profiles obtained in this study were similar for both the skim and full-cream milk. The only apparent difference was that the skim milk samples containing 10 g of grains all had lower acetaldehyde concentrations than the corresponding samples prepared with full-cream milk.

#### *Metabolite production - acetone*

Acetone is not an important Kefir flavour contributor (Blanc, 1984), hence no reference can be found for optimum or average levels in the literature. It is usually produced from citric acid and lactose by strains of *Streptococcus thermophilus*, *L. bulgaricus*, *L. jugurti* and *L. acidophilus* (Blanc, 1984).

The production of acetone over time for Kefir produced using full-cream and skim milk is shown in Fig. 3A and 3B, respectively. From the data, it is clear that acetone occurs at low concentrations in fresh skim and full-cream milk (approximately  $3 \text{ mg.l}^{-1}$ ), and is formed in significant quantities as a metabolite during Kefir production (Gorner *et al.*, 1972; Marshall, 1984a; Ulberth & Kneifel, 1992; Vedamuthu, 1982).

It is also evident from the data (Fig. 3A and 3B) that more acetone is produced in full-cream milk, where maximum levels of  $52 \text{ mg.l}^{-1}$  were reached after 18 h of



**Figure 3.** Acetone concentration changes for Kefir prepared with different inoculum concentrations and incubation temperatures.

incubation for the sample containing a 4 g grain inoculum incubated at 30°C. The corresponding skim milk sample (4 g grains incubated at 30°C) also had the highest overall acetone concentration of 34.5 mg.l<sup>-1</sup>, however, this level was reached after an extended (24 h) incubation period. The fact that full-cream milk favours the formation of acetone is congruent with the finding that acetone is usually produced from citric acid or lactose, but can also be produced from the thermal degradation of fats during fermentation (Blanc, 1984).

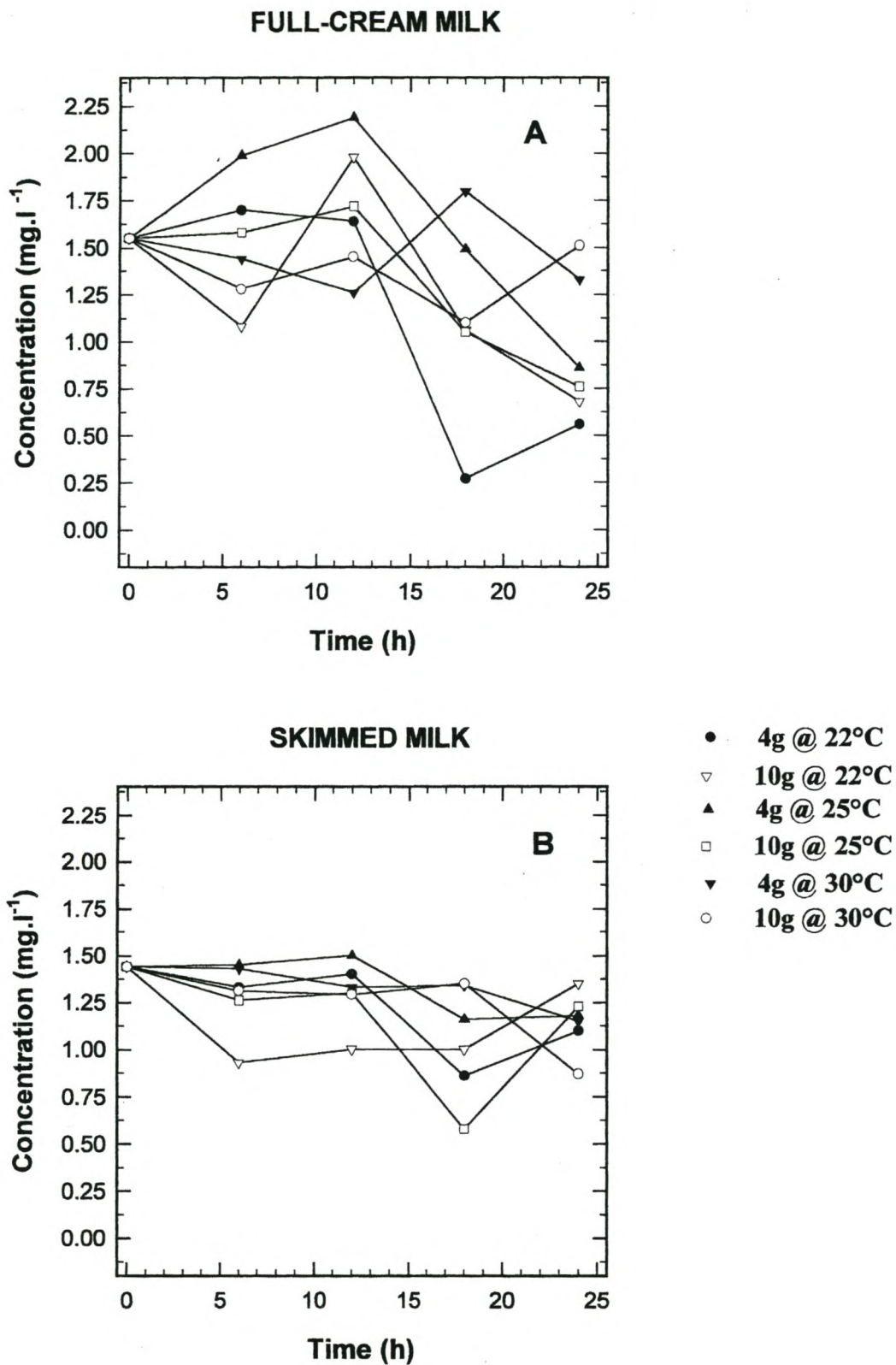
In both milk types, the samples containing 10 g of Kefir grains incubated at 30°C contained the second highest maximum level of acetone, however, it was also evident that acetone was produced fastest in these samples overall. Conversely, the 4 g inoculum concentration samples for both skim and full-cream milk incubated at 22°C had the lowest acetone production (approximately 10.5 mg.l<sup>-1</sup>), with the 10 g samples incubated at 22°C containing only slightly higher levels. It is thus evident from both Fig. 3A and 3B that the higher the incubation temperature, the higher the acetone levels for each inoculum concentration at each time interval. It was thus concluded that acetone production is proportional to the incubation temperature in the temperature range used in this study. This is in accordance with the finding that acetone is produced by the strains of *L. bulgaricus*, *L. jugurti*, *S. thermophilus* and *L. acidophilus*, which are, according to Blanc (1984), all in the upper-mesophilic range of microorganisms. It is also apparent from Fig. 3A and 3B and the findings above, that acetone production is proportional to inoculum concentration at each discrete temperature interval investigated in this study. It appears that temperature therefore has a primary effect on acetone production, with inoculum concentration having a secondary effect at each temperature.

#### *Metabolite production - 2-Butanone*

2-Butanone is seldom referred to in the literature as a major metabolite in Kefir and it has been concluded to be of no importance to Kefir flavour, hence optimum levels are not cited (Blanc, 1984). It is usually produced from lactose, but can also occasionally be obtained during the thermal degradation of fats (Blanc, 1984).

The metabolite profiles for 2-butanone production in full-cream and skim milk Kefir are shown in Fig. 4A and 4B, respectively. As is evident from the data, the initial concentrations of 2-butanone in full-cream and skim milk are similar (approximately





**Figure 4.** 2-Butanone concentration changes for Kefir prepared with different inoculum concentrations and incubation temperatures.

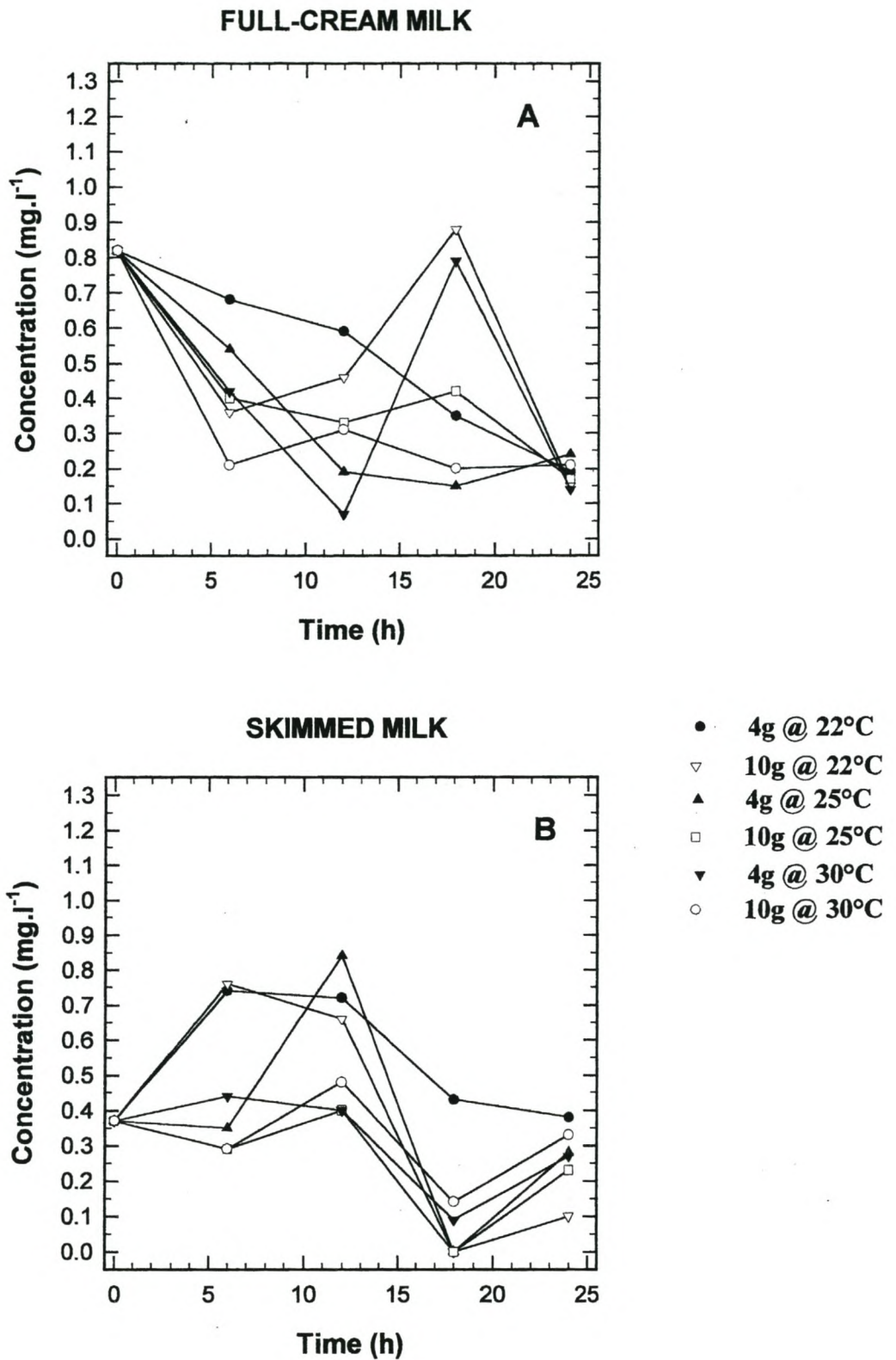
1.50 mg.l<sup>-1</sup>). There was initially a slight increase in 2-butanone concentration in full-cream milk Kefir (Fig. 4A) incubated at 22° and 25°C, with the maximum concentrations for all samples occurring after 12 h of incubation. The 2-butanone concentration then decreased fairly rapidly during further incubation, resulting in a 2-butanone concentration in the final product that was lower than in the original milk.

As can be seen from the data (Fig. 4), 2-butanone was not a major metabolite produced during the Kefir fermentation, with the concentration over the 24 h incubation period staying relatively constant, or decreasing (especially for the skim milk Kefir). This finding explains why only a few studies mention 2-butanone as a metabolite produced during Kefir fermentation, and is consistent with other research findings that this metabolite is only occasionally present in fermented Kefir (Gomer *et al.*, 1972; Palo, 1971). Ulberth & Kneifel (1992) also specifically excluded 2-butanone from their study due to the fact that its concentration values were almost constant throughout their trial.

#### *Metabolite production - Diacetyl*

Diacetyl is usually produced in Kefir by strains of "*Streptococcus lactis*", *L. brevis* and, in some instances, specific yeasts (Marshall, 1984a). It is often formed from citrate, but can also be formed as the result of a reaction between acetaldehyde and acetyl coenzyme-A (Vedamuthu, 1982). It is an extremely important contributor to flavour in fermented milks, with its flavour being described as a buttery, nutty flavour (Vedamuthu, 1982).

The data in Fig. 5A and 5B show the diacetyl concentration profiles for both full-cream and skim milk. It is evident from these profiles that full-cream milk had an original diacetyl concentration that was approximately double the original concentration of diacetyl in skim milk (0.8 mg.l<sup>-1</sup> in full-cream vs 0.4 mg.l<sup>-1</sup> for skim milk). Changes in the diacetyl concentration in the samples during the study, however, resulted in final diacetyl concentrations in a similar range (0.1 – 0.3 mg.l<sup>-1</sup>) for all the full-cream (Fig. 5A) and skim milk (Fig. 5B) samples (after 24 h). These concentrations are below the optimum values cited in the literature of approximately 0.48 mg.l<sup>-1</sup> (Gawel & Gromadka, 1978; Kramkowska *et al.*, 1982) to 3 mg.l<sup>-1</sup> (Marshall, 1982; Marshall, 1984a; Vedamuthu, 1982). This observation highlights the fact that care must be taken to optimise the fermentation time for optimum flavour



**Figure 5.** Diacetyl concentration changes for Kefir prepared with different inoculum concentrations and inoculum temperatures.

metabolite production, as, with an extended fermentation, diacetyl can be further metabolised.

The maximum diacetyl levels reached were  $0.9 \text{ mg.l}^{-1}$  for full-cream milk Kefir (for the 10 g inoculum concentration sample incubated at  $22^\circ\text{C}$  for 18 h) and  $0.85 \text{ mg.l}^{-1}$  for skim milk Kefir (for the 4 g inoculum concentration sample incubated at  $25^\circ\text{C}$  for 12 h). These values are congruent with optimum levels cited in the literature of approximately  $0.48 \text{ mg.l}^{-1}$  (Gawel & Gromadka, 1978; Kramkowska *et al.*, 1982) to  $3 \text{ mg.l}^{-1}$  (Marshall, 1982; Marshall, 1984a; Vedamuthu, 1982).

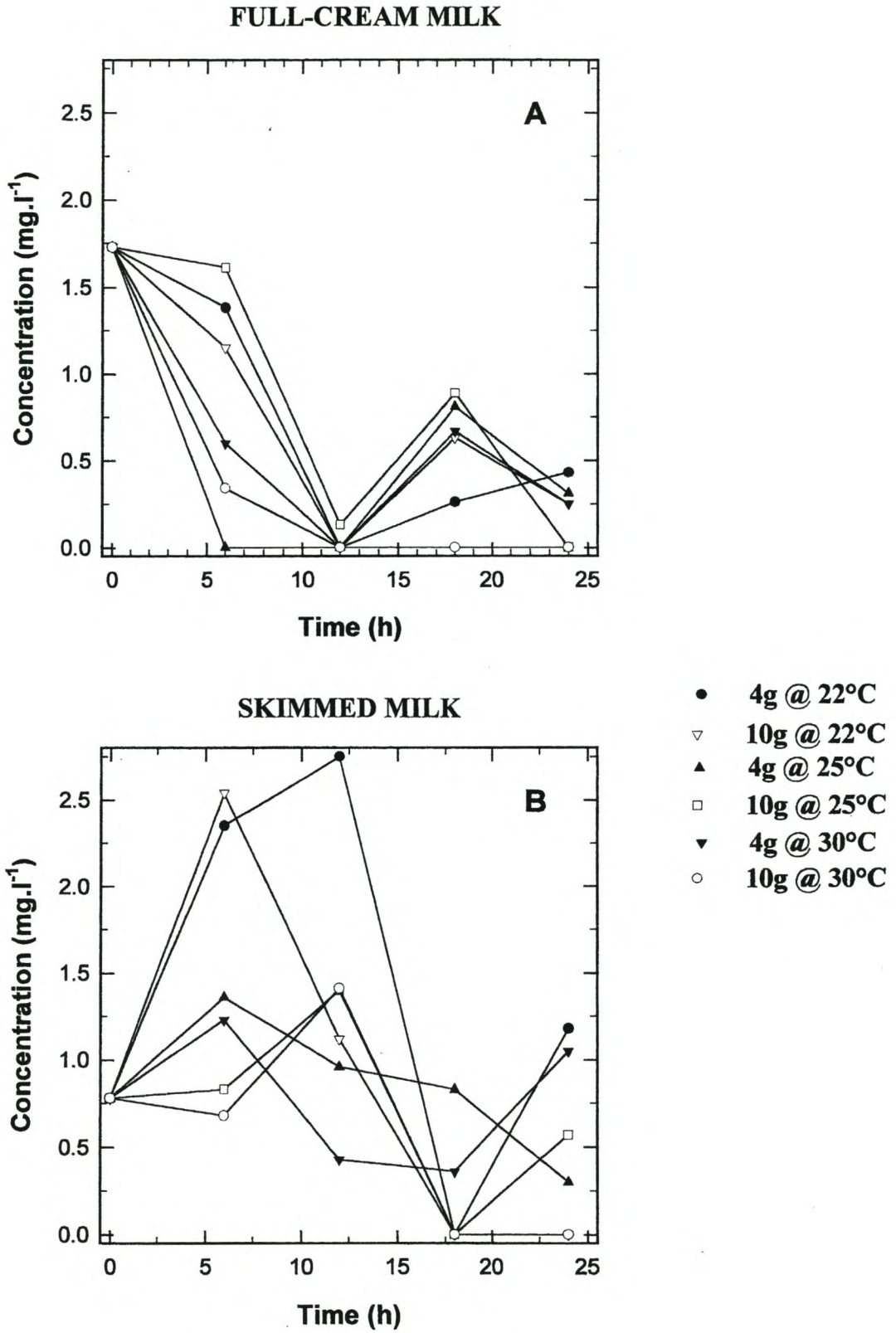
During this investigation, the diacetyl concentration in the full-cream milk Kefir samples (Fig. 5A) decreased rapidly in all six samples during the first 6 h of incubation. This decrease continued in all 4 g inoculum concentration samples until 12 h of incubation, whilst there was a slight increase in diacetyl concentration in all the 10 g inoculum concentration samples during the same period. The diacetyl concentration in the 4 g sample incubated at  $22^\circ\text{C}$  continued falling for the duration of the study (24 h) to a final level of approximately  $0.2 \text{ mg.l}^{-1}$ , whilst in the remaining samples, slight increases in the diacetyl concentration were observed. It is therefore apparent that in full-cream milk, inoculum concentration exerts a direct influence on diacetyl concentration, with incubation temperature also directly affecting diacetyl production at each inoculum concentration. Incubation time also has a significant effect, with maximum concentrations noted after 18 h of fermentation, and minimum concentrations evident after 24 h of incubation. Inoculum concentration was thus the primary influence, with incubation temperature and length of incubation playing a secondary role in diacetyl production. This is in accordance with previous findings that diacetyl does not usually accumulate in cultures until the pH drops to about 5.5 (Collins, 1972). In Chapter 3 of this study, it was found that inoculum concentration had a direct influence on the rate of acidification, as did incubation temperature in the range  $18^\circ - 25^\circ\text{C}$ . According to these findings, the pH of the samples containing 10 g of Kefir grains would therefore drop faster than the pH of the samples containing only 4 g of Kefir grains for each discrete temperature interval up to  $25^\circ\text{C}$ , resulting in earlier diacetyl production (Fig. 5A). This pattern is not followed in the samples incubated at  $30^\circ\text{C}$  due to the slower acid production encountered with higher incubation temperatures ( $30^\circ\text{C}$ ), as documented in Chapter 3 of this thesis.

The diacetyl concentration in the skim milk samples (Fig. 5B) remained more constant during the fermentation period, with the final diacetyl concentration (24 h) in all the skim milk samples being similar to the original concentration of  $0.38 \text{ mg.l}^{-1}$ . There were initially slight increases in diacetyl concentration ( $0.73 \text{ mg.l}^{-1}$ ) in the samples incubated at  $22^\circ\text{C}$  after 6 h of incubation, whilst the levels of diacetyl in the other samples remained constant at between  $0.28$  and  $0.4 \text{ mg.l}^{-1}$  during this same period. The diacetyl concentration reached a maximum level of  $0.8 - 0.9 \text{ mg.l}^{-1}$  for the sample containing 4 g of grains incubated at  $25^\circ\text{C}$  after 12 h of incubation, however the diacetyl concentration in all the samples dropped dramatically between 12 and 18 h of incubation. There does not appear to be the same direct correlation between diacetyl production and inoculum concentration, linked to pH, in skim milk Kefir as was evident in full-cream milk Kefir (Fig. 5A).

#### *Metabolite production - Ethanol*

Ethanol is an essential flavour constituent of Kefir and is usually produced by the yeasts present in the grains, as well as by certain lactic acid bacteria. It imparts a "yeasty"-sour, mildly alcoholic flavour to Kefir (Marshall, 1984a; Vedamuthu, 1982). Ethanol is usually produced from lactose (Blanc, 1984), but can be produced from acetaldehyde by some yeast species (Marshall, 1984a). Ethanol content in Kefir is very variable (Marshall, 1984a), with reports of typical levels of between 0.1 and 2.0% (Duitschaeffer, 1989; Kneifel & Mayer, 1991; Marshall, 1984b; Rasic, 1987), and an optimum level of about 1.0% (Marshall, 1984a; Kosikowski, 1982; Vedamuthu, 1982). It has been reported that ethanol in Polish Kefir rarely exceeds 0.4%, and the concentration is even lower in Finnish and Norwegian Kefir, with levels of 0.05% and 0.15%, respectively (Marshall, 1984a). Swedish Kefir also has very low levels of ethanol ( $< 0.2\%$ ) (Alm, 1982), and one study cited typical Kefir ethanol content to be as low as 0.023% (Gawel & Gromadka, 1978).

The ethanol concentrations produced in full-cream and skim milk, respectively, are shown in Fig. 6A and 6B. The initial ethanol content of the full-cream milk in this study ( $1.75 \text{ mg.l}^{-1}$ ) was higher than that of the skim milk ( $0.75 \text{ mg.l}^{-1}$ ). The ethanol concentration in the full-cream milk samples was found to decrease rapidly to zero after 12 h of incubation. This was followed by an increase, to a maximum level of  $0.85 \text{ mg.l}^{-1}$  for the sample containing 10 g of grains that was incubated at  $25^\circ\text{C}$  for



**Figure 6.** Ethanol concentration changes for Kefir prepared with different inoculum concentrations and incubation temperatures.

18 h. This is in accordance with a study where the highest rate of alcohol production was noted at a temperature of 25°C (Korovkina *et al.*, 1978). Subsequently, a further decrease in ethanol concentration was noted until the end of the study (24 h of incubation). The final ethanol concentrations of four of the samples (0.25 – 0.45 mg.l<sup>-1</sup>) were lower than the initial ethanol concentration in the full-cream milk. The two samples containing 10 g of grains incubated at either 25° or 30°C had a final ethanol concentration of zero. These final ethanol concentrations are in accordance with the findings that the ethanol concentration in Kefir is very variable, with typical levels of between 0.1 and 2.0% (Duitschaeffer, 1989; Kneifel & Mayer, 1991; Marshall, 1984b; Rasic, 1987), and in some instances, even as low as 0.023% (Gawel and Gromadka, 1978).

The profiles of ethanol concentration obtained for the skim milk Kefir (Fig. 6B) were different to those obtained with full-cream milk. There was an initial increase in ethanol concentration in the skim milk samples, with the maximum ethanol production found in the 4 g and 10 g samples incubated at 22°C. The peak maximum (2.55 mg.l<sup>-1</sup>) for the 10 g sample occurred after 6 h of incubation, whereas the maximum for the 4 g sample (2.77 mg.l<sup>-1</sup>) occurred after 12 of incubation. Once these maximum ethanol concentrations had been reached, the concentrations decreased rapidly and by 18 h of incubation, both of these samples had no remaining ethanol content. The other skim milk samples had ethanol concentrations that remained relatively constant throughout the 24 h incubation period, although it does appear that the ethanol content of the lower inoculum concentration samples (4 g) at each temperature and incubation time was marginally higher than for the corresponding larger inoculum concentration sample (10 g). The final ethanol content (24 h) of all the skim milk samples was in the range 0.26 – 1.15 mg.l<sup>-1</sup>. These levels are in accordance with the typical ethanol levels of 0.1 - 2.0% (Duitschaeffer, 1989; Kneifel & Mayer, 1991; Marshall, 1984b; Rasic, 1987) reported in the literature. It is evident from the above that skim milk favours the production of ethanol more than full-cream milk.

Yeast species are often the main ethanol producers during Kefir fermentation, although *L. acidophilus* can also be responsible for ethanol production (Marshall, 1984b). *Leuconostoc mesenteroides* and *Leuc. cremoris* have both also been isolated from Kefir (Marshall, 1984a). All these microbial species possess an alcohol

dehydrogenase that metabolises any produced acetaldehyde to ethanol. This conversion usually occurs during Kefir ripening for 1 - 3 d at 5° - 10°C (Marshall, 1984a), a step that was not performed in this study. This probably explains the observation that the lower incubation temperature samples had a slightly increased ethanol concentration, especially during the first half of the fermentation when using skim milk as growth substrate.

## Conclusions

The Kefir produced in this study were found to have the same metabolites as those cited in the literature as playing the major role in Kefir flavour, namely, acetaldehyde, ethanol and diacetyl. It was also found that the Kefir samples contained high concentrations of the non-flavour enhancing metabolite acetone. Also in accordance with other literature findings, 2-butanone and acetoin did not appear to be important metabolites in the studied Kefir samples (Gorner *et al.*, 1972; Palo, 1971; Ulberth & Kneifel, 1992).

Acetaldehyde was found to be a major metabolite in the studied Kefir, and the final concentrations of acetaldehyde in both skim and full-cream milk were similar to the literature optimums of 1 - 2 mg.l<sup>-1</sup> (Marshall, 1982; Marshall, 1984b; Vedamuthu, 1982). In this study, acetaldehyde production was found to be inversely related to inoculum concentration at a specific incubation temperature, with incubation at 25°C, followed by incubation at 30°C, being the most favourable. Prolonged incubation resulted in a decrease in acetaldehyde concentration which was probably due to the metabolism of acetaldehyde to acetoin, diacetyl or ethanol (Marshall, 1982).

Acetone was produced in large quantities during this study, and it was concluded that acetone production was proportional to incubation temperature in the temperature range studied. Inoculum concentration was also proportional to acetone production, but appeared to play a secondary role at each temperature interval. The fat content of the milk also appeared to have an influence on acetone production, with full-cream milk Kefir having higher acetone levels than skim milk Kefir.

2-Butanone was found to not be a major metabolite in this study, with the concentration remaining almost constant throughout the trial.



Diacetyl was also produced during this study, however, the produced diacetyl was further metabolised during subsequent fermentation, resulting in ultimate diacetyl concentrations below the optimum values cited in the literature (Gawel & Gromadka, 1978; Kramowska *et al.*, 1982; Marshall, 1982; Marshall, 1984a; Vedamuthu, 1982). As diacetyl is such a major contributor to flavour in Kefir, care must be taken to optimise the fermentation time for optimum diacetyl production. Some researchers have suggested the addition of citrate or citric acid to further enhance diacetyl content (Marshall, 1982) as diacetyl is usually produced primarily from these compounds. In full-cream milk, inoculum concentration was found to have the primary influence on diacetyl production, with incubation temperature and incubation period playing a secondary role. From the observations in Chapter 3 of this thesis that inoculum concentration, incubation temperature and length of incubation all have a direct influence on the rate of acidification and thus the subsequent pH drop in Kefir in the temperature range of 22° -25°C, and in accordance with the fact that diacetyl does not accumulate in cultures until the pH drops to about 5.5 (Collins, 1972), it was surmised that acid production and hence a lower pH have a direct influence on diacetyl production in full-cream milk Kefir. The data obtained suggests that there does not appear to be the same direct correlation between diacetyl production and inoculum concentration, linked to a lower pH in skim milk.

Ethanol was also found to be a major metabolite in this study, and the findings were in accordance with the observation that the ethanol concentrations in Kefir are very variable. More ethanol was produced in skim milk than in full-cream milk Kefir, and the actual ethanol concentrations were in line with the cited literature values of between 0.1 and 2.0% (Duitschaeffer, 1989; Kneifel & Mayer, 1991; Marshall, 1984b; Rasic, 1987), with some values as low as the cited 0.023% value (Gawel & Gromadka, 1978). Ethanol production also appeared to be favoured slightly by lower incubation temperatures, especially during the first half of the fermentation using skim milk as growth substrate. This is best explained by the fact that yeast species are often the main ethanol producers, and it is known that they are more active at lower temperatures than most mesophilic and thermophilic bacteria species (Marshall, 1984a).

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## CHAPTER 5

### SENSORY EVALUATION AND OPTIMISATION OF SENSORY CHARACTERISTICS OF KEFIR

#### Summary

During fermentation of Kefir grains in milk, volatile compounds such as acetaldehyde, ethanol, diacetyl and acetone are produced by the metabolic activities of the microorganisms. These flavours, along with lactic acid and carbon dioxide, are responsible for the classic effervescent, yeasty-sour Kefir flavour. Kefir was prepared using 15 different combinations of incubation temperature (22°, 25° and 30°C), length of incubation (12, 18 and 24 h) and inoculum concentration (4 g or 10 g of grains per 500 ml full-cream milk). Two sensory panels consisting of five trained panellists each were used to evaluate three Kefir samples per daily tasting session. The samples were rated on a scale from zero to 10 with regards to sourness, sweetness, yoghurt (green apple) flavour, buttery flavour, yeasty (cheesy) flavour, cowy (barny) flavour, creaminess, effervescence, smoothness and overall acceptability. Statistical analysis consisted of variance analysis (F-test) followed by pairwise t-testing (Student's t-test) of the 15 combinations. The primary effects (temperature, time and concentration) had a significant influence on sourness, sweetness and buttery flavour, whilst incubation time had an influence on creaminess and yoghurt flavour. Inoculum concentration affected cowy flavour, and overall acceptability was affected by incubation temperature and time ( $P \leq 0.05$ ).

#### Introduction

Kefir is generally characterised by a creamy consistency and specific, refreshing lactic flavour (Korovkina *et al.*, 1978; Marshall, 1982) which is also rather "cheesy", and is sometimes described as rancid (Marshall, 1984b). The flavour may also be described as mildly alcoholic, yeasty-sour, with a tangy effervescence (Liu & Moon,

1983; Vedamuthu, 1982) resulting in a “prickly” sensation in the mouth (Duitschaeffer, 1989). Good quality Kefir should foam like beer (Marshall, 1982) and should have a smooth, pourable consistency (Marshall, 1984a). Low acidity values generally result in assessments such as “mild” or “insipid”, and high values as “acid” or “too acid” (Marshall, 1982).

During the fermentation process, lactose is predominantly fermented to lactic acid, which is responsible for the sharp, refreshing taste of all fermented milks. Although lactic acid is non-volatile, it serves as an excellent background for the more distinctive flavours and aromas characteristic of each fermented milk (Marshall, 1984a). Diacetyl contributes to the delicate aroma of dairy products (Blanc, 1984), and is the single most important and essential flavour compound that imparts a characteristic “buttery”, “nut meat”-like aroma and flavour (Vedamuthu, 1982). Diacetyl can be converted into acetoin, which is a flavourless compound sometimes also present in Kefir (Vedamuthu, 1982). Acetaldehyde plays a leading role among the factors contributing to the aroma of dairy products (Blanc, 1984; Vedamuthu, 1982), and imparts a “yoghurt-like” or “green” flavour to dairy products (Marshall, 1984a; Vedamuthu, 1982). Low acetaldehyde concentrations ( $< 10 \text{ mg.l}^{-1}$ ) are associated with “little or no yoghurt flavour” (Marshall, 1982). Ethanol is usually responsible for the “yeasty”, fermented flavour typical in Kefir, but an excessively yeasty flavour is undesirable (Marshall, 1982; Marshall, 1984a). The effervescent nature is a result of carbon dioxide, and is a requirement of Kefir as it imparts a sparkling character that makes this product refreshing (Liu & Moon, 1983; Marshall, 1984a; Vedamuthu, 1982). Consistency can, however, be destroyed by gas production if fermentation is heterofermentative (Marshall, 1984a). Acetone and 2-butanone do not appear to play an important role in Kefir flavour (Blanc, 1984; Kosikowski, 1982).

The concentrations of lactic acid, alcohol and carbon dioxide formed in Kefir can be regulated by several physical factors and the microorganisms in the starter culture. Both the ratio and type of microorganisms present will influence the organoleptic properties of the resultant product (Gawel & Gromadka, 1978; Liu & Moon, 1983), with the temperature of fermentation having a direct influence on viscosity (Korovkina *et al.*, 1978). Incubation at 22°C produces better consistency, flavour and aroma, as well as a firmer, higher viscosity coagulum more resistant to

they separation than incubation at 18°C (Mann, 1979). Most studies recommend incubation of Kefir at 25°C to give maximum production of ethanol and volatile acids as well as good specific flavour and consistency (Liu & Moon, 1983; Mann, 1979; Korovkina *et al.*, 1978). Organoleptic scores in Kefir rose significantly with increased fat content from 0.06% to 2.0% (Mann, 1979), although in a study of yoghurt and bioghurt, the fat content had no influence on the flavour (Hild, 1980). According to Marshall (1982), fat content makes a large difference to both flavour and texture, although rancidity can be a problem.

This study was undertaken to investigate the influence of incubation temperature, length of incubation and initial Kefir grain inoculum concentration on the flavour and texture of Kefir in order to make recommendations for optimal Kefir production.

## **Material and methods**

### *Kefir grain activation*

Frozen Kefir grains were obtained from the ARC-Animal Nutrition and Animal Products Institute, Irene, Pretoria, South Africa (Dr J.F. Mostert, 1996, Personal Communication). These grains were originally obtained from various South African households, and were without specifications of characteristics or composition. Prior to aseptic inoculation into fresh pasteurised milk, the grains were defrosted at ambient temperature and then placed in a temperature-controlled waterbath (22°C) for 24 h. The grains were retrieved using a stainless steel sieve sterilised in a *Milton* solution [1.25% (v/v)] for 30 min and then rinsed using sterile distilled water. These grains were then re-inoculated into a fresh skim milk sample using a sterilised stainless steel laboratory spoon-type spatula. This process was repeated three times over a period of three days in order to fully activate the grains. Locally purchased milk (although pasteurised) was re-pasteurised at 80°C for 10 min in order to ensure a good and consistent microbiological quality.

### *Flavour identification and panellist training*

Fresh full-cream milk was purchased and spiked with the most typically mentioned flavour compounds found in Kefir, namely acetaldehyde, 2-butanone,

acetone, ethanol and diacetyl (Hild, 1980; Marshall, 1984a). These spiked samples were compared to unflavoured milk by a pool of 15 panellists, and comments were made about the resultant aroma and flavour of the samples. From the comments, as well as literature descriptions of actual metabolite flavours (Blanc, 1984; Marshall, 1982; Marshall, 1984a; Vedamuthu, 1982), the typical flavour associations were obtained and each metabolite was assigned its corresponding specific descriptive term. As 2-butanone had no apparent flavour, it was not measured during this study.

The above descriptive terms, together with other attributes important to Kefir quality were then used to craft a generic sensory evaluation form (Fig. 1) containing each descriptive term linked to a scale ranging from 0 (none) to 10 (extreme).

Two further sessions were held with the 15 panellists, where the crafted form and each specific descriptive term was introduced to the panellists with the corresponding spiked fresh milk sample. These spiked samples were again compared to unflavoured fresh milk to ensure that each panellist was comfortable with the terminology and able to recognise each characteristic flavour and make their own associations.

A final session was held with the 15 panellists to standardise the panel's response as far as possible. For this purpose, milk spiked with different levels of each flavour compound was given to each panellist and discussed as a panel so that agreement was obtained on how each perceived flavour intensity should be translated onto the 10-point scale on the sample evaluation form.

#### *Kefir production and testing procedure*

As discussed in Chapter 3 of this thesis, there is little correlation in the literature regarding optimum inoculum concentrations and incubation temperatures for Kefir production. Commonly cited inoculum concentrations of between 0.5 – 10% and incubation temperatures of 22° - 30°C have been used as guidelines for this study (Kneifel & Mayer, 1991; Marshall, 1984b; Merin & Rosenthal, 1986). It was decided to use full-cream milk in this study, due to possible improvements in flavour with higher fat content (Mann, 1979; Marshall, 1982).

Kefir was prepared using 15 different combinations of incubation temperature (22°, 25° and 30°C), length of incubation (12, 18 and 24 h) and inoculum concentration (4 g or 10 g per 500 ml milk). The milk was purchased fresh daily from



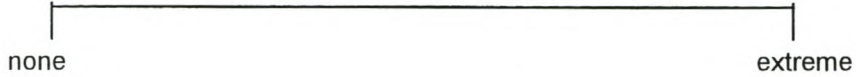
**Panellist:** \_\_\_\_\_ **Date:** \_\_\_\_\_ **Sample:** \_\_\_\_\_

**A. FLAVOUR**

**1. Sourness**



**2. Sweetness**



**3. Yoghurt (green apple)**



**4. Buttery (caramel)**



**5. Yeasty (cheesy)**



**6. Cowy (barny)**



**B. BODY (TEXTURE)**

**7. Creaminess**



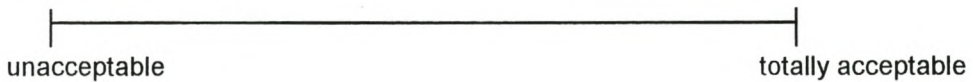
**8. Effervescence (gassiness)**



**9. Smoothness**



**C. OVERALL ACCEPTABILITY**



**Figure 1.** Sample evaluation form for sensory evaluation of Kefir.



local supermarkets and re-pasteurised prior to Kefir grain addition to limit possible variables as much as possible. Once fermentation for the appropriate time was completed, the Kefir beverage containing the grains was cooled at 4°C for 30 min prior to sieving, to ensure no loss of volatiles. Further cooling at 4°C for 1 h was performed prior to evaluation to enhance product acceptability.

Two sensory panels consisting of five trained panellists each were used to sample three Kefir samples per tasting session. The samples tasted were arranged according to a statistical block design such that each sample was tasted in combination with each other sample, resulting in each sample being tasted 10 times by each panellist.

The tasting sessions were held daily between 10h30 and 11h30, as is best practise for optimum discrimination in sensory analyses (Bodyfelt *et al.*, 1988). Special individual sensory booths were used and tasting was done under red light to ensure that colour did not affect the panellist's responses. Each panellist was given approximately 100 ml of each Kefir sample and fresh water and carrots were provided as palate cleansers.

Statistical analysis consisted of variance analysis (F-test) followed by pairwise t-testing (Student's t-test) of the 15 combinations (Larmond, 1982).

## **Results and discussion**

### *Flavour identification*

Table 1 contains a list of each metabolite and the corresponding flavour/attribute description used in this study, as agreed with the trained sensory panel.

### *Variance testing: statistical probability*

Table 2 shows the statistically significant probability results of the influence of time, temperature and inoculum concentration on tested attributes. These results were obtained from variance testing performed on the raw data. Statistically significant variance test results are those with a  $\geq 95\%$  certainty level (underlined in Table 2). These results were analysed further to obtain more detail using Student's t-test. Certainty level results of  $\geq 90\%$  have been included as indicators of probability for the sake of completion.

**Table 1.** Relevant metabolites responsible for perception of flavour and other attributes.

<b>Flavour/Attribute</b>	<b>Metabolite Responsible</b>
1. Sour	Lactic acid
2. Sweet	Lactose
3. Yoghurt (green apple)	Acetaldehyde
4. Buttery (caramel)	Diacetyl
5. Yeasty (cheesy)	Ethanol
6. Cowy (barny)	Acetone
7. Effervescent (gassy)	Carbon dioxide

**Table 2.** Probability (P) values for statistically significant variance test results (95% and 90% certainty levels).

<b>Attribute</b>	<b>Temperature</b>	<b>Incubation Time</b>	<b>Concentration</b>
Sour	<u>0.0093</u>	<u>0.0001</u>	<u>0.0329</u>
Sweet	<u>0.0004</u>	<u>0.0001</u>	<u>0.0339</u>
Buttery	<u>0.0026</u>	<u>0.0001</u>	<u>0.0017</u>
Creamy	-	<u>0.0001</u>	-
Yoghurt	0.0918	<u>0.0001</u>	0.0552
Cowy	-	-	<u>0.0003</u>
Effervescent	-	0.0758	-
Yeasty	-	0.0943	-
Smoothness	-	-	-
Acceptability	<u>0.0008</u>	<u>0.0032</u>	-

All three primary effects (temperature, time and concentration) had an influence on sourness, sweetness and buttery flavour ( $P \leq 0.05$ ). Incubation time had a meaningful influence on creaminess and yoghurt flavour ( $P \leq 0.05$ ). Incubation time was also statistically meaningful for gas and yeasty flavour production at the lower probability level ( $P \leq 0.1$ ), as were incubation temperature and inoculum concentration on yoghurt flavour production ( $P \leq 0.1$ ). Inoculum concentration was the only meaningful parameter influencing cowy flavour ( $P \leq 0.05$ ), whilst there were no meaningful correlations between smoothness and any of the main effects, or combinations of main effects. In terms of overall acceptability, both temperature and time exerted a meaningful influence ( $P \leq 0.05$ ).

#### *Pairwise t-testing: Student's t-test*

Only results that were statistically significant at the 95% certainty level were analysed further, with the results discussed below.

##### (i) Attribute - sourness:

T-testing for meaningful ( $P \leq 0.05$ ) differences using adjusted averages showed that there was a meaningful difference between incubation at 22°C (4.5826) and 25° (5.1311) or 30°C (5.3622) on product sourness (lactic acid). A difference was also noted between 12 h (3.5043) of incubation and 18 h (5.0851) or 24 h (5.8559). The difference in average values between the two inoculum concentrations were not quite as large (4.9950 for 4 g and 5.1493 for 10 g), but were still significant. From these results, it is evident that increases in incubation temperature, length of incubation and inoculum concentration are all directly related to increases in product sourness or acid production. These findings are in accordance with the findings in Chapter 3 of this study, where it was determined that incubation temperature, inoculum concentration and length of incubation were all directly related to acid production during Kefir production.

##### (ii) Attribute - sweetness

For product sweetness (lactose) the opposite effect was evident, namely, increases in temperature, time or concentration had a lowering effect on sweetness. Incubation at 25° (0.8448) and 30°C (0.9053) yielded a less sweet product than

incubation at 22°C (1.3570), as did incubation for 18 h (0.9452) or 24 h (0.8321) in comparison to incubation for 12 h (1.4725). In terms of inoculum concentration, 4 g (1.0807) yielded a sweeter product than 10 g (0.9396). This observation is congruent with the finding for sourness, above, as sweetness in Kefir is a result of lactose content. During fermentation, lactose is converted to lactic acid (Marshall, 1984a), with a subsequent reduction in sweetness and paired increase in sourness (acidity).

(iii) Attribute - yoghurt

Yoghurt flavour (acetaldehyde) strength was proportional to length of incubation, with incubation for 12 h resulting in far lower averages (1.5038) than incubation for 18 h (2.5583) or 24 h (2.8867), although none of the samples had an exceptionally strong yoghurt flavour. The findings for acetaldehyde concentration in Chapter 4 of this study were not in accordance with this finding. In Chapter 4, maximum acetaldehyde concentration occurred after either 12 h or 18 h of incubation, depending on inoculum concentration and incubation temperature, and decreased significantly with further incubation to 24 h. A possible explanation for this discrepancy is the fact that these two trials were not performed simultaneously, but rather sequentially. Due to the dynamic nature of Kefir grains and their microbiological consortium, changes in product characteristics are continually occurring (Duitschaeffer, 1989; Kroger, 1993; Mann, 1979; Marshall, 1984a; Rasic, 1987). A change in Kefir flavour can only be ascribed to changes of metabolism of the organisms (Marshall, 1982). It is also well documented that acetaldehyde plays a central role in flavour metabolism, with acetoin, diacetyl and ethanol all produced from this compound (Marshall, 1982; Marshall, 1984a; Vedamuthu, 1982). This means that slight changes could alter the fermentation pathways and thus final metabolites in Kefir (Marshall, 1982; Marshall 1984a). It would therefore be preferable to perform these trials simultaneously in the future.

(iv) Attribute - buttery

Length of incubation exerted a direct influence on buttery flavour (diacetyl) production. Incubation for 18 h resulted in the highest buttery flavour (1.8747) and 24 h the lowest (1.0922) with 12 h in between (1.6674). This is in accordance with the finding that maximum diacetyl concentration was noted after 18 h of fermentation,

with the lowest concentration being evident after 24 h of incubation for full-cream milk in Chapter 4 of this study. This observation again highlights the fact that care must be taken to optimise the fermentation time for optimum flavour metabolite production, as, with extended fermentation, diacetyl can be further metabolised to acetoin (Vedamuthu, 1982).

Inoculum concentration also had a slight effect, with 4 g resulting in a stronger butter flavour (1.7670) than 10 g (1.3043). Buttery flavour also appeared to decrease with increasing incubation temperature (1.8559 for 22°C; 1.6299 for 25°C and 1.2047 for 30°C). These findings are not in accordance with the findings in Chapter 4 of this study, where higher incubation temperatures resulted in higher diacetyl production, as did higher inoculum concentrations at each temperature. This discrepancy can only be explained by a change in the metabolism of the microorganisms in the grains between Chapter 4 and Chapter 5 of this study, as was evident for acetaldehyde concentration (Marshall, 1982). The fact that diacetyl can be formed from acetaldehyde (Marshall, 1982; Marshall, 1984a; Vedamuthu, 1982) and perceived concentrations for both compounds were different from the results obtained in Chapter 4, further supports this explanation. This again highlights the importance of performing future studies simultaneously where possible.

(v) Attribute - cowy

The defective cowy flavour (acetone) appears to occur only rarely, and is then still not very noticeable or strong. Only inoculum concentration appears to play a meaningful role ( $P \leq 0.05$ ), and 10 g appears to result in a stronger flavour (1.6358) than 4 g (1.0717). This is in accordance with the findings in Chapter 4 of this study that acetone production is proportional to inoculum concentration at the temperatures investigated in this study.

(vi) Attribute - creamy

It appears that creaminess is influenced by length of incubation, with incubation for 12 h resulting in a less creamy (4.2056) product than incubation for 18 h (6.1117). When incubation was extended to 24 h, a slight decrease in creaminess was observed (5.6682). No analytical measurements of creaminess were performed in this study as reference, however, the creaminess of Kefir affects the appeal of the

product. As creaminess is affected mainly by the texture of the coagulum (Kroger, 1993), the acidity of the product is important. For good quality Kefir, the recommended pH range is 4.0 - 4.6 (Kosikowski, 1982; Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983; Vedamuthu, 1982). This finding corroborates the finding above that incubation for 18 h resulted in the creamiest product, with shorter and longer incubation periods resulting in less creamy products.

(vii) Attribute – overall acceptability

Overall acceptability of Kefir is defined by the recommended pH range for good quality Kefir of 4.0 - 4.6 (Kosikowski, 1982; Libudzisz & Piatkiewicz, 1990; Liu & Moon, 1983; Vedamuthu, 1982). In this study, lower incubation temperatures resulted in more acceptable samples, with incubation at 22°C (5.3303) giving similar results to incubation at 25°C (5.1304), which were more favourable than for incubation at 30°C (3.9165). This in accordance with the findings in Chapter 3 of this thesis, that moderate temperatures (22° and 25° C) resulted in superior quality Kefir in terms of pH and TA. At the higher end of the temperature scale (30°C), Kefir grain growth was found to be faster (Chapter 3 (I)), however Kefir quality was compromised due to rapid and excessive acidification (Chapter 3 (II) and Chapter 3 (III)). Literature references are also in accordance with this finding, as incubation at 22°C apparently produces a product with better consistency, flavour and aroma, as well as a firmer, higher viscosity coagulum more resistant to whey separation (Mann, 1979). Most other studies recommend incubation of Kefir at 25°C to give maximum production of ethanol and volatile acids as well as good specific flavour and consistency (Liu & Moon, 1983; Mann, 1979; Korovkina *et al.*, 1978).

In terms of incubation time, 18 h appears to yield a superior product (5.4741) to 24 h (4.5014), and both are preferable to 12 h (3.598). This finding is also in accordance with the finding in Chapter 3 (IV) of this study that optimal pH levels were reached by all Kefir samples within a 20 h incubation period. Extended fermentation resulted in the pH falling below the desirable range, leading to the conclusion that lengthened incubation periods for fully activated Kefir grains resulted in a poor quality product. This finding also corroborates the finding for creaminess, above, that incubation for 18 h resulted in the creamiest product, with shorter and longer incubation periods resulting in less creamy products.

## Conclusions

From the spiking of the milk during the training of the panellists, and the results obtained above, it is evident that the metabolites studied in Chapters 3 and 4 of this thesis, namely, ethanol, diacetyl, acetaldehyde, lactic acid and acetone, are all of great importance to Kefir quality and flavour. This is in agreement with the literature cited (Hild, 1980; Marshall, 1984a). In accordance with the findings in Chapter 4 of this study and other literature reports, 2-butanone and acetoin did not appear to be important flavour metabolites in the studied Kefir samples (Gorner *et al.*, 1972; Palo, 1971; Ulberth & Kneifel, 1992). Other attributes that were not analytically studied, but were also important to quality and appeal are creaminess, sweetness (lactose content), smoothness and carbon dioxide concentration. In future studies, it would be of great benefit to measure these attributes analytically so that more meaningful comparisons and conclusions can be made.

All three primary effects (temperature, time and concentration) had a directly proportional influence on perceived sourness, with a concurrent inverse proportionality to sweetness. This is in accordance with the findings in Chapter 3 that these three effects had a direct influence on lactic acid production from lactose.

In agreement with the findings in Chapter 4 of this study, strength of the cowy flavour (acetone) was proportional to the size of the inoculum concentration, although this flavour defect appeared to occur only rarely, and was not very noticeable.

Incubation for 18 h resulted in the creamiest products, with the best overall appeal. Lower incubation temperatures (22° and 25°C) also resulted in superior products. These findings were in accordance with the findings in Chapter 3 of this thesis, that extended fermentation resulted in pH drops that negatively affected product quality, whilst moderate temperatures (22° and 25° C) resulted in superior quality Kefir.

Buttery flavour (diacetyl) was affected by temperature, time and inoculum concentration. Maximum concentrations were evident after 18 h, and minimum concentrations after 24 h of incubation, in accordance with findings for full-cream milk in Chapter 4 of this study. As diacetyl is such an important flavour compound, care must be taken to optimise the fermentation time for optimum flavour metabolite



production, as, with extended fermentation, diacetyl can be further metabolised to acetoin (Vedamuthu, 1982). To improve diacetyl content, its precursor citrate or citric acid can be added (Marshall, 1982; Vedamuthu, 1982).

Converse to the findings in Chapter 4, however, were the results that smaller inoculum concentrations and decreased incubation temperatures resulted in increased butter flavour. The result that strength was proportional to length of incubation for yoghurt flavour was also in conflict with the results obtained in Chapter 4 of this study. These discrepancies can only be explained by a change in the metabolism of the microorganisms due to the dynamic nature of Kefir grains and their microbiological consortium (Duitschaeffer, 1989; Kroger, 1993; Mann, 1979; Marshall, 1982; Marshall, 1984a; Rasic, 1987). The fact that diacetyl can be formed from acetaldehyde (Marshall, 1982; Marshall, 1984a; Vedamuthu, 1982) and perceived concentrations for both compounds were different from the results obtained in Chapter 4, further supports this explanation. This highlights the importance of performing future studies simultaneously where possible for comparability and reproducibility.

There were also indications that incubation temperature and inoculum concentration have an effect on yoghurt flavour production at the 90% certainty level, however, as these interactions were not analysed further, the actual effects are not known.

There were no statistically meaningful effects on smoothness, ethanol content and gassiness at the 95% certainty level, however, incubation time was statistically meaningful for gas and yeasty flavour production at the lower probability level ( $P \leq 0.1$ ). To better understand these results, further statistical analysis would be necessary.

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## CHAPTER 6

### GENERAL DISCUSSION AND CONCLUSIONS

Fermented milk beverages such as Kefir hold great potential for developing countries in need of cheap nutrition, such as South Africa, due to the numerous advantages of fermented milks over fresh milk. These include prolonged shelf-life due to raised acidity, suitability to be stored at room temperature, improved digestibility for the many lactose-intolerant people, improved aroma, flavour and textural characteristics, improved nutritional value and inhibitory action against pathogenic microorganisms. Very small start-up costs are required, and the system is self-perpetuating and easy to perform, with little equipment, skill and training necessary. This study was performed to assist in optimising Kefir grain biomass increases to rapidly yield sufficient Kefir grains for larger-scale production, and to optimise fermentation conditions to result in favourable metabolite production, and thus favourable sensory characteristics of the Kefir beverage.

The first research chapter in this thesis (Chapter 3) included a study into daily biomass increases of Kefir grains over a 10 d period, using different inoculum concentrations and temperatures. The results of this study indicated that biomass increases were proportional to incubation temperature in the range investigated (18°, 22°, 25° and 30°C), with the smallest increases of approximately 200% over the 10 d period encountered for the samples incubated at 18°C. The largest biomass increase was approximately 500% over the 10 d trial for the samples incubated at 30°C, with biomass increases in a similar range for the samples incubated at 22° and 25°C. Due to the daily washing of the grains, necessary for accuracy of measurement, the actual biomass increases could be significantly higher during genuine grain cultivation. These increases are in accordance with documented literature findings, and the proportionality to temperature is consistent with the fact that metabolic processes usually occur faster at elevated physiological temperatures. For optimal active Kefir grain cultivation, it is therefore recommended that incubation temperature be maintained at 30°C, with a minimum incubation temperature of 22°C.

During the fermentation of Kefir grains in milk, lactose is converted to lactic acid by the grain microorganisms. Lactic acid is responsible for lowering the pH of the milk, resulting in coagulation, and hence the characteristic texture and flavour of Kefir, as well as the preservative effect mentioned above. Due to its importance in Kefir, lactic acid production was also measured in terms of pH and TA in Chapter 3 of this thesis. Lactic acid production was found to be strongly proportional to incubation temperature and inoculum concentration, and was inhibited at low incubation temperatures (18° and 22°C) and inoculum concentrations (0.8% w/v). In this study, optimum final acid production (pH of 4.4 – 4.6 and TA of 1.0 – 1.15%) occurred in the samples containing a 2% (w/v) inoculum concentration that were incubated at 25°C for 24 h. It was also found that it is not beneficial for Kefir quality to extend the fermentation period beyond 30 h or to have exceptionally large inoculum concentrations due the fermentation extending too far, with over-acidification as a consequence. This over-acidification with large inoculum concentrations can, however, be overcome by reducing the fermentation period to 16 – 18 h). One unfortunate aspect about this study was the regular removal of the fermentation vessels from the incubators and regular handling which may have had a detrimental effect on the results obtained due to interrupted fermentation and coagulum separation into curds and whey, making accurate measurement difficult. This will need to be overcome in future studies by increasing the number of samples dramatically so that there is one sample that is removed at each sampling point during the study, and then discarded. There may, however, also be issues attached to this method, in that different vessels may undergo acidification at different rates, which would result in curves that are not smooth over the duration of the trial. Also, the amount of samples needed initially for such a trial becomes inhibitive.

Other major metabolites and volatile compounds such as acetaldehyde, ethanol, diacetyl and acetone are also produced by the metabolic activities of the microorganisms during fermentation. These metabolites were investigated in Chapter 4 of this thesis, during a 24 h Kefir fermentation using headspace gas chromatography. This method of analysis resulted in good resolution and separation of all possible important flavour compounds, including acetaldehyde, acetone, butanol, ethanol and diacetyl. However, in this study, only acetaldehyde, ethanol and acetone were found to be major end-metabolites, with diacetyl and 2-butanone being

produced during the fermentation, but subsequently being further metabolised, resulting in low final concentrations.

Acetaldehyde concentrations were similar for skim and full-cream milk, and were higher than those cited in the literature. Final acetaldehyde concentration was inversely related to inoculum concentration at a specific incubation temperature, with incubation at 25°C being optimal.

Acetone does not contribute much to flavour, but was nevertheless measured during this study. It was found that acetone production was favoured at higher incubation temperatures for each inoculum concentration in the range studied, and was formed in significant quantities in the 0.8% (w/v) Kefir grains full-cream milk sample incubated at 30°C.

The final concentrations of diacetyl were similar for skim and full-cream milk Kefir, however were well below cited literature concentrations due to further metabolism during extended fermentation. The maximum concentration was reached in the sample containing 2% (w/v) Kefir grains incubated in full-cream milk at 22°C after 18 h of incubation. A method to improve final diacetyl concentration suggested in the literature is to add sodium citrate or citric acid to the substrate, and it would be very interesting to perform a future study analysing the effect of this fortification on the final diacetyl concentration, with a concurrent correlation to sensory appeal

In accordance with literature references, ethanol concentrations in the investigated samples were very variable, with ethanol production favoured in skim milk Kefir. The highest rate of ethanol production during this study was found at 25°C, as was also documented by other researchers. An important method cited in the literature to ensure adequate ethanol production is to ripen the produced Kefir beverage at 5° - 10°C for 1 - 3 d. This step was omitted during this study, however, it would be beneficial to see the effect a ripening step such as this would have on final ethanol concentrations as part of a future study.

This study into metabolite production (Chapter 4 of this thesis) had the same sampling issues as those encountered in Chapter 3, including interrupted fermentation due to the repeated removal of the fermenting samples and in this case, placing in the refrigerator to capture all the volatiles. As per the recommendations discussed above, it would therefore be worthwhile to perform this study again with

sufficient samples initially to allow each sample to be removed for measurement and then discarded. This would, however, again result in inhibitive amounts of initial samples being needed, as well as the possibility of concentration profiles with large discrepancies from one measurement to the next due to the dynamic nature of the grains and the resultant possible fermentation of different duplicate samples at different rates. There was no separation of the coagulum into curds and whey for the duration of this study, so homogeneity of samples did not affect the results as highlighted as an area for concern in the previous study. This was probably due to the fact that the samples were handled less regularly than the samples in the previous study (Chapter 3 of this thesis).

These metabolites discussed above, together with carbon dioxide, are responsible for the classic effervescent, yeasty-sour Kefir flavour. In the final research chapter of this thesis (Chapter 5), a detailed sensory evaluation of Kefir prepared using different incubation temperature (22°, 25° and 30°C), length of incubation (12, 18 and 24 h) and inoculum concentration (4 or 10 g of Kefir grains per 500 ml full-cream milk) combinations was undertaken. The results obtained from this study were correlated to the analytical findings for the metabolites measured in Chapters 3 and 4 of this thesis where possible. Although it is usually difficult to correlate sensory parameters with analytical data, this is a feedback loop that is usually encouraged by researchers, and does help to corroborate analytical findings. In future studies, it would be highly advantageous to verify sensory findings with analytical results for the attributes not analytically measured, but evaluated by the panellists, including creaminess, sweetness, smoothness and effervescence (CO<sub>2</sub> content). Suitable methods need to be further investigated. In this study, it was heartening to note that the organoleptic properties of the Kefir were generally related to the chemical evaluation, as has been found in similar Kefir studies. This was particularly true for the attribute sourness, where the panellists could detect acidity levels accurately.

From the results obtained, it was found that the primary effects (temperature, time and concentration) had a significant influence on sourness, sweetness and buttery flavour, with incubation time also affecting creaminess and yoghurt flavour. Inoculum concentration also influenced the perception of the rare, defective cowy flavour, and overall acceptability was affected by incubation temperature and time (P

$\leq 0.05$ ). There were no statistically meaningful correlations between smoothness and any of the main effects, or combinations of main effects. In terms of overall acceptability, both temperature and time exerted a meaningful influence ( $P \leq 0.05$ ).

These initial results were then further analysed using the Student's t-test, and the findings were that yoghurt flavour (acetaldehyde) was not particularly strong in any of the samples, and was only noticed after extended fermentation periods. This finding was different to the analytical finding in Chapter 4 of this thesis, where prolonged incubation resulted in a decrease in acetaldehyde concentration, which is of concern. The findings for buttery (diacetyl) flavour were the same as for Chapter 4 of this study in terms of an optimum incubation period of 18 h for maximum diacetyl production, however, a further discrepancy was noted in that incubation temperature was inversely related to diacetyl production in this study. A possible explanation for these discrepancies is the fact that the two studies were performed sequentially, rather than simultaneously, resulting in possible micro-organism fluctuations in the grains and thus possible different fermentation products at different time intervals. This possibility is further justifiable in that acetaldehyde and diacetyl concentrations are inter-linked in Kefir, as diacetyl can be produced from acetaldehyde. It would therefore be of great benefit to perform future studies into organoleptic and analytical characteristics simultaneously on the same samples. Creaminess and overall acceptability appeared to be linked to pH, with optimum creaminess scores for Kefir incubated for 18 h. Incubation temperature also influenced overall acceptability, with moderate temperature incubation (preferably 22°C, although incubation at 25°C yielded similar results) being optimal. This observation was probably also linked to desirable pH and TA ranges for good quality Kefir, as discussed in Chapter 3 of this thesis, and is in accordance with literature findings. There may, therefore be an important link between perceived creaminess and overall acceptability in Kefir, with creaminess possibly being the most important attribute for desirable Kefir. This would re-affirm the literature findings that optimal Kefir flavour is obtained with full-cream rather than skim milk Kefir. This insight would, however, need to be further analysed statistically to ensure that this finding is not just circumstantial.

It is therefore evident that for optimal sensory appeal, Kefir should be incubated for 18 h at moderate incubation temperatures (22° or 25°C) and grain inoculum concentrations (0.8% w/v).