PRODUCTION OF KEPI GRAINS USING PURE CULTURES AS STARTERS

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DECLARATION

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

Marise Cronjé

ABSTRACT

Kepi is a refreshing, fermented dairy beverage that differs from other fermented milk products in that it is produced with a mixed microbial community which is confined to discrete grains. These grains can be recovered as a solid matrix at the end of the fermentation and then be re-utilised as a starter to ferment the next batch of milk. The grain microbial community consists of a symbiotic association of yeasts and lactic acid bacteria, but the overall composition of the grains has not been completely elucidated. The microbes in the grains are embedded in a protein-polysaccharide Kefiran matrix, which appears essential for grain formation. The mechanism of grain formation is still not fully understood and it thus remains undecided which organism is really responsible for the production of this protein-polysaccharide matrix. The aim of this study was to isolate, characterise and identify the microbes present in Kefiran from mass cultured South African grains and then to evaluate grain formation with these purified cultures isolated from Kefiran strings using a mass cultivation process.

Sixteen strains of lactic acid bacteria and one yeast strain were isolated from Kefiran strings produced during the mass cultivation of South African Kepi grains. API technology, numerical clustering and DNA sequence comparisons were used to identify the purified isolates. The isolates were grouped into seven clusters by numerical clustering and clustering distance from selected reference and marker strains. The heterofermentative lactobacilli were identified as *Lactobacillus parakefiri* and *Lb. kefiri* and the homofermentative strains as *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. gallinarum, Lb. acidophilus* and *Lb. bavaricus.* One isolate was found to be a member of the genus *Lactobacillus*, but was not positively identified to species level.

Cultures isolated from Kefiran were evaluated for ability to grain formation by adding 1 x 10^9 cfu.ml^{-1} bacteria and 1 x 10^8 cfu.ml^{-1} yeast to double pasteurised, full cream milk during the mass cultivation process. It was found that the control and all the cultures in double pasteurised milk showed grain accumulation indicating that other microbes were present in pasteurised and double pasteurised milk which had an influence on the grain forming ability. The cultures isolated from pasteurised and double pasteurised milk included members of the species *Pediococcus, Acinetobacter, Lactococcus lactis* ssp. *lactis, Candida lipolytica, C. guilliermondii, Chryseobacterium meningosepticum*, *Pseudomonas putida* and four isolates of the *Bacillus cereus* group. It was found that these rod-shaped "milk isolates" resulted in grain accumulation when inoculated into UHT milk and it was concluded that the "milk isolates" did contribute to grain formation. These isolates were then combined with the

Kefiran cultures and this resulted in grains very similar to the traditional Kepi grains. These grains were made from *Lb. gallinarum* in double pasteurised milk as well with a combination of *Lb. gallinarum, Lb. acidophilus, Lb. kefiri, Lb. delbrueckii* ssp. *bulgaricus, Candida lambica* and *Pseudomonas putida* in UHT milk. The grains were firm, elastic and did not dissolve in water but kept their structure and were retained when sieved. An acceptable Kepi beverage was produced from these grains.

From these typically traditional grain characteristics it was concluded that, even though the microbial compositions were probably not the same, the general appearance was similar to traditional grains and that it is thus possible to produce grains from pure single strain Kefiran cultures and "milk isolates". Furthermore, it was possible to produce a Kepi-like beverage from these grains, which included similar characteristics as the traditional Kepi beverage.

UITTREKSEL

Kepi is `n verfrissende, gefermenteerde suiweldrankie wat van ander gefermenteerde produkte verskil in die opsig dat dit vervaardig word deur Kepi korrels in melk te inkubeer. Die Kepi korrels kan aan die einde van die fermentasie herwin word en weer gebruik word om die volgende lot melk te fermenteer. Die korrels bestaan uit `n simbiotiese samestelling van giste en melksuurbakterieë, maar die presiese samestelling van die korrels is steeds onbekend. Die mikro-organismes is vasgevang in `n proteïen-polisakkaried Kefiran matriks en die Kefiran word as essensieel beskou vir korrelvorming. Die meganisme van korrelvorming bly steeds onbekend en daar is nog nie tot `n gevolgtrekking gekom oor watter organisme die Kefiran produseerder is nie. Die doel van die studie was om die mikro-organismes in Kefiran te isoleer en te identifiseer deur Suid-Afrikaanse Kepi korrels te massa kweek. Hierdie mikro-organismes was dan verder geëvalueer ten opsigte van korrel vorming.

Sestien melksuurbakterieë isolate en een gis isolaat is geïsoleer vanuit die Kefiran. API tegnologie, numeriese groepering en DNA volgorde vergelykings was gebruik om die isolate te identifiseer. Die isolate is in sewe groepe verdeel volgens numeriese groepering. Die afstand van verwysings en merker organismes is ook in ag geneem. Die heterofermentatiewe organismes is geïdentifiseer as *Lactobacillus parakefiri* en *Lb. kefiri* en die heterofermentatiewe organismes as *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. gallinarum, Lb. acidophilus* en *Lb. bavaricus.* Een isolaat kon nie geïdentifiseer word tot op spesie vlak nie, maar is verwant aan die genus *Lactobacillus*.

Hierdie geïsoleerde Kefiran kulture is geëvalueer ten op sigte van korrelvorming, deur 1 x 10^9 kve.ml^{-1} van die bakterieë en 1 x 10^8 kve.ml^{-1} van die gis by dubbel gepasteuriseerde volroom melk te voeg tydens die massakwekings proses. Die kontrole wat geen bygevoegde kulture bevat nie, sowel as die wat wel bygevoegde kulture bevat, het korrel vorming getoon. Laasgenoemde toon dat daar organismes teenwoordig is in gepasteuriseerde en dubbel gepasteuriseerde melk wat `n rol kan speel tydens korrelvorming. Die kulture wat geïsoleer is vanuit gepasteuriseerde en dubbel gepasteuriseerde melk, sluit in: *Pediococcus, Acinetobacter, Lactococcus lactis* ssp. *lactis, Candida lipolytica, C. guilliermondii, Chryseobacterium menigosepticum*, *Pseudomonas putida* en vier isolate van die *Bacillus cereus* groep. Hierdie organismes wat uit melk geïsoleer is, het korrelvorming getoon in UHT melk en die gevolgtrekking kan gemaak word dat die "melk organismes" wel `n rol speel tydens korrel vorming. Hierdie "melk isolate" in kombinasie met die Kefiran kulture het korrels tot gevolg gehad wat baie dieselfde was as tradisionele Kepi korrels. Laasgenoemde korrels is gemaak deur *Lb. gallinarum* in dubbel gepasteuriseerde melk, sowel as deur `n

kombinasie van *Lb. gallinarum, Lb. acidophilus, Lb. kefiri, Lb. delbrueckii* ssp. *bulgaricus, Candida lambica* en *Pseudomonas putida* in UHT melk. Die korrels was stewig, elasties, het nie opgelos in water nie en het hulle struktuur behou wanneer gesif.

Wanneer hierdie tipiese tradisionele korrels se eienskappe in ag geneem word, kan die gevolgtrekking gemaak word dat alhoewel die mikrobiese samestelling van die korrels nie dieselfde is as die tradisionele korrel nie, is die algemene voorkoms en eienskappe dieselfde en dat dit wel moontlik is om korrels te produseer deur isolate geïsoleer vanuit Kefiran en melk. Verder was dit moontlik om `n drankie te vervaardig met die korrels wat baie dieselfde is as tradisionele Kepi.

Dedicated to my parents, Wessel and Susan,
for their endless love.

CONTENTS

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

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CHAPTER 1

INTRODUCTION

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A substantial proportion of the South African population is extremely poor and in addition also has a high incidence of lactose intolerance (Buttriss, 1997). They prefer traditional fermented milks, which are cheaper, more nutritious and with a low lactose content compared to other commercially available dairy products. Traditional fermented milk products are prepared by a spontaneous natural fermentation resulting from the activity of microbes inherent to raw milk or other substrates under normal environmental conditions (Mutukumira *et al.,* 1996). Natural milk fermentation by lactic acid bacteria (LAB) is one of the oldest methods of food bio-preservation and plays an important role in the production of traditional fermented milks.

Maas (Amazi) is such a natural traditional fermented milk product which has been produced for many generations by just allowing unpasteurised milk to sour (Keller & Jordaan, 1990). A recent food consumption study conducted in the rural areas of the Eastern Cape showed an average consumption of 1.4 litres of home-made Maas per day per adult equivalent (M. Nomakaya, 1999, Department of Agricultural Economics, University of Fort Hare, personal communication). Maas is presently commercially manufactured but is too expensive for most members of the low income communities to purchase.

Commercial dairy products in South Africa are normally developed and produced for sophisticated and affluent consumers (Van Wyk *et al.,* 2002). Thus both the price and technology make commercial Maas unsuited to the majority of South Africa`s population with their very low purchasing power and their specific living conditions. Furthermore, a new South African law came into effect in 1999, and stipulates that nobody may sell raw (unpasteurised) milk or raw cream unless it is to be used for further processing (Anon., 1997; Viall, 1999). This results in a situation where urban, low-income African consumers are distanced from a highly nutritional traditional food product. Based on these facts, Van Wyk *et al.* (2002) concluded that for the low income market, low cost dairy products combined with low cost relatively simple technologies will have to be developed. There is thus a special need for a fermented milk product which is cheap, easy to produce, with the taste and aroma attractive to the African low income consumers. Kepi, also known as Kefir, is a product that fits this description.

Kepi is a self carbonated, fermented beverage with a pH of about 4.0, a clean, pleasant acid taste without any bitterness, a prickling and sparkling of CO_2, a slight yeasty taste and aroma and an homogeneous consistency - altogether a very refreshing beverage (Kemp, 1984; Kwak *et al.,* 1996). The Kepi beverage is manufactured by fermenting milk with a starter culture

in the form of Kepi grains. These Kepi grains are a symbiotic association of yeasts, LAB and sometimes acetic acid bacteria embedded in a resilient protein-polysaccharide Kefiran matrix, which leads to an acid-alcoholic fermentation of the milk substrate (Marshall, 1984; Garrote *et al.*, 1997).

Kepi beverage production is a low-cost method of preserving milk where Kepi grains are placed in either pasteurised or raw, full or skimmed milk in a clean container and incubated at room temperature for approximately 24 h or until the desired consistency is reached (Schoevers, 1999). The mixture is then strained into a bowl to separate and retrieve the grains, which can immediately be used as starter to ferment the next milk batch. Thus Kepi differs from other fermented dairy products due to the fact that the Kepi grains can be recovered as a solid matrix at the end of the fermentation. The only way to increase the mass of these grains, is to let the grain biomass grow and multiply in milk and impart their properties and structure to the new grains.

The production of Kepi grains through the normal cultivation process is somewhat laborious and time consuming, and especially on an industrial scale, it is an expensive way to increase Kepi grains (Marshall, 1987). The production process is also dependent on previous fermentations to obtain Kepi grains. In spite of much research over many years, a follow-up fermentation is still the only way to obtain more Kepi grains. There is thus a need to produce Kepi grains using a cheap, rapid and effective method (Duitschaever, 1989).

According to literature reports, attempts to reproduce the Kepi grains from the constituent microbes have invariably failed (Lipatov, 1978; Koroleva, 1991; Rea *et al.*, 1996). Whether this is the result of failure to isolate the true key organism/s responsible for the maintenance of the structure of the grain, or due to the complex structure and arrangement of the microbes in the grains, is not known. It has been suggested that the Kefiran, in which the microbes are embedded, is essential for grain formation and integrity (Marshall, 1984), but the bacterium or bacteria responsible for the production of the Kefiran in the grains have not yet been positively identified (Özer & Özer, 2000).

With these facts in mind, a more practical solution for the mass culturing of Kepi grains might be to use pure defined cultures which play a role in the production of Kefiran, as starters. This method would allow better control of the microbes and metabolic activities involved, as well as simplify the production and lead to a more consistent beverage quality. The use of defined cultures would also permit modifications of the product to achieve certain additional health and nutritional benefits (Saloff-Coste, 2000). Producing Kepi grains from pure cultures could thus be an important contribution to the fermented milk industry.

The main objective of this study was to evaluate the use of pure cultures as starter/s for the production of Kepi grains. This will be done firstly, by isolating the organisms present in the

production of the protein-polysaccharide matrix, and secondly, evaluating the isolates singly or in combination for the mass cultivation of the Kepi grains.

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CHAPTER 2

LITERATURE REVIEW

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LITERATURE REVIEW

A. BACKGROUND

Fermentation of milk is an ancient practice (Roginski, 1988) and has been an important part of the traditional diets of most European, Asian and African countries (Koroleva, 1988b). Fermented milk products are a palatable and economical source of a wide range of nutrients (Gurr, 1987). The nutrient composition is similar to that of milk, but concentrations of vitamins are generally lower, with the possible exception of folic acid. Concentrations of lactic acid, galactose, free amino acids and fatty acids are increased as a result of the fermentation.

Fermented milk products have characteristic flavours, aromas and consistencies, and can be stored unspoilt for a longer time than untreated fresh milk (Roginski, 1988). Based on the metabolism of the microbes, fermented milks can generally be divided into three groups: lactic, yeast-lactic and mould-lactic fermentations (Johnson & Steele, 1997). The first group can be subdivided according to the characteristics of the lactic microbes into mesophilic and thermophilic fermentations. Lactic acid bacteria can further be divided into homofermentative and heterofermentative groups (Marshall, 1984b), where the homofermentative produce lactic acid as major end-product and the heterofermentative organisms lactic acid, carbon dioxide, ethanol, acetoin and diacetyl.

The ready-to-serve Kepi drink (also known as Kefir) is characterised by a compact and uniform curd, creamy consistency and a sour, bracing and slightly effervescing taste (Libudzisz & Piatkiewicz, 1990). The production of Kepi involves yeast-lactic fermentation with two steps: first by fermentation of lactose to lactic acid by bacteria; and secondly, the fermentation of lactose or lactate to alcohol and carbon dioxide by yeasts (Marshall, 1984b). Carbon dioxide leads to carbonation, which lends a refreshing feature especially in products like Kepi. Lactic acid gives a slightly acidic taste, and its mixture with ethanol and other flavour products create the exotic flavour of Kepi (Duitschaever *et al.,* 1987; Guzel-Seydim *et al.,* 2000). The sensoric characteristics of Kepi are: pH of about 4; a clean, pleasant, acidic taste without any bitterness; prickling and sparkling, a slight taste of yeast; alcohol content varies from 0.5 to 2%; a smooth texture and altogether a very refreshing beverage (Kemp, 1984; Duitschaever *et al.*, 1987).

The history of Kepi is lost in time (Koroleva, 1988b) as there is no record of the date or time when Kepi grains were first made (Kemp, 1984). The grains were probably developed accidentally and consequently their function and value were recognised (Kurmann *et al.*, 1992).

Traditionally fresh milk was fermented in leather bags made from goat skins, which were hung in the house during winter and outside during summer (Ayres *et al.*, 1980; Kurmann *et al.*, 1992). Whenever some of the fermented drink was taken from the bags a new batch of fresh milk was added. Currently the traditional method of making Kepi is by adding Kepi grains directly as a starter to milk that has been pasteurised and cooled to 20° - 25°C (Koroleva, 1991). After a 26 h fermentation period, the grains are removed by filtration and the beverage is ready for consumption.

At the end of the 18th century after the publication of research on the healthy properties of Kepi, Kepi grains were brought from the Caucasus with great difficulty and, in 1908, its production in small quantities was organised (Koroleva, 1991). The industrial production of Kepi started in the 1930s, after Russian medical doctors stated that Kepi had beneficial effects in the treatment of intestinal and stomach diseases (Koroleva, 1988b). The first Kepi produced was a set type product of bad quality. In the 1950s specialists developed a method for the manufacturing of Kepi, which provided a product with properties close to those of the traditional product.

Today fermented milk foods can be counted among the most important contributors to human welfare (Marshall, 1982; Heller, 2001). Kepi as a fermented product proved to be useful to control several diseases (Zubillaga *et al.*, 2001). Kepi possesses a high antimicrobial activity against extraneous intestinal microbes, due to the presence of acetic acid bacteria and yeasts as part of the microbial consortium (Koroleva, 1988b; Roginski, 1988). Lactose-intolerant individuals (Cheeseman, 1991) tolerate lactose when it is consumed as a fermented milk product like Kepi, as a result of the conversion of a percentage of the lactose to organic acids and other compounds (Gurr, 1987; Keller & Jordaan, 1990). Kepi contributes to a more even food movement in the intestine, which is recommended for patients with gastro-intestinal and metabolic diseases. Kefiran, a water-soluble polysaccharide found in Kepi grains, has been shown to have the property of retarding tumour growth *in vivo* when administered orally in mice (Zubillaga *et al.*, 2001). Other therapeutic benefits, compared to milk, include an increase in the vitamin B_1, B_2 and folic acid content and higher values of non-protein and free amino-nitrogen content after pepsin digestion *in vitro* which probably lead to better digestibility of protein. It is also generally known that various Kepi cultures are able to assimilate cholesterol in different ranges to produce a low-cholesterol type beverage (Vujičić *et al.*, 1992).

Kepi has the potential to expand the fermented milk market in that it has therapeutic benefits as well as a novel characteristic of fizziness. This makes an appealing refreshing drink in combination with snack foods, dips and dressings (Marshall, 1987).

B. KEPI GRAINS

Kepi grains (Fig. 1) are characterised by an irregular form, folded or uneven surface, white or yellowish colour, elastic consistency, quite tough (Bottazzi & Bianchi, 1980; Koroleva, 1991) and are insoluble in water and ordinary solvents (Kosikowski, 1982). In a dry form (Fig. 2) the grains are yellow and during active growth they are almost white. The diameter may be in the range of 1 - 6 mm, depending on the intensity of the agitation during their growth. The more intense the agitation, the smaller the Kepi grains are in size. This has an advantage as a small grain size provides a larger surface area in contact with the milk and thus leads to a higher activity of the microbes. In addition to the white cauliflower-like structures, smaller thinner sheet-like structures and scroll-like grain forms can also be present (Marshall, 1984a; Marshall *et al.*, 1984). The cauliflower structures measure approximately 20 x 10 x 10 mm and are white, while the sheet-like structure contains a smooth, flat side and the other side is convoluted and rough (Bottazzi & Bianchi; 1980, Marshall *et al.*, 1984). Kepi grains probably arise from the curling of flat sheet-like structures with subsequent folding and re-folding accompanied by increased thickening as the microbes multiply and insoluble carbohydrate accumulates (Marshall *et al.*, 1984). The grains are composed mostly of proteins and polysaccharides in which the complex microbial community is enclosed (Garrote *et al.*, 1998). The granules contain about 10.3% ($m.m^{-1}$) dry matter of which the solids consist of 3.5% fat, 32.6% protein, 6% ash and 56.2% carbohydrate (Ayres *et al.*, 1980).

In the production of Kepi, the starter is in the form of grains (Marshall, 1984a). These grains settle to the bottom when added to milk, but in time the fermentation process may carry the grain structure to the top as a result of the gas development (Hammer & Babel, 1959). They grow and multiply in milk and impart their properties and structure to the new granules (Ayres *et al.*, 1980). The granules which develop during fermentation, float on top and can be removed and dried. In the dry state these grains resemble small seeds, and when added to milk, they swell and become active in bringing about fermentation (Burke, 1938). Microbes will continuously be shed from the grain into the milk medium, but Kepi milk cannot be used as a starter, due to the loss of the balance of the microbes (Marshall, 1984a).

For propagation of the starter culture, the presence of all the microbes composing the Kepi grain in the desirable and adequate proportion, is required (Kosikowski, 1982). Dried Kepi grains are known to be able to retain activity for 12 - 18 months, whereas wet grains retain activity for only 8 - 10 days. Garrote *et al.* (1997) concluded that freezing can be used to preserve the Kepi grain. Grains stored at -20° and -80°C showed a greater increase in grain weight by successive subculturing, but those stored at 4°C did not increase their weight after

Figure 1. A sample of Kepi grains.

Figure 2. A sample of dried Kepi grains (Cilliers, 2001).

eight milk transfers. The growth rate of the Kepi grains at 4°C means that the microbes present in the grain are actively multiplying (Yokoi *et al.,* 1990). The products obtained with Kepi grains stored at -20° and -80°C were similar and storage in a freezer at -20°C can be used to maintain the grains for Kepi production.

C. MICROBIAL COMMUNITY

The Kepi grains contain a complex microbial community consisting of lactic acid bacteria (LAB) and yeasts (Rea *et al.,* 1996; Marshall, 1984a). Yeasts, especially non-fermenting yeasts, have a close symbiosis with the lactobacilli (Marshall, 1984a) as the bacteria control lactic acid production and the yeasts subsequently produce alcohol.

According to Pintado *et al.* (1996), Portuguese Kefir grains consisted predominantly of *Saccharomyces delbrueckii* and *Lactobacillus kefir*, while Rea *et al.* (1996) studied Irish Kefir grains and found that lactococci were the dominant microbes and that acetic acid bacteria (AAB) were absent in all their Kepi grains. Garrote *et al.* (1997) found two yeasts (*Sac. cerevisiae* and *Sac. lipolytic*), a mycelial fungus (*Geotrichum candidum)*, two cocci (*Lactococcus lactis* ssp. *diacetylactis* and *Lac. lactis* ssp. *lactis*) and four lactobacilli (*Lb. kefiranofaciens*, *Lb. kefirgranum, Lb. kefir* and *Lb. parakefir*) as the main microbes in Kepi grains from Argentina. Schoeman (2001) found *Lb. fermentum*, *Leuconostoc mesenteroides*, *Lb. delbrueckii* and *Candida kefir* in South African Kepi grains after Kepi was continuously produced over a period of 30 days, while *Lb. plantarum*, *Lc. lactis*, *Candida lambica* and *Cand. krusei* were found in the South African Kepi grains after 10 days of mass production (Shoevers, 1999).

Various organisms have been isolated from Kepi and it is well known that the species present are not constant (Hammer & Babel, 1959). The composition of the microbes can also differ according to the place of origin, storage conditions and handling pattern (Iwasawa *et al.,* 1982; Pintado *et al.,* 1996). La Rivière *et al.* (1967) suggested that the washing of the grain might lead to the loss of some microbes, particularly those which are loosely attached to the grain. La Rivière *et al.* (1967) also concluded that at least part of the microbial composition of the grain is specific and constant, and capable of surviving among numerous different microbes that can develop as a result of the lack of hygienic conditions. Differences in microbial composition, for example the inclusion of species of *Acetobacter* and *Enterobacteriaceae* may be explained by the lack of hygiene during treatment (Rosi & Rossi, 1978; Marshall *et al.* 1984; Takizawa *et al.* 1998). Different microbiological qualities of the milks used or lack of asepsis during product manufacture of the product might lead to the isolation of species of *Bacillus*, *Micrococcus* or *Pediococcus* from raw or untreated milk (Angulo *et al.,* 1993).

The numerous species that have been reported to be associated with Kepi and Kepi grains are summarised in Table 1. The current nomenclature of the LAB relevant to fermented milk products are given in Table 2. The distribution of microbes in the Kepi grain and milk are listed in Table 3. According to Wood (1990), the lactobacilli (homo- and heterofermentative, meso- or thermophilic) constitute about 65 - 80% ($m.m^{-1}$) of the microbial content, the lactococci 20% and the yeasts 5%. The LAB include lactobacilli, lactococci, leuconostoc and streptococcus.

Takizawa *et al.* (1998) divided the lactobacillus population into four groups based on taxonomical characteristics, *Lb. kefirgranum* accounting for 49% ($m.m^{-1}$), *Lb. kefiranofaciens* for 43%, *Lb. kefir* for 6% and *Lb. parakefir* for 2% of the isolates. The main microbes of Kepi grains determined were homofermentative lactobacilli (*Lb. kefirgranum* and *Lb. kefiranofaciens*), while heterofermentative lactobacilli (*Lb. kefir* and *Lb. parakefir*) were minor populations. Kandler & Kunath (1983) reached a similar conclusion and reported that the homofermentative lactobacilli constitute about 90% of the total lactobacilli and the heterofermentative lactobacilli 10%.

According to Neve (1992), lactococci are scarce in the grain-associated populations. This scarcity of lactococci in Kepi grains has also been reported in other studies (Bottazzi & Bianchi, 1980; Kandler & Kunath, 1983; Duitschaever *et al.,* 1988; Toba *et al.,* 1990). However, lactococci can be isolated from the Kepi beverages in high numbers, indicating that a microbial shift occurs during fermentation of milk (Kandler & Kunath, 1983). Simultaneously, Rea *et al.* (1996) concluded that lactococci will dominate during the Kepi fermentation in the fermented milk and not in the Kepi grain itself.

The presence of acetic acid bacteria have not often been reported as part of the grain structure, but it is known that they produce vitamin B_{12} and may therefore stimulate other organisms in the grain (Rea *et al.,* 1996). Acetic acid bacteria have a highly aerobic nature and their cessation of growth after about 20 h is probably as a result of oxygen depletion (Koroleva, 1988a). These microbes are very active in maintaining symbiosis between the Kepi starter microbial populations (Koroleva, 1988a) and improve the Kepi consistency by increasing its viscosity (Libudzisz & Piatkiewicz, 1990).

The yeasts play an important role by promoting symbiosis among the microbes, CO_2 formation and the development of characteristic taste and aroma (Koroleva, 1988b). Both budding and elongated yeasts have been reported and due to the leading role of lactose fermentable yeasts in the alcoholic fermentation, they are considered characteristic of the Kepi population (Iwasawa *et al.,* 1982), but non-fermentable yeasts have also been found in Kepi.

It has been reported that the surface of Kepi grains is often covered with a white mycelial fungus, *Geotrichum candidum*, which does not detract much from the beverage quality

Table 1. Microbes associated with the Kepi beverage and the Kepi grains.

Microbes	Reference
Enterococcus durans	Marshall, 1993
Lactic acid bacteria	
Lactobacillus acidophilus	Libudzisz & Piatkiewicz, 1990; Kwak *et al.*, 1996; Marshall, 1987
Lactobacillus brevis	Rosi & Rossi, 1978; Angulo *et al.*, 1993
Lactobacillus casei	
ssp. *alactosus*	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
ssp. *pseuoplantrum*	Angulo *et al.*, 1993
ssp. *rhamnosus*	Koroleva, 1998a; Libudzisz & Piatkiewicz, 1990; Marshall, 1993
ssp. *tolerans*	Angulo *et al.*, 1993
Lactobacillus cellobiosus	Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Kwak *et al.*, 1996
*Lactobacillus bulgaricus**	Koroleva, 1988a; Marshall, 1993; Kwak *et al.*, 1996
*Lactobacillus lactis**	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
Lactobacillus fermentum	Angulo *et al.*, 1993
Lactobacillus helveticus	
ssp. *jugurti*	Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990; Kwak *et al.*, 1996
ssp. *lactis*	Marshall, 1993
Lactobacillus ssp. KPB-167B	Yokoi *et al.*, 1990
*Lactobacillus kefir**	Kandler & Kunath, 1983; Marshall, 1993; Kwak *et al.*, 1996; Pintado *et al.*, 1996
Lactobacillus kefiranofaciens	Toba *et al.*, 1986; Fujisawa *et al.*, 1988; Mukai *et al.*, 1990a
Lactobacillus kefirgranum	Takizawa *et al.*, 1998
Lactobacillus kefis	Pintado *et al.*, 1996
Lactobacillus lactis ssp. *lactis*	Kwak *et al.*, 1996
*Lactobacillus parakefir**	Takizawa *et al.*, 1998
Lactobacillus plantarum	Kwak *et al.*, 1996
Lactococcus filant	Kwak *et al.*, 1996
*Streptococcus lactis**	
ssp. *cremoris*	Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990; Marshall, 1993
ssp. *lactis*	Koroleva, 1988a; Kwak *et al.*, 1996
ssp. *lactis* biovar. *diacetylactis*	Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990
Leuconostoc kefir	Kwak *et al.*, 1996
Leuconostoc mesenteroides	
ssp. *cremoris*	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
ssp. *dextranicum*	Koroleva, 1988a; Kwak *et al.*, 1996
ssp. *mesenteriodes*	Marshall, 1993; Kwak *et al.*, 1996
Streptococcus durans	Marshall, 1987; Kwak *et al.*, 1996
Streptococcus salivarius ssp. *thermophilus*	Libudzisz & Piatkiewicz, 1990; Kwak *et al.*, 1996
Streptococcus filant	Libudzisz & Piatkiewicz, 1990

Table 1. *(cont.)*

Acetic acid bacteria	
Acetobacter aceti	Angulo *et al.*,1993
Acetobacter rancens	Angulo *et al.*,1993
Yeasts	
Brettanomyces anomalus	Mann, 1979; Garrote *et al.*, 1997; Lin *et al.*, 1999
Candida colliculosa	Wyder *et al.*, 1999
Candida friedricchii	Mann, 1979; Garrote *et al.*, 1997; Lin *et al.*, 1999
Candida holmii	Marshall, 1993; Brialy *et al.*, 1995
Candida kefir	Marshall, 1987; Roginski, 1988; Libudzisz & Piatkiewicz, 1990; Marshall, 1993
Candida lambica	Mann, 1979; Garrote *et al.*, 1997; Lin *et al.*, 1999
Candida pseudotropicalis	Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Pintado *et al.*, 1996
Candida tenuis	Pintado *et al.*, 1996
Candida valida	Mann, 1979; Garrote *et al.*, 1997; Lin *et al.*, 1999
Kluyveromyces bulgaricus	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
Kluyveromyces lactis	Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Kwak *et al.*, 1996
Kluyveromyces marxianus	Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Kwak *et al.*, 1996
ssp. *marxianus*	Roginski, 1988; Koroleva, 1988a; Marshall, 1993; Kwak *et al.*, 1996
Saccharomyces carlsbergensis	Libudzisz & Piatkiewicz, 1990; Kwak *et al.*, 1996
Saccharomyces cerevisiae	Marshall, 1987; Koroleva, 1988a; Kwak *et al.*, 1996
Saccharomyces exiguus	Wyder *et al.*, 1999
Saccharomyces turicensis	Wyder *et al.*, 1999
Saccharomyces florentinus	Libudzisz & Piatkiewicz, 1990; Brialy *et al.*, 1995
Saccharomyces globosus	Libudzisz & Piatkiewicz, 1990
Saccharomyces lipolytic	Mann, 1979; Garrote *et al.*, 1997; Lin *et al.*, 1999
Saccharomyces lactis	Pintado *et al.*, 1996
Saccharomyces unispores	Libudzisz & Piatkiewicz, 1990
Torulopsis holmii	Kwak *et al.*, 1996
Torulopsis kefir	Marshall, 1987; Libudzisz & Piatkiewicz, 1990
Torulaspora delbrueckii	Iwasawa *et al.*, 1982
Mycelial fungus	
Geotrichum candidum	Marshall, 1987; Roginski, 1988

* For current nomenclature, see Table 2.

Table 2. Current nomenclature of the LAB relevant to fermented milk products (Marshall, 1987; Roginski, 1988, Truper & De'Clari, 1997, 1998).

New nomenclature	Old nomenclature
Lactic acid bacteria	
Lactococcus	*Streptococcus*
L. lactis ssp. *lactis*	*S. lactis* ssp. *lactis*
L. lactis ssp. *lactis* biovar. *diacetylactis*	*S. lactis* ssp. *diacetylactis*
L. lactis ssp. *cremoris*	*S. lactis* ssp. *cremoris*
Lactobacillus	
Lb. delbrueckii ssp. *delbrueckii*	*Lb. delbrueckii*
Lb. delbrueckii ssp. *leichmannii*	*Lb. leichmannii*
Lb. delbrueckii ssp. *bulgaricus*	*Lb. bulgaricus*
Lb. delbrueckii ssp. *lactis*	*Lb. lactis*
Lb. kefir	*Lb. caucasicus*
Lb. kefiri	*Lb. kefir*
Lb. parakefiri	*Lb. parakefir*
Leuconostoc	
Leuc. mesenteroides ssp. *mesenteroides*	*Leuc. mesenteroides*
Leuc. mesenteroides ssp. *cremoris*	*Leuc. cremoris*
Streptococcus	
S. salivarius ssp. *thermophilus*	*S. thermophilus*
Yeasts	
Torulaspora delbrueckii	*Saccharomyces delbrueckii*
Kluyveromyces marxianus spp. *marxianus*	*Saccharomyces kefir, Saccharomyces fragilis, Kluyveromyces fragilis*
Kluyveromyces marxianus	*Saccharomyces fragilis* spp. *bulgaricus*
Candida kefir	*Saccharomyces kefir, Torulopsis kefir, Candida pseudotropicalis* var. *lactosa*
Candida lambica	*Pichia fermentans*

Table 3. Distribution of microbes in Kepi grains (cfu.g $^{-1}$) and fermented milk (cfu.ml^{-1})

	Lactococci	Leuconostoc	Acetic acid bacteria	Lactobacilli	Yeast	Reference
Grain	-	$1x10^6$	$1x10^6$	$1x10^9$	$1x10^8$	Marshall, 1993
	$1x10^7$	-	-	$1x10^9$	$1x10^8$	Garrote *et al.*, 1997
	-	-	-	-	$1x10^5$- $1x10^7$	Schoeman, 2001
	$1x10^7$- $1x10^8$	-	-	$1x10^7$- $1x10^8$	$1x10^8$	Wood, 1990
Beverage	$1x10^9$	$1x10^8$	$1x10^5$	$1x10^8$	$1x10^6$	Marshall, 1993
	$1x10^9$	-	-	$5 x 10^6$	$1x10^6$	Rea *et al.*, 1996
	$1x10^9$- $1x10^{10}$	-	-	$1x10^9$- $1x10^{10}$	$1x10^6$- $1x10^7$	Garrote *et al.*, 1997

(Kosikowski, 1982). Bacilli and micrococci, which are not desired and hasten spoilage of the product, may also contaminate the grains.

The importance of propionibacterium in Kepi is its capability to produce vitamin B_{12} (Lyon & Glatz, 1995). Studies determining the influence of the growth of LAB without *Propionibacterium* on the level of vitamin B_{12} have shown that the concentration of this vitamin decreased between 15 and 95% during lactic acid fermentation of milk (Lui & Moon, 1983). Kepi produced using Kepi grains together with "*Propionibacterium shermanii*" was found to be a product with a high food value, rich in proteins and vitamins, including vitamin B_{12} (Lui & Moon, 1983).

D. GRANULE MICROBIAL MICROSTRUCTURE

The mixed microbes present in the Kepi grain are not indiscriminately intermingled, but appear to have a particular arrangement (Marshall *et al.*, 1984; Özer & Özer, 2000). Light (Marshall *et al.*, 1984) and electron microscopy (Bottazzi & Bianchi, 1980; Marshall *et al.*, 1984; Duitschaever *et al.*, 1987) of Kepi grains have demonstrated particular arrangements of LAB and yeasts.

Bottazzi & Bianchi (1980) studied the microstructure of cauliflower Kepi granules (Fig. 3) by means of scanning electron microscopy and found that the peripheral part of the granule was densely populated by a microbial population composed almost exclusively of short, rod-shaped bacteria. The internal part of the granule was characterised by yeasts arranged on a rather porous structure. In this central part, the microbial population was smaller than that in the more peripheral part of the granule. Toba *et al.* (1990) also reported the same special localisation of microbes in non-propagable grains. Rea *et al.* (1996) concluded that in areas where yeasts predominate there were few lactobacilli, and where lactobacilli predominate, there were few yeasts. Toba *et al.* (1990) concluded that they could not observe a particular arrangement of microbes in propagable grains, except that encapsulated bacteria resided in the inner parts. It has also been reported that the Kefiran producing lactobacilli are imbedded inside the sheet-like structures, where grain growth is favoured by anaerobic conditions and the presence of ethanol (Varnam & Sutherland, 1994).

Marshall *et al.* (1984) concluded that a population of lactobacilli, which reside within the matrix, produces the matrix in which the microbes are embedded. Non-kefiran producing lactobacilli and yeasts each predominate on separate sides of the sheets. Toba *et al.* (1990) also found that rod-shaped bacteria and yeasts colonised separately on a spongy material on the inside of the grain. According to Korovela (1988a), Roginski (1988) and Neve (1992) non-lactose

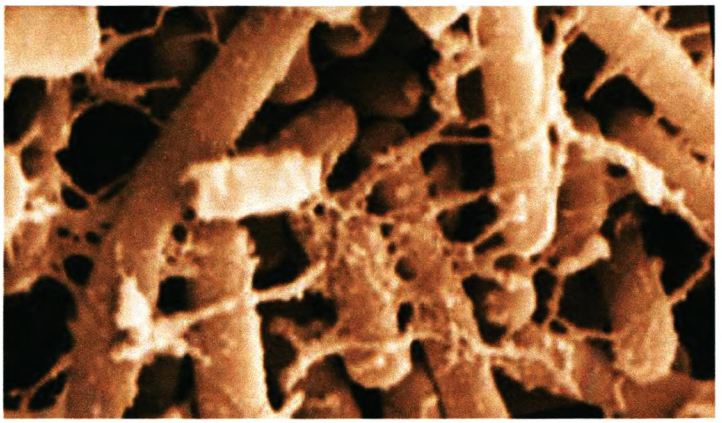

Figure 3. Figure of Kepi granule microstructure.

fermenting yeasts are found in the deep layers of the Kepi grains. Korovela (1988a) found that lactose fermenting yeasts are present mainly in the peripheral layers, while Roginski (1988) and Neve (1992) found the lactose fermenting yeasts in the fermented milk.

According to Korovela (1988a), Roginski (1988) and Neve (1992) non-lactose fermenting yeasts are found in the deep layers of the Kepi grains. Korovela (1988a) found that lactose fermenting yeasts are present mainly in the peripheral layers, while Roginski (1988) and Neve (1992) found the lactose fermenting yeasts in the fermented milk.

E. FROM MILK TO KEPI

Cow's milk contains about 3 - 4% ($m.m^{-1}$) protein, 3 - 6% fat, 5% lactose and 0.7% ash, giving a total solids content of 11.5 - 15.5% (Cheeseman, 1991). During the production of Kepi, some of the lactose is depleted and as lactic acid is produced, the pH decreases (Kwak *et al.,* 1996). Lactose, a disaccharide composed of glucose and galactose, is the only free-form sugar present in milk, and the first step in the transformation of lactose to lactic acid is the hydrolysis of lactose to glucose and galactose through homofermentative or heterofermentative pathways (Hammer & Babel, 1959).

Lactose metabolism

Lactic acid bacteria can be divided into two groups based on metabolic end-products of glucose metabolism (Johnson & Steele, 1997). The LAB that produce lactic acid as the major product of glucose fermentation are designated **homofermentative** and include the genera *Pediococcus*, *Streptococcus*, *Lactococcus*, *Vagococcus* along with some of the lactobacilli. Lactose is translocated into the cell by a phosphoenolpyruvate phosphotransferase system (Kandler, 1983). As depicted in Fig. 4. the lactose is phosphorylated during translocation and cleaved into glucose and galactose-6-phosphate. The glucose moiety enters the glycolitic pathway and the galactose-6-phosphate is converted to tagatose-6-phosphate via the tagatose pathway. Both sugars are cleaved by specific aldolases into trioses phosphates, which are converted to pyruvic acid at the expense of NAD^+ (Kandler, 1983). For continued energy production, NAD^+ must be regenerated. This is usually accomplished by the reduction of pyruvic acid to lactic acid.

Streptococcus thermophilus and some thermophilic lactobacilli (*Lb. helveticus* and *Lb. delbrueckii*) transport lactose via a lactose-galactose antiport system driven by an electrochemical proton gradient (Poolman, 1993). Lactose is not phosphorylated but is cleaved by β-galactosidases to yield glucose and galactose. The glucose moiety enters the glycolitic pathway, but galactose is excreted from the cells and accumulates in the milk. Thermophilic

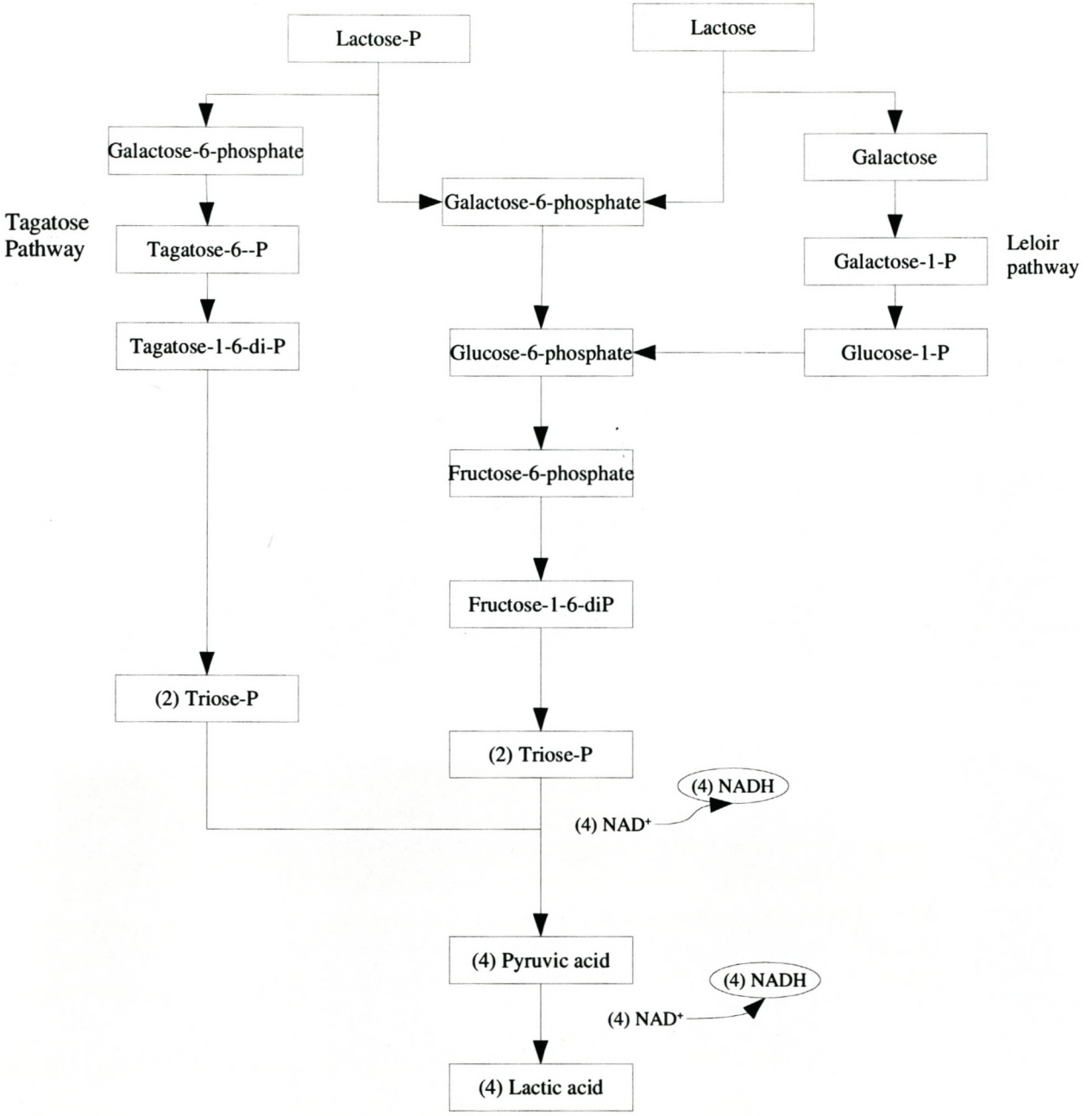

Figure 4. Lactose metabolism in homofermentative LAB (Johnson & Steele, 1997).

lactobacilli that do not excrete galactose and *Lb. helveticus* strains able to transport excreted galactose, utilise the Leloir-pathway to metabolise galactose. *Lb. delbrueckii* and *S. thermophilus* cannot metabolise galactose. These residual sugar can be metabolised heterofermentatively by other bacteria and cause rapid production of carbon dioxide. Lactococci can also be included in the starter to ensure that all the sugar is fermented (Poolman, 1993).

The LAB that produce equal molar amounts of lactic acid, carbon dioxide and ethanol from hexoses are designated **heterofermentative** and comprise the *Leuconostoc*, *Oenococcus*, *Carnobacterium* and some lactobacilli. Lactose is transport by a specific permease and followed by hydrolysis of lactose to D-glucose and D-galactose by ß-galactosidase (Fig. 5) (Kandler, 1983; Johnson & Steele, 1997). The galactose moiety is transformed into glucose-6-phosphate through the Leloir-pathway and, together with glucose, is metabolised through the phosphoketolase-pathway.

Heterofermentative LAB lack aldolases to transform pentoses to hexoses or trioses via transaldolase and transketolase reactions. A pentose sugar xylose-5-phosphate and carbon dioxide are formed through a dehydrogenation-decarboxylation system, and the xylulose-5-phosphate is then cleaved by phosphoketolase to yield glyceraldehyde and acetyl-phosphate. Lactic acid and ethanol are formed from these intermediates, facilitating the regeneration of NAD^+ (Kandler, 1983).

The heterofermentative lactobacilli are more important than the homofermentative lactobacilli in producing flavour and aroma components (Kandler, 1983). The extent to which flavourous compounds (acetylaldehyde and diacetyl) or flavourless compounds (acetoin, butyleneglycol and ethanol) are formed, depends on the oxidation-reduction balance of the system. The flavourless compounds are more reduced structures than the flavourous compounds.

Utilisation of pyruvate

An essential feature of bacterial fermentations is the oxidation of a substrate to generate energy-rich intermediates, which subsequently can be used for ATP production by substrate-level phosphorylation (Fig. 6) (Kandler, 1983). The oxidation results in the formation of NADH from NAD^+, which has to be regenerated in order for the cells to continue the fermentation. Pyruvate serves as an electron acceptor for this regeneration step, and under certain circumstances, LAB use alternative ways of utilising pyruvate than the reduction to lactic acid.

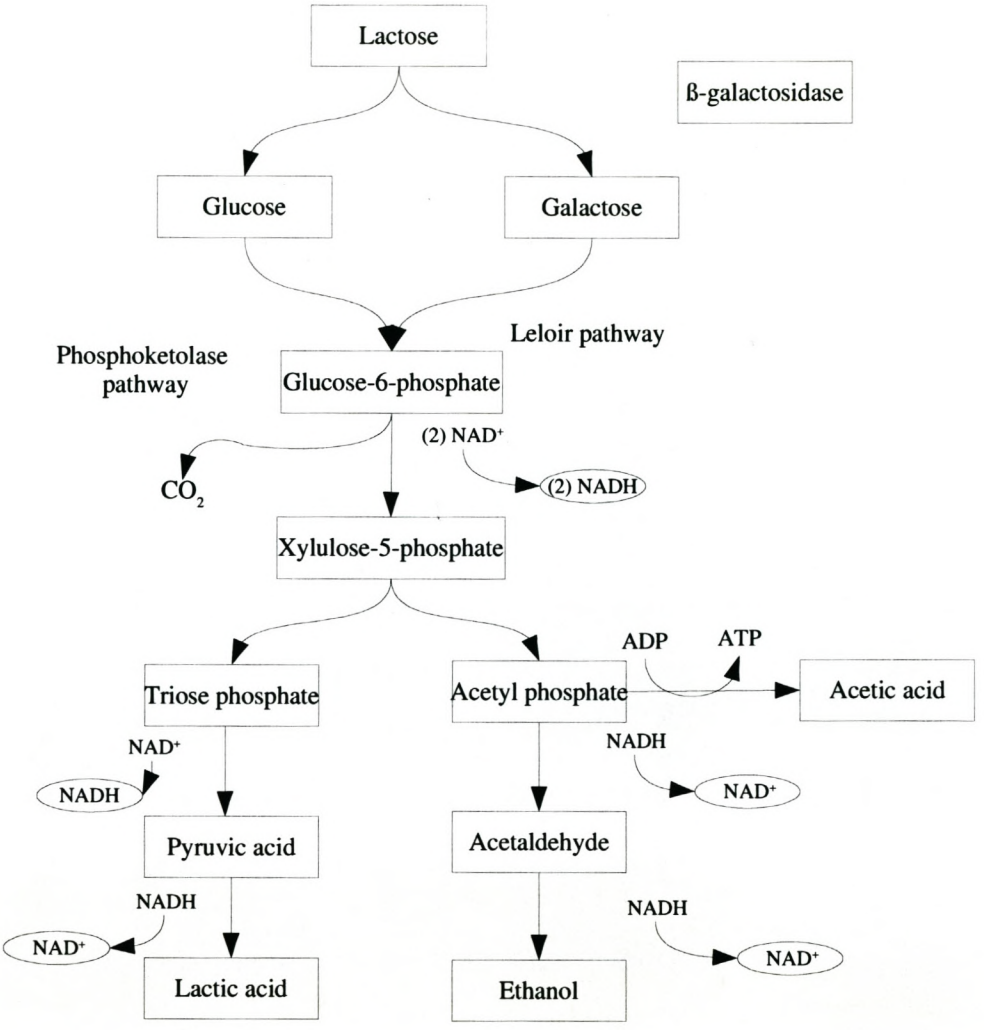

Figure 5. Lactose metabolism in heterofermentative LAB (Johnson & Steele, 1997).

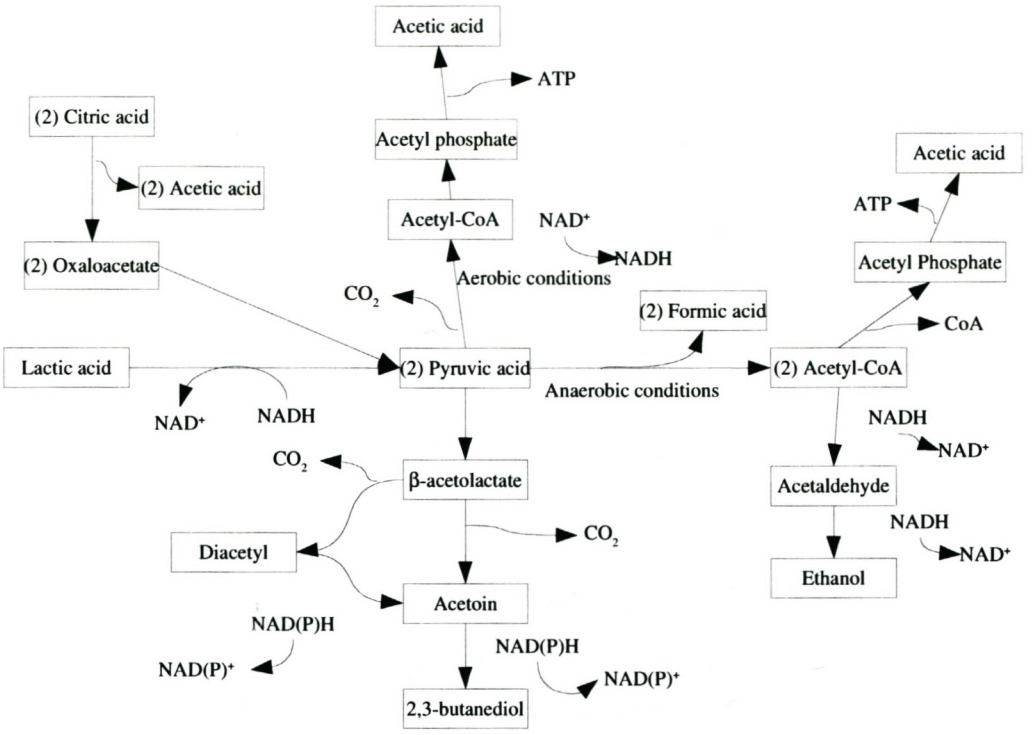

Figure 6. Utilisation of pyruvate (Johnson & Steele, 1997).

Citric acid metabolism

Citrate is considered to be the principal precursor of diacetyl in fermented dairy products (Hugenholtz, 1993). Milk contains 0.15 - 0.2% ($m.m^{-1}$) citric acid, but not all LAB can metabolise this compound. Citric acid is transported into the cell by a citric acid permease, which is plasmid encoded in *Lactococcus* and *Leuconostoc* species and metabolised to pyruvic acid without generation of NADH (Johnson & Steele, 1997). The result is an excess of pyruvic acid which does not have to be reduced to lactic acid to regenerate NAD^+, therefore this excess of pyruvic acid is available for other reactions. *Leuconostoc* species metabolise citric acid during growth but do not form diacetyl until the pH is below 5.4, due to the fact that citrate permease is more active at a low pH (Harvey & Collins, 1962). When diacetyl is not formed, *Leuconostoc* species form lactic acid from pyruvic acid derived from citric acid and regenerate NAD^+. Diacetyl can be reduced by 2,3-butanediol dehydrogenase to acetoin and 2,3-butanediol, both flavourless compounds. The presence of citric acid inhibits these reactions, but reduction begins when citric acid is exhausted. Diacetyl and acetoin are of little metabolic use to the microbes that produce them.

Acetaldehyde production

Acetaldehyde is considered as the centre of flavour production as acetoin, diacetyl and ethanol can all be catabolised from this compound (Lees & Jago, 1976). Ethanol is formed by the reduction of acetaldehyde, a reaction catalysed by alcohol dehydrogenases (Lees & Jago, 1976). *Lactobacillus brevis* produces CO_2, ethanol, acetate, acetoin and diacetyl, while *Lb. bulgaricus* produces large quantities of acetaldehyde, and appears to lack or only to have low levels of the enzymes that metabolises the acetaldehyde to other flavour compounds. *Leuconostoc* species can also prevent an excessive amount of acetaldehyde accumulating through the metabolism of acetaldehyde to ethanol (Johnson & Steele, 1997).

The enzyme pyruvate-formate lyase, favoured under **anaerobic** conditions, catalyses the reaction of pyruvate to formate and acetyl-CoA (Kandler, 1983). Acetyl-CoA may be used either as electron acceptor resulting in ethanol formation, as a precursor for substrate-level phosphorylation via acetyl phosphate, or both. End-products include acetate, formate and ethanol. The enzyme pyruvate oxidase, favoured under **aerobic** conditions, converts pyruvate to CO_2 and acetyl phosphate with the formation of H_2O_2. Significant amounts of acetic acid are formed.

The major end-products of Kepi organisms are lactic acid (about 0.8 – 0.9% ($m.m^{-1}$) and mainly the L(+) form), ethyl alcohol (about 0.035 - 2%) and carbon dioxide (0.08 - 0.2%)

(Kosikowski, 1982; Wood, 1990). The typical Kepi flavour is due mainly to an optimum ratio (3:1) of diacetyl and acetaldehyde (Libudzisz & Piatkiewicz, 1990).

In Table 4 the main metabolic products produced by microbes found in Kepi, are summarised. When seen as a complete synergenic fermentation, it can generally be concluded that the bacteria control the acid production and the yeast produce the alcohol. During alcohol fermentation by lactose fermenting yeasts, lactose and other sugars are utilised to produce ethanol and carbon dioxide (Hammer & Babel, 1959). Ueda *et al.* (1982) found that *Torulopsis holmii*, a lactose non-fermentable yeast, consumes galactose faster than glucose in a galactose-glucose medium. *Saccharomyces* sp. are also able to ferment galactose. Formation of diacetyl during yeast fermentations has been known for a long time, and acetaldehyde can also be produced directly form pyruvate catalysed by pyruvate decarboxylase, an enzyme not present in the other starter organisms (Iwasawa *et al.,* 1982). Yeasts also possess an alcoholic dehydrogenase to convert acetaldehyde to ethanol and *Sac. cerevisiae* may combine acetaldehyde with "active aldehyde" to produce acetoin. Non-lactose fermenting yeasts and AAB can grow in milk only when LAB are present to hydrolyse the lactose.

Members of the *Acetobacter* are obligate aerobes which oxidise ethanol to acetic acid (Roginski, 1988). They also oxidise acetate and lactate to CO_2 and H_2O_2.

F. KEFIRAN

The complex microbial community in the Kepi grain is held together by a matrix of water-insoluble material (Rea *et al.,* 1996), which is essential for grain formation and integrity (Marshall *et al.*, 1984). La Rivière *et al.* (1967) reported that almost half of the material embedding the microbial community of the Kepi grain consists of a polysaccharide with equal amounts of the carbohydrates, galactose and glucose. The carbohydrate is of bacterial origin and is produced by the starter bacteria (Iwasawa *et al.,* 1982). The polysaccharide found in Kepi grains has never been encountered elsewhere in nature, and La Rivière *et al.* in 1967, proposed the name Kefiran. According to La Rivière *et al.* (1967), Delft Kepi grains have a dry weight of 12 - 14% ($m.m^{-1}$), of which 24% consists of the water-soluble polysaccharide, 13% of protein, 46% is insoluble in cold water (cells and debris), and 17% remains undetermined.

The polysaccharides produced by the bacteria have relevant ecological and physiological functions (Micheli *et al.,* 1999), of which the role in the bacteria is not clearly defined (Cerning, 1990; Gamar-Nourani *et al.,* 1998). The capsular structure may protect the cell against unfavourable environmental conditions such as desiccation, phagocytosis and phage attack as well as providing higher oxygen tension, participating in the uptake of metal ions, functioning as

Table 4. Metabolic products of microbes present during Kepi manufacturing (Marshall, 1982; Litopoulou-Tzanetaki & Tzanetakis, 2000)

Organism	Type	Fermentation type	Major end-products	Secondary end-products
Lactococcus lactis ssp. *lactis*	Mesophilic (25°-30°C)	Homofermentative	L(+) lactic acid	Acetaldehyde, Acetone, Diacetyl, Ethanol
Lactococcus lactis ssp. *diacetylactis*	Mesophilic (25°-30°C)	Homofermentative	L(+) lactic acid, Acetaldehyde, Diacetyl, Acetoin, CO_2	Acetone, Ethanol
Streptococcus salivarius ssp. *thermophilus*	Thermophilic (40°-44°C)	Homofermentative	L(+) lactic acid	Acetaldehyde, Acetone, Acetoin, Diacetyl, [Ethanol]
Leuconostoc mesenteroides ssp. *mesenteroides*	Mesophilic (25°-30°C)	Heterofermentative	D(-) lactic acid, Acetoin, Diacetyl, CO_2	Ethanol
Leuconostoc mesenteroides ssp. *cremoris*	Mesophilic (25°-30°C)	Heterofermentative	D(-) lactic acid, Acetoin, Diacetyl, CO_2	Ethanol
Leuconostoc mesenteroides ssp. *dextranicum*	Mesophilic (25°-30°C)	Heterofermentative	D(-) lactic acid, Acetoin, Diacetyl, CO_2	Ethanol
Lactobacillus delbrueckii ssp. *lactis*	Thermophilic (40°-44°C)	Homofermentative	D(-) lactic acid	Acetaldehyde, Acetone, Diacetyl, [Ethanol]
Lactobacillus delbrueckii ssp. *bulgaricus*	Thermophilic (40°-44°C)	Homofermentative	D(-) lactic acid	Acetaldehyde, Acetone, Acetoin, Diacetyl, [Ethanol]
Lactobacillus helveticus	Thermophilic (40°-44°C)	Homofermentative	DL lactic acid	Acetaldehyde, Acetic acid, Diacetyl, [Ethanol]
Lactobacillus acidophilus	Thermophilic (40°-44°C)	Homofermentative	DL lactic acid	Acetaldehyde, [Ethanol]
Lactobacillus kefir	Mesophilic (25°-30°C)	Heterofermentative	DL lactic acid	Acetaldehyde, Acetic acid,[Ethanol], CO_2
Lactobacillus kefiranofaciens	Mesophilic (25°-30°C)	Homofermentative	DL lactic acid	
Lactobacillus brevis	Mesophilic (25°-30°C)	Heterofermentative	Lactic acid, Acetate, CO_2	Acetoin, Diacetyl

Trace end-products are indicated in brackets[]

adhesive agents, and the development systems such as those found in myxobacteria (Cerning, 1990). The presence of the excreted polysaccharides probably favours the interaction between physical supports and the bacteria, resulting in the appearance of biofilms. The polysaccharide does not appear to function as an energy source, since slime-forming bacteria are usually not capable of catabolising polymers.

Excreted microbial polysaccharides are known to have potential applications in food and pharmaceutical productions (Cerning, 1990; Gamar-Nourani *et al.,* 1998). Pharmaceutical applications include antibacterial, antifungal and antitumoral activity. Polysaccharides play an important role in the rheological behaviour and the texture of fermented milks and in the prevention of syneresis. The popularity of "100% natural" food products without any additives, has increased. The utilisation of slime-producing strains in the manufacture of yoghurt has been of particularly interest in France and The Netherlands, since the addition of stabilisers to unfruited yoghurt is prohibited in these countries. Robijn *et al.* (1995) concluded that microbial polysaccharides may form a new generation of food thickeners.

Kefiran can be characterised by means of viscosity, optical rotatory power and infrared (IR) spectral measurements (Micheli *et al.,* 1999). These measures are a good index for determining the structural identity between polymers extracted and classified in literature as Kefiran (La Rivière *et al.*, 1967; Kooiman, 1968). Infrared analysis normally gives an indication of the purity and the structure of samples, where the absence of protein absorption indicates a good degree of purity.

The chemical structure of Kefiran (Fig. 7) is generally described as a branched hexa- or heptasaccharide repeating unit that is composed of a regular pentasaccharide unit to which one or two sugars are randomly linked (Kooiman, 1968; Mukai *et al.*, 1990a). Methylation of Kefiran by the Haworth procedure yielded four products on paper chromatography after hydrolysis, which included: 2,3,4,6-tetra-O-methyl-D-glucose (13%); tri-O-methylglucoses (33%); tri-O-methylgalactoses (38%) and 3,4-di-O-methyl-D-galactose (16%) (Kooiman, 1968). The tri-O-methylglucoses were separated on a carbon-Celite column by using a gradient of butanone-water and yielded equal amounts of 2,3,4- and 2,3,6-tri-O-methyl-D-glucose. Tri-O-methylgalactoses yielded equal amounts of 2,3,6- and 2,4,6-tri-O-methyl-D-galactose. These results indicate that Kefiran has a main chain with branch points at O-2 and O-6 of some of the D-galactose residues; the branches are terminated by D-glucose residues. The remaining D-galactose residues are linked through positions 4 and 3, and the remaining D-glucose residues are linked through positions 4 and 6. Due to the diversity of linkage types, Kefiran is resistant to polysaccharide-hydrolysis (Mukai *et al.,* 1990b) and enzymatic attack (Kooiman, 1968). According to Kooiman (1968), several crude enzyme-preparations from various sources failed to hydrolyse Kefiran. A

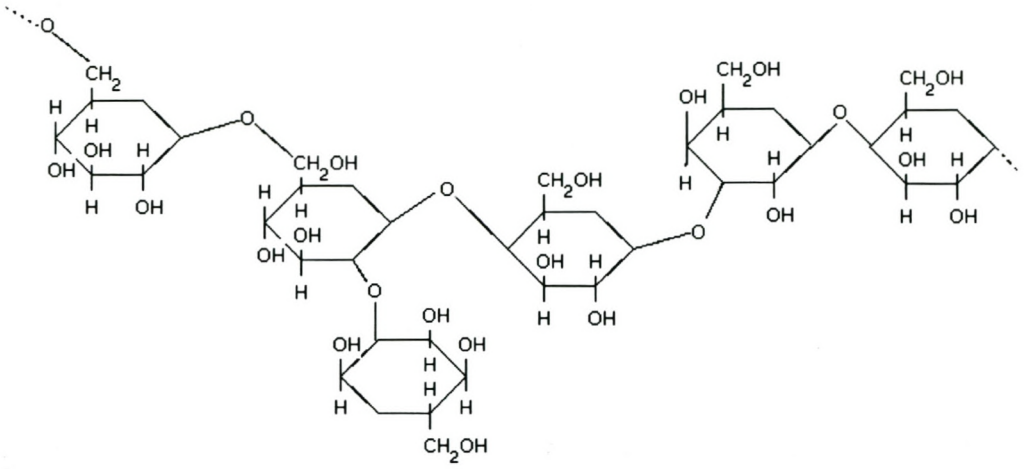

Figure 7. The chemical structure of Kefiran (Kooiman, 1968; Mukai *et al.*, 1990a).

crude cellulase from *Trichoderma viride* was able to degrade the polysaccharide to give glucose and a pentasaccharide, in equimolar quantities. Kefiran has been found to dissolves slowly in cold water and quickly in hot water and a 2% solution of Kefiran is a viscous liquid (Koroleva, 1991). Rheological studies have shown that Kefiran has a very low viscosity in solution and can form weak gels in conditions of low water activity. It is unable to form rigid gels in the absence of ethanol (Mukai *et al.,* 1990a; Mukai *et al.,* 1991).

Kefiran can be used commercially as a food thickener or gelling agent in the food industry (Kooiman, 1968; Mukai *et al.,* 1990b). Mukai *et al.* (1990b) also performed chemical modifications on the Kefiran by preparing carboxymethyl-Kefiran to increase viscosity by the introduction of an ionic group to the neutral polysaccharide. Although the viscosity increased fourteen fold, it was still much less than that of xanthan gum and it was concluded that the commercial use of Kefiran for food thickening will be more restricted than that of presently used polysaccharides.

According to Schellhaass (1983), all ropy mesophilic and thermophilic LAB strains are able to grow and produce slime in milk. Heteropolysaccharides are produced by slime-forming mesophilic and thermophilic LAB (Cerning, 1990). The composition of heteropolysaccharides produced by mesophilic and thermophilic LAB which are also present in Kepi, is given in Tables 5 and 6.

There is little agreement regarding the chemical composition of polysaccharides that have been isolated from thermophilic LAB (Cerning, 1990) such as *Lb. delbrueckii* ssp. *bulgaricus*, *Lac. lactis* ssp. *cremoris* and *S. salivarius* ssp. *thermophilus*. When a ropy strain of *S. salivarius* ssp. *thermophilus* is grown in association with a non-ropy strain of *Lb. delbrueckii* ssp. *bulgaricus,* almost 800 mg polysaccharides are produced per litre of culture medium. Ropy strains of mesophilic *Lac. lactis* ssp. *lactis*, *Lac. lactis* ssp. *cremoris* and *Lb. casei* ssp. *casei* produced heteropolysaccharides containing primarily galactose and glucose, with the former predominating (Cerning *et al.,* 1992). Small amounts of mannose, rhamnose and pentoses were also identified. La Rivière *et al.* (1967) concluded that the polysaccharide of Kefiran consists of equal amounts of galactose and glucose. When based on the known composition of Kefiran, it is possible to compare the composition of polysaccharides produced by microbes with the polysaccharide composition of Kefiran in Kepi. These results could be used to identify the major Kefiran producer.

Table 5. The composition of heteropolysaccharides produced by mesophilic LAB.

Species	Monomers in polysaccharides						Reference
	Galactose	Glucose	Arabinose	Rhamnose	Mannose	Ratio	
Lactococcus lactis ssp. *cremoris*	+	+	-	+	-	1.75:1.45:1 (gal glu:rham)	Cerning *et al.*, 1992
Lactococcus lactis ssp. *cremoris*	+	+	+	+	+		Cerning *et al.*, 1992
Lactobacillus casei ssp. *casei*	+	+	+	-	+		Cerning *et al.*, 1992
Lactobacillus casei ssp. *casei*	+	+	-	+	+		Cerning *et al.*, 1994
Lactobacillus kefiranofaciens	+	+	-	-	-	1:1 (gal:glu)	Mukai *et al.*, 1990a
Lactococcus lactis spp. *lactis*	+	+	-	+	-		Schellhaass, 1983
Lactococcus lactis spp. *lactis*	+	+	-	-	-		Schellhaass, 1983

gal = galactose
glu = glucose
rham = rhamnose

Table 6. The composition of heteropolysaccharides produced by thermophilic LAB.

Species	Monomers in polysaccharides									Reference
	Galactose	Glucose	Fructose	Rhamnose	Mannose	Xylose	Arabinose	Galactos amine	Neuramic acid	
*Lactobacillus bulgaricus**	+	+	-	-	+	-	+	-	-	Cerning *et al.*,1986
	+	+	-	-	-	-	-	-	-	Oda *et al.*, 1983
	-	+	+	-	-	-	-	-	-	Schellhaass, 1983
	+	+	-	+	-	-	-	-	-	Cerning *et al.*,1986
	+	+	-	-	-	-	-	-	-	Schellhaass, 1983
*Streptococcus thermophilus**	+	+	-	tr	+	tr	tr	-	-	Cerning *et al.*,1988
	+	+	-	-	-	-	-	+	-	Doco *et al.*, 1990
	+	+	-	-	-	-	-	-	-	Schellhaass, 1983
	+	+	-	-	-	+	-	-	+	Marshall, 1987
	+	+	-	tr	tr	-	tr	-	-	Cerning *et al.*, 1988

+ = Monomer produced
tr = Only traces of monomer produced
- = Monomer not produced
* For current nomenclature, see Table 2.

G. KEFIRAN PRODUCERS

The bacteria responsible for the production of Kefiran have not yet been positively identified (Özer & Özer, 2000). According to La Rivière *et al.* (1967), only one natural polysaccharide is reported to consist of equal amounts of glucose and galactose. This is the capsular polysaccharide produced by a strain of *Bacillus megaterium*, but they found that the polysaccharide had a ratio of 0.57 (glucose : galactose) and is not identical to the polysaccharide found in Kepi grains.

La Rivière *et al.* (1967) found that heterofermentative rods form capsules of Kefiran, a polysaccharide constituting about 25% of the dry weight of the grains. They described this organism as a member of the species *Lb. brevis* but the capsule formation ability was already lost with the first transfer after the strain had been isolated. In contrast, Teixeira (2000) concluded that not only one main microbe is responsible for the production of the polysaccharide, but that Kefiran is produced by all the predominating bacterial species, including *Lb. brevis.* Rosi & Rossi (1978) suggested that polysaccharides of Kepi grains be produced by homofermentative lactobacilli, which they called "atypical *Streptobacterium*". Kandler & Kunath (1983) concluded that *Lb. brevis* is not responsible for the Kefiran production, but that a capsular forming bacterium is responsible for the polysaccharide production. Toba *et al.* (1987) reached a similar conclusion when they compared the polysaccharide characteristics from Kepi grains, *Lb. kefir* and an encapsulated homofermentative *Lactobacillus* species, in order to identify the main polysaccharide producer in the grains. Comparative studies were performed by gel filtration, sugar composition analysis and methylation analysis. They found that a homofermentative *Lactobacillus* strain produced a polysaccharide identical to the one extracted from Kepi grains. They thus concluded that it is an encapsulated homofermentative *Lactobacillus* species that is responsible for the polysaccharide production in the grains.

Toba *et al.* (1990) found that encapsulated bacteria reside in the inner parts of propagable grains, and that encapsulated bacteria are absent in non-propagable grains. They concluded that encapsulated bacteria are thus responsible for the propagation of Kepi grains. It remains undecided which organism is the Kefiran producer, because capsular bacteria are difficult to isolate or need complex media for growth. Toba *et al.* (1986) developed a selective media (KPL-medium), which contains wine, for the isolation of homofermentative lactobacilli. The bacterium cannot be used for mass production of the polysaccharide, because of the expensive medium (Yokoi *et al.,* 1990). MRS-broth was found to strongly interfere with the polysaccharide isolation procedures and an improved media, RCW (Rogosa Cheese Whey), developed by Kojima *et al.* (1993), was found to be more selective.

Fujisawa *et al.* (1988) further characterised the homofermentative *Lactobacillus* species described by Toba *et al.* (1986) as the producer of Kefiran and proposed the formation of a new species, *Lb. kefiranofaciens,* for this strain. The species was characterised as a slime-forming, homofermentative, capsulated, rod-shaped LAB. Mukai *et al.* (1992) found that Kefiran is the main accessory polymer in the cell wall of *Lb. kefiranofaciens.* A comparison of the biochemical and physiological characteristics, morphology, DNA base composition and DNA homology indicated that this species differs from other homofermentative *Lactobacillus* species which have lower G+C contents (Fujisawa *et al.,* 1988). Mukai *et al.* (1990a) reported that the capsular polysaccharide from *Lb. kefiranofaciens* was composed of only D-glucose and D-galactose in the molar ratio of 0.9 : 1.1. The purified polysaccharide was obtained in a yield of 63 $mg.l^{-1}$ from the supernatant.

According to Takizawa *et al.* (1998), the count of bacteria in Kepi grains was 5.5 x 10^{10} per gram wet grains and 40 - 57% of the viable bacterial count was capsular bacteria producing polysaccharides. They isolated *Lb. kefiranofaciens* as a dominant member of the *Lactobacillus* population, and reported that this specific species produced large amounts of polysaccharide and probably is responsible for grain formation. Yokoi *et al.* (1991) also isolated a Kefiran-producing homofermentative bacterium, *Lactobacillus* sp. KPB 167B, from Kepi grains with a newly developed milk whey medium, but concluded that it could still be related to the *Lb. kefiranofaciens* species.

Micheli *et al.* (1999) isolated a slime forming, rod-shaped *Lactobacillus* from Kepi grains, using a modified MRSL medium. The Kepi grains were washed with sterile distilled water and homogenised with a Waring blender. Ropy strains were isolated by plating diluted homogenated Kepi grains on the medium. Mucoid colonies morphologically different on the medium were purified by subculturing. Cycloheximide (200 $mg.l^{-1}$) was added to the medium to inhibit the growth of yeasts (Angulo *et al.,* 1993; Lin *et al.,* 1999). Potato dextrose agar was also employed for the isolation of yeasts (Lin *et al.,* 1999). All the colonies morphologically different from each other were purified by streaking on the same media.

According to ^{13}C- and ^{1}H-NMR spectra data the primary structures of the polysaccharide extracted from the Kepi grains and extracted polysaccharide produced by the isolated slime-forming, rod-shaped *Lactobacillus* strain, were identical (Micheli *et al.,* 1999). The bacterial strain was characterised by determining the nucleotide sequence of the gene coding for the 16S ribosomal RNA and the strain was placed in the *Lactobacillus* genus in the *Lb. casei* group. The most probable producers of polysaccharides in the Kepi grain are thus representatives of the genus *Lactobacillus* (Koroleva, 1991), although it is possible that the geographical origin of the starting Kepi grains will eventually determine the population structure.

H. DISCUSSION

Kepi is different from other fermented milk products in that it is the result of fermentation of mixed microbes (LAB and yeasts) confined to a matrix of discrete Kepi grains. Attempts at making Kepi grains form pure cultures or a mixed culture of microbes present in the original grains have so far not been successful. Kefiran appear essential for grain formation and integrity and many studies have been done on the identification of the Kefiran producer but it still remains undecided which organism is really responsible for the production of this polysaccharide matrix.

It is known that the microbial community in the Kepi grain differs according to the spesific origin of the grains. The possibility exists that the Kefiran producer of South African Kepi grains differs from grains of a different origin. It is therefore necessary to identify the producer of Kefiran in Kepi grains from South Africa.

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CHAPTER 3

ISOLATION, CHARACTERISATION AND IDENTIFICATION OF KEFIRAN-PRODUCING MICROBES FROM KEPI GRAINS

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Abstract

Sixteen strains of lactic acid bacteria and one yeast strain were isolated from Kefiran strings produced during the mass cultivation of South African Kepi grains. API technology, numerical clustering and DNA sequence comparisons were used to identify the purified isolates. The isolates were grouped into seven clusters by numerical clustering and clustering distance from selected reference and marker strains. The heterofermentative lactobacilli were identified as *Lactobacillus parakefiri* and *Lb. kefiri* and the homofermentative strains as *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. gallinarum, Lb. acidophilus* and *Lb. bavaricus.* One isolate was found to be a member of the genus *Lactobacillus*, but was not positively identified to species level.

Introduction

Kepi differs from other fermented milk products in that it is produced with a mixed microbial community which is confined to discrete grains that can be recovered as a solid matrix at the end of the fermentation (Marshall *et al.,* 1984). The Kepi grains can then be re-utilised as starter to ferment the next batch of milk, or stored for later use (Rea *et al.,* 1996).

The grain microbial community consists of a symbiotic association of yeasts and lactic acid bacteria (LAB), but the overall composition of the grains has not been completely elucidated (Saloff-Coste, 2000). It is well known that there is a large diversity of different microbial types and species found in Kepi grains (Saloff-Coste, 2000), with batches of Kepi grains produced in other countries and from different milk sources varying widely in microbial composition.

The microbes in the grains are embedded in a protein-polysaccharide Kefiran matrix, which appears essential for grain formation (La Rivière *et al.,* 1967; Lin *et al.,* 1999). Many studies have been done on the identification of the major Kefiran producer and Fujisawa *et al.* (1988) described a capsule-forming homofermentative lactobacilli, *Lactobacillus kefiranofaciens,* as the main Kefiran producer. Takizawa *et al.* (1998) concluded that although *Lb. kefiranofaciens* probably is the major Kefiran contributor, there could also be other species which produce Kefiran or other types of polysaccharides. This was confirmed by Saloff-Coste

(2000) who showed that several *Lactobacillus* species, including *Lb. kefiranofaciens* and *Lb. kefir*, produce Kefiran and are an integral part of the Kepi grain. He also concluded that without their presence, Kepi grains cannot be propagated. It is generally considered that the mechanism of grain formation is not fully understood. It thus remains undecided which organism is really responsible for the production of this protein-polysaccharide matrix (Özer & Özer, 2000).

The aim of this study was to isolate, characterise and identify the microbes present in Kefiran from mass cultured South African grains.

Materials and methods

Source and mass cultivation of Kepi grains

The Kepi grains used as starter cultures for the mass cultivation were originally obtained from Dr. J.F. Mostert (Animal Nutrition & Animal Products Institute, Irene, South Africa) and stored at -18°C. The mass cultivation of Kepi grains was done according to the method developed by Schoevers (1999). A 10% (m/v) inoculum of Kepi grains was added to 400 ml of a double pasteurised milk mixture and incubated in a shake waterbath (rpm = 130) at 25°C. The grains were sieved out and the milk mixture replaced every 24 h.

Isolation method

The mass cultivation process was continued until Kefiran was visible between the grains. Kefiran strings were then removed and plated onto MRSL medium (Yokoi & Watanabe, 1992; Lin *et al.*, 1999; Micheli *et al.*, 1999). The use of the MRSL medium was based on the findings of Micheli *et al.* (1999) on the isolation of the main Kefiran producer, *Lb. kefiranofaciens*. Cycloheximide (BDH Laboratory Supplies) was added to the media (0.5% (m/v)) to inhibit the growth of yeasts. Kefiran was also streaked out on potato dextrose agar (PDA) (Merck) to isolate yeasts present in Kefiran and the pH adjusted to 4.5 with tartaric acid to inhibit the growth of lactobacilli.

Morphologically different colonies were purified by subculturing on the same media. The lactobacilli were incubated anaerobically using the Anaerocult-A system (Merck) for 5 - 10 d at 25°C, and the yeasts aerobically for 2 - 4 d at 25°C. The strains isolated from the Kefiran strings were cultured under the same conditions and on the same media as for the isolation procedure. Once purified, all the cultures were lyophilised using the method of Joubert & Britz (1987).

Isolate characteristics

The Gram-stain, oxidase and catalase tests (Gerhardt *et al.,* 1994) were performed on each isolate, and the production of gas from glucose was determined. Growth at 15° and 45°C in MRS broth (Merck) was measured spectrophotometrically (Spectronic 20, Spectronic Instruments, USA) at 550 nm after 24 h. To compensate for differences in temperature during measuring and inoculation, a variation of less than 20% between the before and after value was taken to represent no change in growth. The growth response (change in biomass production) was determined at different pH (5.2 and 6.5) and temperature combinations (20°, 25°, 30° and 35°C) every 6 h for 30 h. The fermentation profiles of the isolates were determined using the API 50 CHL and the API 20 E systems (API system S.A., La alme le Grottes, 38390 Montalieu Vercieu, France). The yeasts were identified using the Rapid ID 32C system.

Numerical clustering

Microlog software (Biolog Inc., USA) was used to facilitate clustering of the microbes isolated from Kefiran. One-dimensional plots were created on the basis of the calculated dendrogram differences (D_D) and the different isolates were clustered in relation to their closest relatives based on the phenotypic characterisation as calculation concept. The data-set consisted of the characteristics of the isolated bacteria as well as the characteristics of reference and marker cultures (Table 1). Numerical clustering was used to interpret the groupings of the isolates after taking into consideration the clustering position of the marker and reference cultures. These reference and marker cultures were selected according to the species previously identified in Kepi (Garrote *et al.,* 2001), as well as microbes previously isolated from South African Kepi grains (Schoeman, 2001). The characteristics of the reference cultures were determined with the same API system used for the isolates. The characteristics of the marker cultures were taken from the literature (Table 1).

Molecular typing

DNA was isolated according to the method of Van Elsas *et al.* (1997) by adding 100 µl of the isolated culture (cultivated in MRS broth) to 120 mM sodium phosphate buffer (120 mM NaH_2PO_4 : 120 mM Na_2HPO_4, pH 8.0 (1:9)), phenol (Merck) and 20% sodium dodecyl sulphate (SDS). The mixture was vortexed for 1 - 2 min and incubated at 60°C for 20 min. This step was repeated twice before samples were centrifuged at 4 000 x g for 5 min. A phenol extraction step was followed by a chloroform:phenol:isoamylalcohol (24:25:1) (v/v) extraction until the interphase was clear. The DNA was precipitated on ice for 1 h with 0.1 volume 3 M sodium acetate (pH 5.5) and 0.6 volume isopropanol before centrifugation at 12 000 x g for 10 min. The

Table 1. Reference and marker cultures used in the numerical clustering.

Culture name	Strain type	Reference
Lb. brevis-R(20054)	Reference	DSMZ[1]
Lb. delbrueckii ssp. *bulgaricus*-R(3)	Reference	FS-US[2]
Lb. fermentum-R(20052)	Reference	DSMZ[1]
Lac. lactis ssp. *lactis*-R(316)	Reference	FS-US[2]
Lb. plantarum-R(1027)	Reference	FS-US[2]
*Lb. acidophilus*1-M	Marker	API system
*Lb. acidophilus*2-M	Marker	API system
Lb. bavaricus-M	Marker	Kandler & Weiss, 1986
*Lb. brevis*1-M	Marker	API system
*Lb. brevis*2-M	Marker	API system
*Lb. brevis*3-M	Marker	API system
*Lb. brevis*4-M	Marker	Kandler & Weiss, 1986
Lb. buchneri-M	Marker	Kandler & Weiss, 1986
Lb. crispatus-M	Marker	Kandler & Weiss, 1986
Lb. fermentum-M	Marker	Kandler & Weiss, 1986
Lb. fructiforans-M	Marker	Kandler & Weiss, 1986
Lb. gallinarum-M	Marker	Hammes & Vogel, 1995
Lb. helveticus-M	Marker	Hammes & Vogel, 1995
Lb. kefirgranum-M	Marker	Takizawa *et al.*, 1994
"Lb. kefir"-M	Marker	Kandler & Weiss, 1986
Lb. kefiranofaciens-M	Marker	Hammes & Vogel, 1995; Fujisawa *et al.*, 1988
"Lb. parakefir"-M	Marker	Takizawa *et al.*, 1994

[1] = Deutsche Sammlung von Mikroorganismen und Zellenkulturen

[2] = Department of Food Science, University of Stellenbosch

pellet was washed with 70% (v/v) ethanol, dried and resuspended in 100 µl TE buffer (10 mM Tris, 1mM EDTA; pH 8.0) and stored at -18°C.

For **pure cultures A(M1), D(M2) and 5(M2)**, the PCR amplification was performed in the Mastercycler (Eppendorf). A 1.5 kilo base pair (kb) fragment of the 5' end of the V3 variable region of the 16S rRNA gene was amplified using the primers F8 (5'-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3') and R1512 (5'-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3') (Felske *et al.*, 1997). PCR was performed in a total reaction volume of 25 µl containing 0.5 µM of each of the primers, 0.5 mM dNTPs (Promega), Dimethyl Sulfoxide (DSMO) (Merck), 1 U *Taq* DNA Polymerase (Boehringer Mannheim), the buffer supplied with the enzyme and 1 µl of the isolated DNA. The reactions were run for 30 cycles; denaturation was at 95°C for 60 s, annealing at 25°C for 45 s and elongation at 72°C for 60 s. An initial 5 min denaturation at 95°C and a final 7 min elongation at 72°C were used. These fragments were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) according to the manufacturer's instructions and sequenced using an ABI PRISM 377 DNA Sequencer at the Sequencing Facility at the University of Stellenbosch. Sequences obtained were aligned with 16S rRNA gene sequences of known bacterial species using the BLAST search in GenBank.

For the **pure culture B(M1)**, the same procedure was followed as above, but this culture was cloned before it was sequenced. The PCR fragments were cloned using the pGemT-Easy Vector System II (Promega). Transformed colonies were screened for the correct sized insert using the primers T7 (5'-GTA ATA CGA CTC ACT ATA GGG-3') and SP6 (5'-TAC GAT TTA GGT GAC ACT ATA G-3'). PCR was performed in a total reaction volume of 100 µl containing 0.5 µM of each of the primers, 0.5 mM dNTPs (Promega), 1 U *Taq* DNA Polymerase (Boehringer Mannheim), the buffer supplied with the enzyme and 1 colony of the transformed cells. The reactions were run for 35 cycles; denaturation was at 92°C for 30 s, annealing at 54°C for 30 s and elongation at 68°C for 60 s. An initial 3 min denaturation at 92°C and a final 7 min elongation at 72°C were used. Amplified fragments were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) according to the manufacturer's instructions and sequenced in a ABI PRISM 377 DNA Sequencer. Sequences obtained were aligned with sequences available by using the BLAST program in GenBank.

Identification of isolates

As a result of the large volume of data generated during the morphological and biochemical characterising of the isolates using the API 50 CHL, API 20 E and Rapid ID 32C systems, together with the results of the additional tests performed, the data is given at the end of this chapter as Appendix A to simplify the discussion section.

Results and discussion

Mass cultured grains

The typical structure of normal Kepi grains before, and grains after mass cultivation as found in this study, are shown in Fig. 1A and 1B, respectively. It was found that the mass of the grains doubled after 12 d and Kefiran strings became visible after about 14 d. These Kefiran strings present between the grains are illustrated in Fig. 1B.

Microbes present were isolated (Fig. 1 D, E, F and G) from the strings of Kefiran visible between the mass cultured grains after the mass cultivation process (Fig. 1B). The isolation was done in duplicate from the Kefiran strings of two different batches of mass cultured Kepi grains.

Sixteen rod-shaped isolates and one yeast strain were obtained (Table 2). No isolates with a coccus-shape morphology were found. The morphology of the yeast isolate is illustrated in Fig. 1D and the isolate was identified as *Candida lambica* (API identification of 99.9%). The results of the Rapid ID 32C test are given in Table A1 in the appendix.

The mixed population of rod-shaped bacteria and exopolysaccharides (EPS) are illustrated in Fig. 1 E, F and G. The general characteristics of the purified bacterial isolates and the reference and marker strains are given in Table 3. All the isolates were found to be Gram-positive rods, and oxidase and catalase negative. Seven of the 16 isolates produced gas from glucose and were taken as heterofermentative, while the rest were homofermentative.

Results of the growth studies at 15° and 45°C before and after the incubation period are given in Table A2. Due to poor growth of isolates A(M1), C(M2), D(M2), K(M1) and 4(M1) during isolation, the effect of different combinations of pH and temperature were monitored on all the isolates every 6 h for a 30 h period to determine the best growth conditions. The summarised results are given in Table 4. The results showed that with the exception of isolate F(M2), all the isolates showed increased growth at the higher temperatures than that was obtained at the isolation temperature of 25°C. It was also found that isolates A(M1), D(M2) and 4(M1) showed a greater increase in biomass at pH 5.2 than at pH 6.5.

The carbohydrate fermentation profiles obtained with the API 50 CHL of the isolates as well as the marker and reference cultures, are given in Table A3. Isolates E(M1), F(M2) and 1(M1) were identified as *Lb. acidophilus* with the API profile (API identification percentage of 60%). Isolates C(M2) and D(M2) was identified to belong to the heterofermentative species *Lb. fermentum* (API identification of 84.4%), but these isolates were found to be homofermentative. The homofermentative isolate H(M2), was identified as *Lb. buchneri*, a heterofermentative rod. Isolate (5M2) was identified as *Leuconostoc* species, but the morphology of the long, thin rod-shaped isolate and that of *Leuconostoc* did not agree. Isolates A(M1), K(M1), 4(M1), B(M1),

(a) Normal Kepi grains

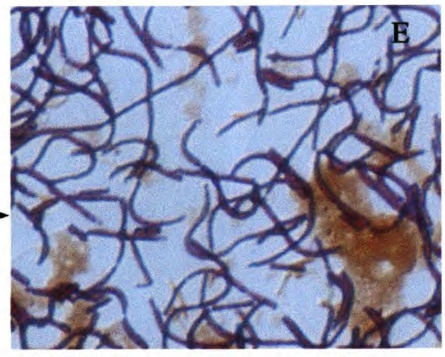
Mass cultivation for 25 d

(b) Mass cultured Kepi grains with Kefiran strings

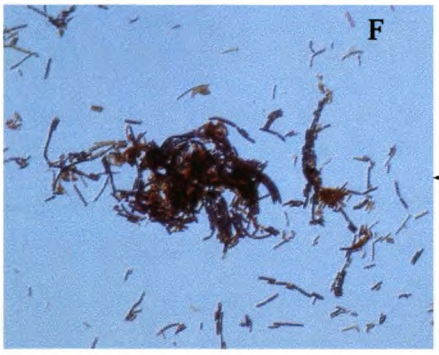
(c) Microbial growth of Kefiran strains on MRSL media after 5 d at 25°C

Purification

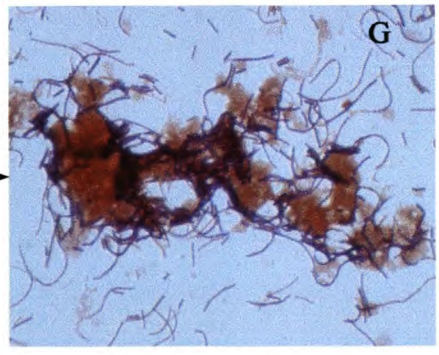
(d) Yeasts



(e) Rod shaped bacteria and ECP



(f) Bacteria and ECP



(g) Bacteria and ECP

Figure 1. Schematic illustration of the protocol used for strain isolation from the Kefiran strings during the mass cultivation of Kepi grains.

Table 2. Isolates from Kefiran strings and identification methods used.

Isolated culture code	Characterisation method
Bacteria	
A(M1)	API 50 CHL + NC + MI
B(M1)	API 50 CHL + NC + MI
C(M2)	API 50 CHL + NC
D(M2)	API 50 CHL + NC + MI
E(M1)	API 50 CHL + NC
F(M2)	API 50 CHL + NC
G(M1)	API 50 CHL + NC
H(M2)	API 50 CHL + NC
I(M2)	API 50 CHL + NC
J(M2)	API 50 CHL + NC
K(M1)	API 50 CHL + NC
1(M1)	API 50 CHL + NC
2(M1)	API 50 CHL + NC
3(M2)	API 50 CHL + NC
4(M1)	API 50 CHL + NC
5(M2)	API 50 CHL + NC + MI
Yeast	
6(P1)	API ID 32 C

Numbering: First digit - isolate number; second digit - isolation medium (M - MRSL; P - PDA); third digit - batch number (1 and 2)
NC = Numerical clustering
MI = Molecular identification

Table 3. General characteristics of the isolates and the reference and marker LAB strains.

Culture	Morphology	Gas from glucose	Oxidase	Catalase	Growth at 15°C	Growth at 45°C
Isolates						
A(M1)	Rod	+	-	-	+	+
B(M1)	Rod	+	-	-	+	-
C(M2)	Rod	-	-	-	-	+
D(M2)	Rod	-	-	-	-	+
E(M1)	Rod	-	-	-	-	+
F(M2)	Rod	-	-	-	-	+
G(M1)	Rod	+	-	-	+	-
H(M2)	Rod	-	-	-	-	-
I(M2)	Rod	+	-	-	+	-
J(M2)	Rod	-	-	-	-	+
K(M1)	Rod	+	-	-	+	-
1(M1)	Rod	-	-	-	-	-
2(M1)	Rod	+	-	-	+	+
3(M2)	Rod	-	-	-	-	+
4(M1)	Rod	+	-	-	+	+
5(M2)	Rod	-	-	-	-	-
Reference and Marker strains						
*Lb. acidophilus*1-M	Rod	-	-	-	-	+
*Lb. acidophilus*2-M	Rod	-	-	-	-	+
Lb. bavaricus-M	Rod	-	-	-	+	-
Lb. brevis-R	Rod	+	-	-	+	-
*Lb. brevis*1-M	Rod	+	-	-	+	-
*Lb. brevis*2-M	Rod	+	-	-	+	-
*Lb. brevis*3-M	Rod	+	-	-	+	-
Lb. brevis4-M	Rod	+	-	-	+	-
Lb. buchneri-M	Rod	+	-	-	+	-
Lb. crispatus-M	Rod	-	-	-	-	+
Lb. delbrueckii ssp. *bulgaricus*-R	Rod	-	-	-	-	+
Lb. fermentum-R	Rod	+	-	-	-	+
Lb. fermentum-M	Rod	+	-	-	-	+
Lb. fructiforans-M	Rod	+	-	-	-	-
Lb. gallinarum-M	Rod	-	-	-	-	+
Lb. helveticus-M	Rod	-	-	-	-	+
Lb. kefirgranum-M	Rod	-	-	-	+	-
'Lb. kefir" -M	Rod	+	-	-	+	-
Lb. kefiranofaciens-M	Rod	-	-	-	-	-
Lc. lactis ssp. *lactis*-R	Cocci	-	-	-	+	+
Lb. plantarum-R	Rod	-	-	-	+	+
'Lb. parakefir" -M	Rod	+	-	-	+	-

Numbering: M = Marker culture
R = Reference culture

Table 4. Growth responses (% increase in biomass) at different combinations of temperature and pH after 30 h of incubation.

Isolate	20°C		25°C		30°C		35°C	
	5.2	**6.5**	**5.2**	**6.5**	**5.2**	**6.5**	**5.2**	**6.5**
A(M1)	329	230	556	201	666	242	678	495
B(M1)	120	92	518	250	1134	586	479	582
C(M2)	117	354	537	198	734	934	604	671
D(M2)	147	234	365	262	569	475	206	516
E(M1)	44	74	165	243	347	576	330	359
F(M2)	905	430	632	1332	475	659	391	730
G(M1)	206	163	264	151	353	223	420	358
H(M2)	127	140	383	326	592	497	592	611
I(M2)	219	137	267	171	492	262	376	376
J(M2)	66	70	308	315	604	591	593	736
K(M1)	153	120	215	233	234	155	111	274
1(M1)	120	83	405	206	578	392	574	917
2(M1)	329	230	556	201	666	242	678	495
3(M2)	120	92	518	250	1134	586	479	582
4(M1)	345	259	500	357	639	424	507	293
5(M2)	127	210	590	541	1031	1061	873	432

G(M1), I(M2) and 2(M1) were all identified as *Lb. fermentum*, but results based on morphology clearly distinguished two groups. Both of these groups need to be confirmed with numerical clustering and DNA sequence analysis. Of all the isolates, only two could be positively identified with API technology. These isolates, J(M2) and 3(M2), were identified as *Lactobacillus delbrueckii* ssp. *bulgaricus* (API identification of 95.2%). It is possible that since the database of the API 50 CHL system consists of 50 general LAB strain representatives, strains representing the LAB isolated from the Kefiran strings were not included in the database and could thus not be positively identified using the API system.

Numerical clustering

Numerical clustering was used to place the isolates, reference and marker cultures into groups based on morphological, physiological and biochemical similarities. Additional characteristics (API methodology) were determined which included: Ortho-nitro-β-D-galactopyranoside (Beta-galactosidase); arginine dihydrolase; lysine decarboxylase; ornithine decarboxylase; citrate utilisation; H_2S production; urea; tryptophane deaminase; indole production; acetoin production; gelatinase; and NO_2 production. These results were used together with the results of the API 50 CHL and general characteristics to facilitate clustering.

The 16 LAB isolates were grouped in seven clusters as illustrated in Fig. 2. **Group 1** contained three strains, C(M2) and D(M2) with 100% similarity, and marker strain *Lb. gallinarum*-M. The clustering position of these isolates showed that they are closely related to the *Lb. gallinarum* species and those strains were taken as representatives of this species. **Group 2** contained only one isolate (5M2). **Group 3** consisted of strains E(M1), F(M2) and 1(M1) with isolates E(M1) and F(M2) at a 100% similarity level. Group 3 strains were related to the marker *Lb. acidophilus*2-M, and those strains were thus taken as representatives of *Lb. acidophilus*. **Group 4** contained one isolate, H(M2) and a reference strain, *Lb. bavaricus*-R. Isolate H(M2) was thus taken as a representative of *Lb. bavaricus* based on the close relatedness of the isolate to this reference strain.

Group 5 consisted of strains J(M2), 3(M2) and the reference strain, *Lb. delbrueckii* ssp. *bulgaricus*-R. The two isolates and the marker strain clustered at the 100% D_D level and strains J(M2) and 3(M2) were thus taken as representatives of the species *Lb. delbrueckii* ssp. *bulgaricus*. **Group 6** consisted of five strains, A(M1), K(M1) and 4(M1), marker "*Lb. kefir*"-M and reference strain *Lb. buchneri*-R. Based on the clustering level, the isolates were considered to be more representative of the species "*Lb. kefir*", but this must be confirmed using DNA sequencing analysis. **Group 7** consisted of four isolates, B(M1), G(M1), I(M2), 2(M1) and marker "*Lb. parakefir*"-M. The clustering position of these isolates indicated that they are

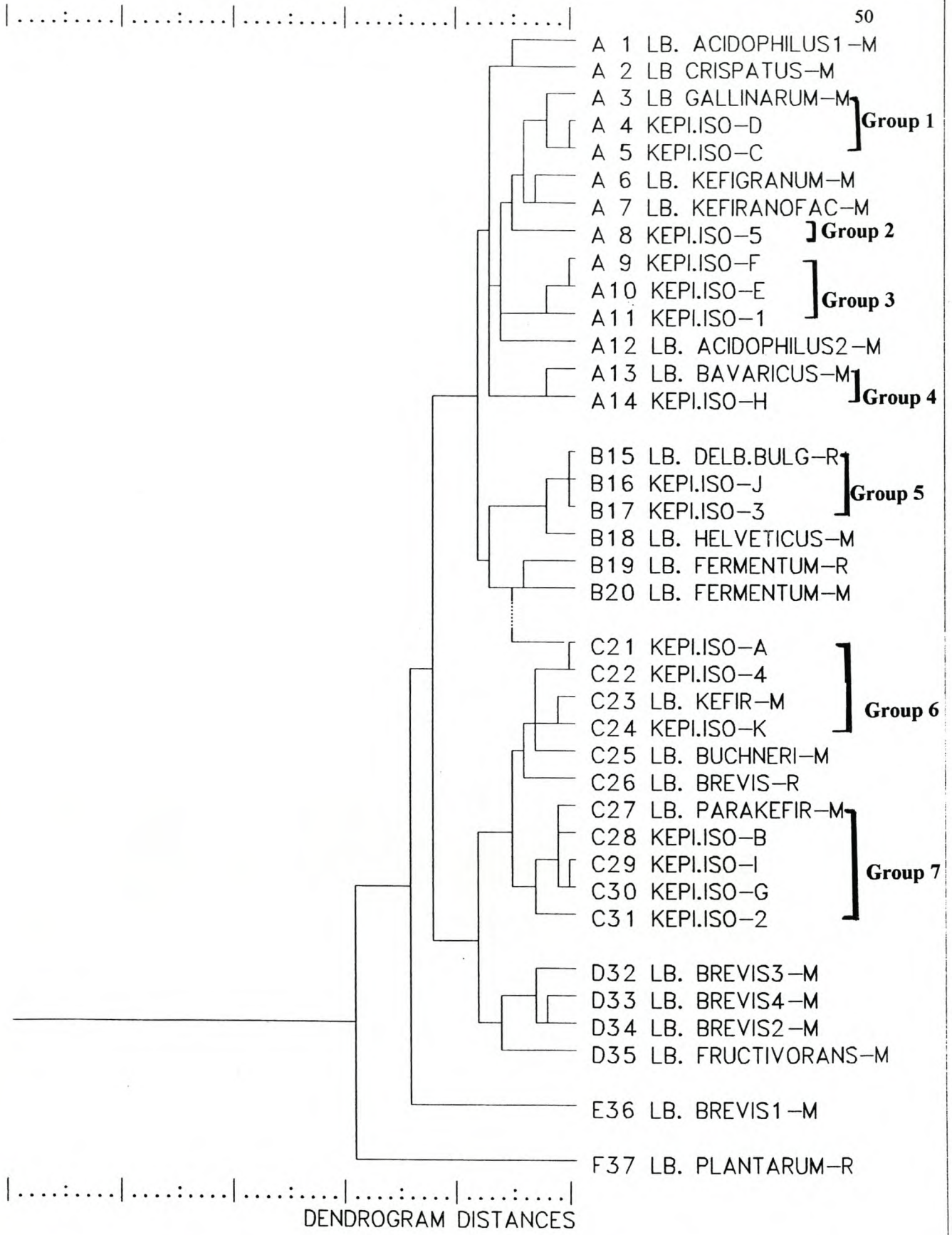


Figure 2. Numerical clustering of reference strains, marker strains and cultures isolated from Kefiran strings

closely related to the *"Lb. parakefir"* species and were thus taken as representative of *"Lb. parakefir"*.

Molecular identification

Molecular identification was used to identify the isolates that could not be positively identified with the API 50 CHL system or by grouping with numerical clustering (Table 5). One isolate was chosen from each numerical group (Fig. 2). The isolates of **Group 5**, which were positively identified with the API 50 CHL system, and the isolates in **Group 3** and **4**, which were positively identified with the numerical clustering, were not confirmed using molecular identification.

Isolate D(M2) from **Group 1** was identified as *Lb. gallinarum* (of the 432 bp aligned, 426 were homologous). Kandler & Weiss (1986), Fujisawa *et al.* (1992), Takizawa *et al.* (1994) and Du Plessis & Dicks (1995) all reported that the species *Lb. acidophilus* consists of a genetically heterogeneous collection of species (*Lb. gallinarum, Lb. crispatus, Lb. amylovorus, Lb. gasseri, Lb. johnsonii* and *Lb. kefirgranum*) that are difficult to differentiate by simple physiological and biochemical tests. This, and the fact that *Lb. gallinarum* is not included in the API data base, is probably the reason why the isolates of Group 1 could not be positively identified with the use of the API system.

The sequences of isolate 5(M2) (of the 592 bp aligned, 575 were homologous to an uncultured bacterial clone p-1340-a2, AF 371470) from **Group 2** was found to be related to the genus *Lactobacillus*, but could not be positively identified to species level.

Isolate A(M1) from **Group 6** was identified as *Lb. kefiri* (former *"Lb. kefir"* - Truper & de`Clari, 1997) (of the 461 bp aligned, 457 were homologous) and based on the sequence alignment and presence of *"Lb. kefir"*-M in the cluster, the group was taken as representatives of the species *Lb. kefiri*. Kandler & Weiss (1986) and Takizawa *et al.* (1994) reported that it is difficult to clearly distinguish between *Lb. brevis, Lb. buchneri, Lb. parabuchneri* and *"Lb. kefir"* by simple physiological and biochemical tests, especially in terms of the pattern of acid production from carbohydrates. In this study the numerical positioning also showed that *Lb. brevis, Lb. buchneri* and *Lb. kefiri* are related. The heterofermentative rod, *"Lb. kefir"* was originally isolated from Kepi by Kandler & Kunath (1983) and they reported that this species is the main LAB present in the Kepi beverage but is only a minor component of the grains. In contrast Saloff-Coste (2000) concluded that *"Lb. kefir"*, together with *Lb. kefiranofaciens*, produce specific or other polysaccharides without which the Kepi grain could not be propagated.

Table 5. Identification of the groups of isolated cultures from Kefiran strings.

Numerical group	Isolates	Identification
1	D(M2), C(M2)	*Lb. gallinarum*
2	5(M2)	Strain could not be identified
3	E(M1), F(M2), 1(M1)	*Lb. acidophilus*-related
4	H(M2)	*Lb. bavaricus*
5	J(M2), 3(M2)	*Lb. delbrueckii* ssp. *bulgaricus*
6	A(M1), K(M1), 4(M1)	*Lb. kefiri*
7	B(M1), G(M1), I(M2), 2(M2)	*Lb. parakefiri*

Isolate B(M1) from **Group 7**, based on the sequencing data, was identified as *Lb. parakefiri* (former *"Lb. parakefir"* - Truper & de`Clari, 1998) (of the 580 bp aligned, 575 were homologous). Thus based on the numerical clustering, the sequencing results and the presence of this marker strain *"Lb. parakefir"*-M this group was taken as representative of the species *Lb. parakefiri*. This species has previously also been isolated from Kepi grains by Takizawa *et al.* (1994).

Conclusions

La Rivière *et al.* (1967) and Lin *et al.* (1999) concluded that Kefiran appears essential for grain formation. As the main objective of this study, microbes from only the Kefiran strings were isolated and not from the Kepi grain as a whole. These isolated microbes were all identified as LAB and one yeast species.

The heterofermentative rods isolated from Kefiran were identified using combinations of the API data system, numerical clustering and DNA sequencing as part of the 16S rRNA gene as *Lb parakefiri* and *Lb. kefiri*, whereas the homofermentative rods included *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. gallinarum*, *Lb. bavaricus, Lb. acidophilus* and an isolate that belongs to the genus *Lactobacillus*. The only yeast strain found was identified as a member of the species *C. lambica*. To our knowledge *Lb. gallinarum* and *Lb. bavaricus* have not previously been isolated from Kepi grains.

All the groups of strains isolated from the Kefiran strings, with the exception of Group 5 (*Lb. delbrueckii* ssp. *bulgaricus*), could not be positively identified with the API system probably because members of these species were not included in the database of the API 50 CHL system. It was found that in certain cases, DNA sequencing comparisons were required to facilitate the identification of specific isolates.

Although *Lb. kefiranofaciens* is found to be the main Kefiran producer in Kepi grains (Fujisawa *et al.*, 1988), Özer & Özer, 2000 concluded that the mechanism of grain formation is still not fully understood and it thus remains undecided which organism is really responsible for the production of Kefiran. In this study, however, *Lb. kefiranofaciens* was not detected with the isolation conditions used. Garrote *et al.* (2001) were also unable to isolate *Lb. kefiranofaciens* form Argentinean Kepi grains. Whether these strains isolated from the Kefiran strings were able to play a role in the mechanism of grain formation during the mass cultivation of Kepi grains or were just caught-up in strings, still needs to be investigated. Furthermore, it is also important to investigate which one of these isolated strains is able to form grains in milk, or if combinations of isolates will be needed to produce Kepi grains.

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APPENDIX A

To Chapter Three

Tables A1 – A5 are given in this appendix. The large amount of data generated was placed in a separate appendix to simplify the discussion section of this chapter.

Table A1. Characterisation of the yeast species isolated from Kefiran, using the Rapid ID 32 C identification system.

Test	Isolate 6(P1)
Galactose	-
ACT	-
Saccharose	-
N Acetyl glucosamine	+
LAT	+
Arabinose	-
Cellobiose	-
Raffinose	-
Maltose	-
Trehalose	-
2 ceto-gluconate	-
Methyl-D-glucoside	-
Sorbitol	-
Xylose	+
Ribose	-
Glycogene	-
RHA	-
PLE	-
Erythritol	-
Melibiose	-
GRT	-
Melezitose	-
Gentibiose	-
LVT	-
Mannitol	-
Lactose	-
Inositol	-
Glucose	+
SBE	-
Gluconate	+
Esculine	-

Table A2. Results of growth response of isolated cultures at 15° and 45°C.

	15°C				45°C			
Culture	Start		End		Start		End	
	OD (nm)	Cfu $(10^6).ml^{-1}$	OD (nm)	Cfu $(10^6).ml^{-1}$	OD (nm)	Cfu $(10^6).ml^{-1}$	OD (nm)	Cfu $(10^6).ml^{-1}$
A(M1)	0.412	4.94	0.609	7.31	0.343	4.12	0.459	5.51
B(M1)	0.336	4.03	0.543	6.52	0.439*	5.27	0.459*	5.51
C(M2)	0.400	4.80	0.400	4.80	0.267	3.20	0.583	7.00
D(M2)	0.523*	6.28	0.584*	7.01	0.471	5.65	0.840	10.08
E(M1)	0.186*	2.23	0.206*	2.47	0.265	3.18	0.321	3.85
F(M2)	0.399	5.04	0.399	4.79	0.362	4.34	0.635	7.62
G(M1)	0.340	4.08	0.517	6.20	0.529	6.35	0.490	5.88
H(M2)	0.328	3.94	0.200	2.40	0.141	1.69	0.175	2.10
I(M2)	0.306	3.67	0.527	6.32	0.779	9.35	0.750	9.00
J(M2)	0.387	4.64	0.352	4.22	0.275	3.30	0.440	5.28
K(M1)	0.425	5.10	0.553	6.64	0.724*	8.69	0.816*	9.79
1(M1)	0.187	2.24	0.185	2.22	0.284	3.41	0.250	3.00
2(M1)	0.480	5.76	0.616	7.39	0.537	6.44	0.863	10.36
3(M2)	0.325*	3.90	0.346*	4.15	0.585	7.02	1.005	12.06
4(M1)	0.374	4.49	0.597	7.16	0.579	6.95	0.812	9.74
5(M2)	0.442	5.30	0.364	4.37	0.401	4.81	0.375	4.50

* = A variation of 20% between the start and end values were taken to represent change in growth.

Table A3. Characterisation of the isolated, reference and marker cultures, using the Rapid API 50 CHL identification system.

Test	A(M1)	B(M1)	C(M2)	D(M2)	E(M1)	F(M2)	G(M1)	H(M2)	I(M2)	J(M2)	K(M1)	1(M1)	2(M1)	3(M2)	4(M1)	5(M2)
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	+	-	-	-	-	+	-	+	-	+	-	+	-	+	-
Ribose	+	+	-	-	-	-	+	+	+	-	+	-	+	-	+	-
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	?	-	+	-	?	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	-	+	+	+	+	-	+	-	+	+	+	-	+	+	+
D-Mannose	-	-	+	+	+	+	-	+	-	?	-	+	-	?	-	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α Methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N Acetyl glucosamine	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
Amygdaline	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-
Arbutine	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Esculine	-	-	+	+	+	+	-	+	-	-	-	+	-	-	-	-
Salicine	-	-	?	?	+	+	-	+	-	-	-	+	-	-	-	-
Cellobiose	-	-	-	-	+	+	-	+	-	-	-	+	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	-	+	+	+	+	?	+	?	-	-	+	-	-	+	+
Saccharose	-	-	+	+	+	+	-	+	-	-	-	+	-	-	-	+
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	+	-	-	-	-	-	-	?	-	-	-	-	-	-	-
D-Raffinose	-	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-
Amidon	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B Gentiobiose	-	-	-	-	+	+	-	+	-	-	-	+	-	-	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Fructose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fructose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A3. (Cont.)

	Lb. brevis-R	*Lb. delbrueckii* ssp. *bulgaricus*-R	*Lb. fermentum*-R	*Lac.lactis* ssp. *lactis*-R	*Lb. plantarum*-R	*Lb. acidophilus*1-M	*Lb. acidophilus*2-M	*Lb. bavaricus*-M	*Lb. brevis* 1-M	*Lb. brevis* 2-M
Glycerol	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	-	-	-	+	-	-	-	-	+
Ribose	+	-	+	+	+	-	-	+	-	+
D-Xylose	+	-	-	+	-	-	-	-	-	+
L-Xylose	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-
B Methyl-xyloside	-	-	-	-	-	-	-	-	-	-
Galactose	-	+	+	+	+	-	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	-	+	+	+	+	+	+	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	+	-	-	-	-	-
Sorbitol	-	-	-	-	+	-	+	-	-	-
α Methyl-D-mannoside	-	-	-	-	+	-	-	-	-	-
α Methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-
N Acetyl glucosamine	-	-	-	+	+	-	+	-	+	-
Amygdaline	-	-	-	+	+	-	-	-	+	-
Arbutine	-	-	-	+	+	-	-	-	+	-
Esculine	-	-	-	+	+	+	+	+	+	-
Salicine	-	-	-	+	+	+	-	+	+	-
Cellobiose	-	-	-	+	+	+	+	+	+	-
Maltose	+	-	+	+	+	+	+	+	+	+
Lactose	-	+	+	+	-	-	+	+	-	-
Melibiose	-	-	+	-	+	-		+	-	+
Saccharose	-	-	+	+	+	+	+	+	+	+
Trehalose	-	-	-	+	+	-	-	-	-	-
Inuline	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	+	-	-	-	-	-
D-Raffinose	-	-	-	-	?	-	+	-	-	+
Amidon	-	-	-	+	-	-	+	-	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-
B Gentiobiose	-	-	+	+	+	+	+	-	-	-
D-Turanose	-	-	-	-	+	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-
D-Fructose	-	-	-	-	-	-	-	-	-	-
L-Fructose	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-
Gluconate	+	-	-	+	+	-	-	+	+	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-	-	-	-	-

Table A3. (Cont.)

	Lb. brevis 3-M	*Lb. brevis* 4-M	*Lb. buchneri*-M	*Lb. fermentum*-M	*Lb. fructivorans*-M	*Lb. helveticus*-M	*Lb. kefirgranum*-M	*Lb. kefiri*-M	*Lb. kefiranofaciens*-M	*Lb. parakefiri*-M
Glycerol	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	+	+	?	-	-	-	+	-	+
Ribose	+	+	+	+	+	-	-	+	-	+
D-Xylose	+	?	-	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-
B Methyl-xyloside	-	-	-	-	-	-	-	-	-	-
Galactose	+	?	?	+	-	+	+	-	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	?	+	+	+	-
D-Mannose	-	-	-	+	-	?	+	-	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	+	-	-	-	-
α Methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-
α Methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-
N Acetyl glucosamine	-	-	-	-	-	-	-	-	-	-
Amygdaline	-	-	-	-	-	-	-	-	-	-
Arbutine	-	-	-	-	-	-	-	-	-	-
Esculine	-	-	-	-	-	-	?	-	-	-
Salicine	-	-	-	-	-	-	?	-	-	-
Cellobiose	-	-	-	-	-	-	?	-	+	-
Maltose	+	+	+	+	?	?	+	+	+	+
Lactose	-	?	?	+	-	+	+	+	+	+
Melibiose	-	+	+	+	-	-	+	+	+	?
Saccharose	-	?	?	+	?	-	?	-	+	-
Trehalose	-	-	-	-	-	?	?	-	-	-
Inuline	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	+	-	-	-	-	-	-	+
D-Raffinose	-	?	?	+	-	-	+	-	+	-
Amidon	-	-	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-
B Gentiobiose	-	-	-	-	-	-	-	-	-	-
D-Turanose	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-
D-Fructose	-	-	-	-	-	-	-	-	-	-
L-Fructose	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-
Gluconate	+	+	+	+	?	-	-	+	-	-
2 ceto-gluconate	-	-	-	-	-	-	-	-	-	-
5 ceto-gluconate	-	-	-	-	-	-	-	-	-	-

Table A4. Characterisation of the isolated, reference and marker cultures, using the Rapid API 20 E identification system.

	A(M1)	B(M1)	C(M2)	D(M2)	E(M1)	F(M2)	G(M1)	H(M2)	I(M2)	J(M2)	K(M1)	1(M1)	2(M1)	3(M2)	4(M1)	5(M2)
ONPG#	+	+	+	+	-	-	+	+	+	-	+	+	+	-	+	-
Arginine	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-
Lysine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tryptophane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP*	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-
Gelatinase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NO_2 production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

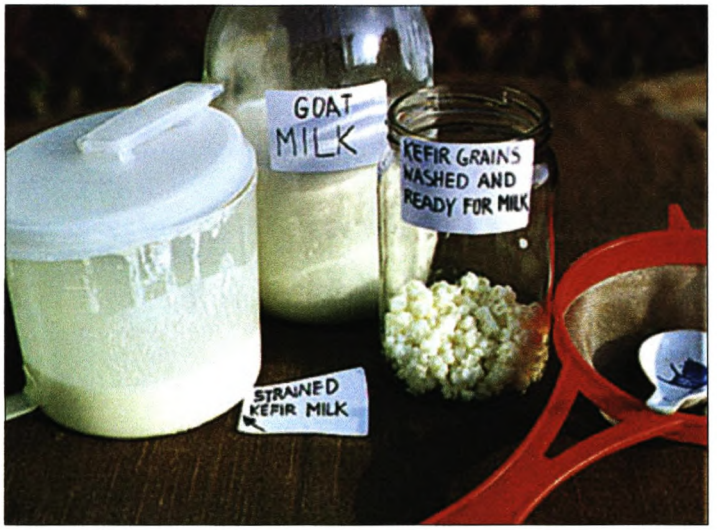
= Ortho-nitro-β-D-galactopyranoside
* = Acetoin production

Table A4. (cont.)

Test	*Lb. brevis*3-R	*Lb. delbrueckii* ssp. *bulgaricus*-R	*Lb. fermentum*-R	*Lac.lactis* ssp. *lactis*-R	*Lb. plantarum*-R	*Lb. acidophilus* 1-M	*Lb. acidophilus* 1-M	*Lb. bavaricus*-M	*Lb. brevis* 1-M	*Lb. brevis* 2-M	*Lb.brevis* 3-M
ONPG*	-	-	+	-	-	-	-	-	-	-	-
Arginine	+	-	+	+	-	-	-	-	+	+	+
Lysine	-	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-	-	-
Tryptophane	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-
VP*	-	-	-	+	+	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-	-	-
NO_2 production	-	-	-	-	-	-	-	-	-	-	-

Table A4. (cont.)

	Lb. brevis 4-M	*Lb. buchneri*-M	*Lb. fermentum*-M	*Lb. fructivorans*-M	*Lb. helveticus*-M	*Lb. kefirgranum*-M	*Lb. kefiri*-M	*Lb. kefiranofaciens*-M	*Lb. parakefiri*-M
ONPG*	-	-	+	-	-	-	-	+	-
Arginine	+	+	+	+	-	-	+	-	+
Lysine	-	-	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-
Tryptophane	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-
VP*	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-
NO_2 production	-	-	-	-	-	-	-	-	-



CHAPTER 4

EVALUATION OF THE GRAIN FORMATION ABILITY OF KEFIRAN CULTURES

CHAPTER 4

EVALUATION OF THE GRAIN FORMATION ABILITY OF KEFIRAN CULTURES

Abstract

Cultures isolated from Kefiran were evaluated for ability to grain formation by adding 1 x 10^9 cfu.ml^{-1} bacteria and 1 x 10^8 cfu.ml^{-1} yeast to double pasteurised, full cream milk during the mass cultivation process. The Kefiran cultures included strains of *Lactobacillus parakefiri, Lb. kefiri, Lb. delbrueckii* ssp. *bulgaricus, Lb. gallinarum, Lb. acidophilus, Lb. bavaricus,* a *Lactobacillus* and *Candida lambica.* It was found that the control and all the cultures in double pasteurised milk showed grain accumulation indicating that other microbes were present in pasteurised and double pasteurised milk which had an influence on the grain forming ability. The cultures isolated from pasteurised and double pasteurised milk included members of the species *Pediococcus, Acinetobacter, Lactococcus lactis* ssp. *lactis, Candida lipolytica, C. guilliermondii, Chryseobacterium meningosepticum, Pseudomonas putida* and four isolates of the *Bacillus cereus* group. These "milk isolates" were also tested for grain formation singly and in combination with the Kefiran cultures in double pasteurised and UHT milk. It was found that it is possible to obtain grain-like structures through the mass cultivation process with single strain starters and furthermore, it was possible to produce an acceptable Kepi-like beverage from these single strain starter grains.

Introduction

The Kepi grain microbial community consists of a symbiotic association of yeasts and lactic acid bacteria (LAB), but the overall composition of the grains has not completely been elucidated (Saloff-Coste, 2000). The microbes in the grains are embedded in a resilient protein-polysaccharide Kefiran matrix, which is essential for grain formation (La Rivière *et al.,* 1967; Lin *et al.,* 1999). Many studies have been done on the identification of the major Kefiran producer and Fujisawa *et al.* (1988) described a capsule-forming homofermentative lactobacilli, *Lactobacillus kefiranofaciens,* as the main Kefiran producer. It is generally considered that the mechanism of grain formation is not fully understood and it remains undecided which organism is really responsible for the production of this protein-polysaccharide matrix (Özer & Özer, 2000).

Apart from the traditional method of Kepi making with Kepi grains, it has also been produced using commercially prepared pure starter cultures, which may be in powdered or liquid forms (Libudzisz & Piatkiewicz, 1990). Commercial starters are used especially for large scale

manufactures, but according to Marshall & Cole (1985), this product cannot be compared with the traditional Kepi produced with the grains as starter. The industrial preparations deliberately contain few and sometimes no yeasts because the production of CO_2 by yeasts have led to "blown" packages which consumers have mistaken as spoiled. These commercial cultures consist almost entirely of "*Streptococcus lactis* ssp. *diacetylactis*", "*Leuconostoc cremoris*" or "*Steptococcus thermophilus*" and "*Lactobacillus bulgaricus*". The resulting product lacks the foaming characteristics of traditional Kepi and contains less than 0.01% ethanol. In another study, Toba *et al.* (1991) prepared a fermented Kepi type milk with *Lactobacillus kefiranofaciens* as starter culture, and although the product had a ropy consistency, it was given lower scores for acceptability by a consumer panel than a similar product made with *Lactobacillus delbrueckii* ssp. *bulgaricus*. According to Saloff-Coste (2000) milk for the production of the Kepi beverage can be inoculated simultaneously with lactic acid bacteria and yeasts, or it can undergo two separate fermentations, the first with lactic acid bacteria and the second with yeast. She concluded that finding the right equilibrium of bacterial and yeast strains to create a product with the characteristic properties of traditional kefir including both the organoleptic qualities and the health benefits, is a difficult task. In contrast, Beshkova *et al.* (2002) concluded that specific microbes isolated from Kefir grains and incorporated into a starter culture can ensure a product with properties that are close to that of traditional kefir.

In the absence of the actual grains, many of the natural properties that are produced by the grains and released into the media, are not found in the commercial product (Anfiteatro, 1999). When using single strain starter cultures to make the Kepi beverage, new starter cultures have to be prepared for inoculations for each beverage production and at the end of the fermentation there are no grains that can be re-used. The incorporation of pure cultures to form grains which could be re-used like traditional Kepi grains would be cheaper than the use of single starters and still have the characteristic appearance, taste and health properties of traditional Kepi. Unfortunately, according to literature, attempts to make new grains by recombining isolated microbes have so far been unsuccessful (Lui & Moon, 1983; Libudzisz & Piatkiewics, 1990).

The aim of this study was to evaluate grain formation with purified cultures isolated from Kefiran strings using a mass cultivation process.

Materials and methods

Study 1 – Grain formation using Kefiran cultures in double pasteurised milk and MRS broth

In this study the ability of single strain starters to form grains using the mass cultivation process (Schoevers, 1999) was examined. The study was done in duplicate. Pasteurised (P) full

cream milk was given a further heat treatment in a temperature-controlled waterbath at 80°C for 30 min and then cooled to 4°C. The purpose of this double pasteurisation (DP) was to obtain milk of a reliable microbiological quality, as previous studies on commercially pasteurised milk showed that pasteurisation was not always performed satisfactorily (Human, 1998). The Kefiran cultures (Table 1) isolated in Chapter 3 of this thesis were inoculated (1 x 10^9 cfu.ml^{-1} for the bacteria and 1 x 10^8 cfu.ml^{-1} for the yeasts) into the double pasteurised milk with added yeast extract (2% (m/v)) and urea (0.5% (m/v)) (dedicated as DP+). The inoculation concentrations were determined spectrophotometrically (Spectronic 20, Spectronic Instruments, USA) at 550 nm. Two hundred ml of milk with the added Kefiran isolate was incubated in 250 ml and 500 ml containers in a shake waterbath (rpm = 130) at 25°C, and after sieving, 50% of the milk was replaced every 24 h for 9 d. The ability of the isolates to form grains were also tested in duplicate in 200 ml MRS broth (pH = 6) in 250 ml and 500 ml containers.

The pH and TA (titratable acidity) were determined daily, and the biomass changes and lactose content were determined at the end of each study. The TA of the samples was determined by the titration of a 9 ml sample with standardised 0.1 M NaOH to the phenolphthalein endpoint (Dixon, 1973). The lactose content was measured colorimetrically with the use of methylamine as an indicator (Katsu *et al.*, 1994).

Study 2 – Effects of different milk heat treatments on grain formation

Based on the results obtained in Study 1 indications were found that there were microbes present that survived the pasteurisation process, which could play a role in grain formation. In this study, the impact of different milk heat treatments (Table 2) were evaluated for grain formation. This study was done in duplicate and consisted of three sub-studies. In **Study 2a**, pasteurised full cream milk (P) was subjected to three different heat treatments, which included double pasteurisation (DP), pasteurisation plus sterilisation (PS) and double pasteurisation plus sterilisation (DPS). In **Study 2b**, the same four milk heat treatments were applied, but additionally yeast extract (2% (m/v)) and urea (0.5% (m/v)) (dedicated as +) were added to the milk. In **Study 2c**, the same milk heat treatments were applied, but the *Lactobacillus gallinarum* culture D (Table 1) was added as inoculum. Culture D was chosen based on the close clustering position to *Lactobacillus kefiranofaciens* on the dendrogram as illustrated in Chapter 3 of this thesis. Furthermore, *Lb. kefiranofaciens* was previously reported to be the main Kefiran producer (Fujisawa *et al.*, 1988) and probably is the major player during grain formation.

Table 1. Isolates from Kefiran strings.

Isolate	Identification
Bacteria	
A	*Lactobacillus kefiri*
B	*Lactobacillus parakefiri*
D	*Lactobacillus gallinarum*
F	*Lactobacillus acidophilus*
H	*Lactobacillus bavaricus*
3	*Lactobacillus delbrueckii* ssp. *bulgaricus*
5	Unidentified homofermentative *Lactobacilli*
Yeast	
6	*Candida lambica*

Table 2. Different milk heat treatments used in Study 2.

Abbreviation	Method	Parameters
P	Pasteurised, full cream	Used direct from commercial sachet
P+	Pasteurised, full cream with added sterile yeast extract and urea	Used direct from sachet
DP	Double pasteurised, full cream	80°C, 30 min
DP+	Double pasteurised, full cream, with added yeast extract and urea	80°C, 30 min
PS	Pasteurised-sterilised milk	121.1°C, 15 min, 100 kPa
PS+	Pasteurised-sterilised milk, with added yeast extract and urea	121.1°C, 15 min, 100 kPa
DPS	Double pasteurised-sterilised milk	80°C, 30 min plus 121.1°C, 15 min, 100 kPa
DPS+	Double pasteurised-sterilised milk, with added yeast extract and urea	80°C, 30 min plus 121.1°C, 15 min, 100 kPa

+ = Indicates that yeast extract (2% (m/v)) and urea (0.5% (m/v)) were added.

Study 3 - Isolation of "milk isolates"

Based on the results obtained in Study 2, where it was found that there were microbes present in P and DP milk, an isolation senario was set up. Two hundred millilitres of the heat treated milks (P, P+, DP, DP+, PS, PS+, DPS, DPS+, UHT and UHT+) were mass cultivated in 250 ml containers in a shake waterbath at 25°C for 2 d. A dilution series of samples from days 0 and 2 of the mass culturing was made from these heat treated milk using MRS (pH 6.0) and NA media (pH 7.0), and the plates incubated aerobically and anaerobically (Anaerocult-A system) at 30°C for 3 to 5 d.

Morphologically different colonies were chosen from P, P+, DP and DP+ milks and purified. The Gram-stain, oxidase and catalase tests (Gerhardt *et al.,* 1994) were performed on each purified isolate, and the production of gas and acid from glucose determined. The biochemical profiles of the isolates were determined using combinations of the API 50 CHL, API 50 CHB and API 20 NE systems (API system S.A., La alme le Grottes, 38390 Montalieu Vercieu, France). Additional characteristics (API methodology) were determined which included: Ortho-nitro-β-D-galactopyranoside (Beta-galactosidase); arginine dihydrolase; lysine decarboxylase; ornithine decarboxylase; citrate utilisation; H_2S production; urea; tryptophane deaminase; indole production; acetoin production; gelatinase and NO_2 production. These results were used together with the results of the API 50 CHL, API 50 CHB, API 20 NE and the general characteristics to identify the isolates. Numerical clustering and DNA sequencing comparisons were performed on the isolates which could not be positively identified with the API systems. The yeasts were identified using the Rapid ID 32C system. Once purified, all the "milk isolates" were lyophilised using the method of Joubert & Britz (1987).

Study 4 – Grain formation using Kefiran cultures in UHT milk

In Study 1 it was found that all the Kefiran cultures including the uninoculated (control) (Table 1) produced a large mass of grains in the DP milk. It was assumed that there were other microbes that survived the double pasteurisation process which could play a role in grain formation. In this study the ability of only the Kefiran isolates to form grains in UHT milk (with added yeast extract and urea = UHT+) was tested. The control was UHT+ milk without added isolates.

Study 5 - Grain formation in UHT milk using the "milk isolates" as inoculum

In Study 4 it was found that the Kefiran cultures did not produce large masses of grain accumulates in UHT milk and in Study 3 it was found that other organisms that survived the heat

treatments could play an important role. In this study the ability of the 12 "milk isolates" from the P and DP milks (S1, S2, S3, S4, S5, S6, C1, C2, C3, C4, G1 and G2) were evaluated as grain formers in UHT+ milk.

Study 6 – Grain formation in UHT milk with four inoculum combinations of the 'milk isolates" and Kefiran cultures

In Study 5, it was found that the different "milk isolates" led to different grain-like accumulations masses. It was therefore deemed necessary to evaluate the effect of combining the Kefiran cultures with the "milk isolates". During this study, combinations of each "milk isolate" and Kefiran cultures were evaluated singly in UHT+ milk to observe the effect of combinations on grain formation. The choice of the "milk isolates" was based on the mass of grains produced in UHT milk during Study 5. In **combination 1**, the yeast "milk isolate" (G2) was combined singly with each of the Kefiran cultures (A, B, D, F, H, 3, 5 and 6). In **combination 2**, the Gram-negative rod "milk isolate" (S6) was combined with each of the eight Kefiran cultures. The Gram-positive rod-shaped "milk isolates" (S3 and S4) were used in **combinations 3** and **4**, respectively.

Study 7 - Final combinations of Kefiran cultures and 'milk isolates"

In the results from Study 6, it was found that combinations of the Kefiran cultures with "milk isolates" S4 (Table A14) and S6 (Table A12) produced a larger mass of grains than found with combinations of the Kefiran cultures and "milk isolates" S3 (Table A13) and G2 (Table A11). "Milk isolates" S4 and S6 were thus used in the final combinations as a result of the larger mass of grains produced by the combinations.

This study consisted of four different final combinations of "milk isolates" and/or Kefiran cultures. In **combination 1**, Kefiran culture D was added to DP+ milk. No "milk isolates" were added in combination 1. Kefiran culture D was chosen due to its position found with numerical clustering to be closely related to *Lactobacillus kefiranofaciens*, which according to Fujisawa *et al.* (1988) can be ascribed as the main Kefiran producer and major role player in grain formation.

Combination 2 consisted of a combination of Kefiran cultures D, A, F, 3 and 6 in DP+ milk. The yeast (6) was added to stimulate the characteristic production of CO_2. *Lactobacillus kefiri* (A) was found to produce Kefiran which contributed to grain formation (Saloff-Coste, 2000). *Lactobacillus acidophilus* (F) and *Lb. delbrueckii* ssp. *bulgaricus* (3) were added due to the stability and mass of the grains produced in Studies 1, 4 and 6.

In **combinations 3 and 4**, "milk isolates" S4 and S6 were added respectively, to the

same Kefiran cultures used in combination 2 (D, A, F, 3 and 6). For these two combinations UHT+ milk was used to investigate the effect of combinations of the "milk isolates" and Kefiran cultures on grain formation.

Study 8 - Beverage production using combinations of single strains

The aim of this study was to use the grain-like accumulations from the previous studies for Kepi beverage production. Four beverages were produced with the grains obtained from the four combinations in Study 7, using the traditional Kepi production method. Five grams of grains from each of the four combinations of Study 7 were added as inoculum to 100 ml DP+ milk and incubated at 22°C for 18 h. The grains were separated by sieving and the beverage was further incubated at 18°C for 6 h and then stored at 2°C. This procedure was repeated 12 times.

The four final beverages were then evaluated by a selectively trained panel according to viscosity, appearance, aroma, taste as well as final pH, TA and lactose. These characteristics were evaluated with traditional Kepi as the reference standard.

Results and discussion

As a result of the large volume of data generated during the study, and to simplify the discussion section, the data are given at the end of this chapter as Appendix A.

Study 1 – Grain formation using Kefiran cultures in double pasteurised milk and MRS broth

In this study the Kefiran cultures were added singly to DP+ milk and MRS broth, in both 250 and 500 ml containers. It was found that there was grain accumulation (Fig. 1A) in the control, as well as when the Kefiran cultures were inoculated singly into DP+ milk in the 250 ml containers (Table A1 in the appendix). No grain formation was observed when the single Kefiran cultures were inoculated into 500 ml DP+ milk or into the 250 ml and 500 ml MRS broth containers. It was, however, found that the biomass did increase in the MRS broth but no grain-like accumulation were formed. Based on the results it was decided that only 250 ml containers will be used for the mass cultivation process in the remaining studies.

These results suggested that there could possibly be microbes present in the DP milk which survived pasteurisation. The effect of different heat treatments of milk thus need to be investigated. It was also concluded from the MRS medium study that the milk medium was necessary for grain formation.

Study 2 – Effects of different milk heat treatments on grain formation

In this study the effect of different milk heat treatments on grain formation was

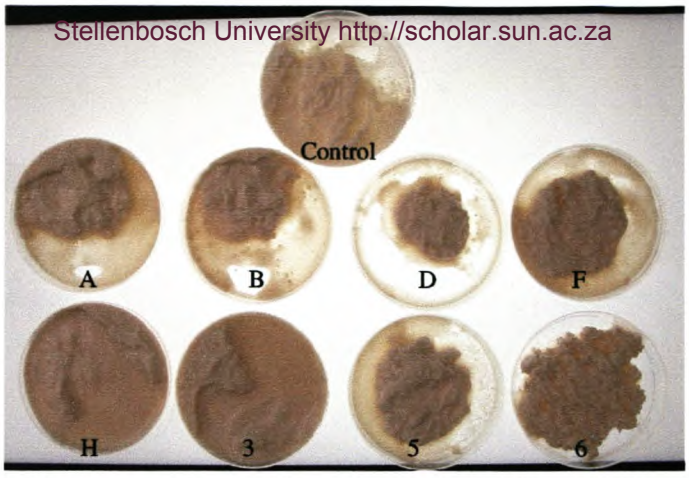


Figure 1A. Grain-like accumulation in DP+ milk after inoculation with the eight Kefiran cultures.

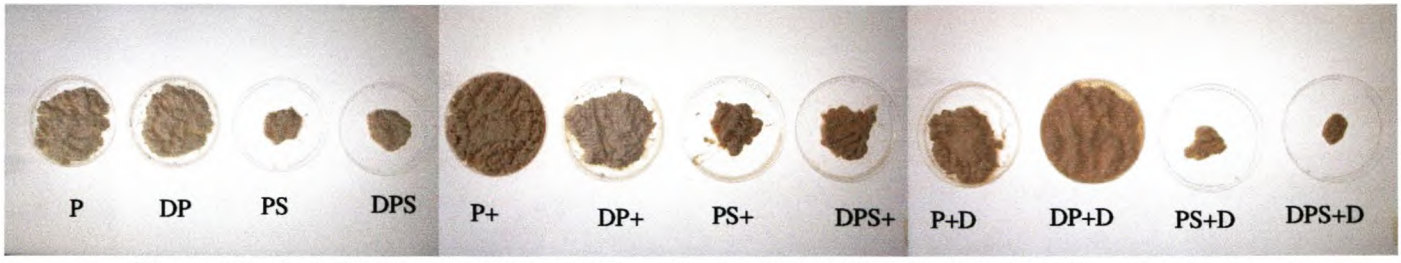


Figure 1B. Grain-like accumulation of different heat treated milks with and without inoculated cultures.

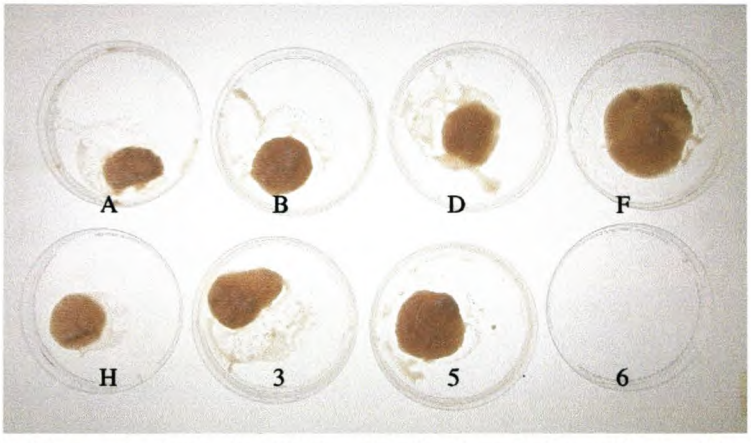


Figure 1C. Grain-like accumulation in UHT+ milk after inoculation with the eight Kefiran cultures.

Figure 1D. Grain-like accumulation in UHT+ milk after inoculation with the 12 "milk isolates".

evaluated and the results for days 0 and 2 are given in Table A2 and A3, respectively, in the appendix, as well as in Fig. 1B. The grain-like formation was found in all three sub-studies in both P and DP milks. The mass of the grains in P, P+, P+ and culture D, DP, DP+ and DP+ and culture D milks after 9 d mass culturing was > 33 g in comparison to the accumulation of < 12 g grains observed for the PS, PS+, PS+ and culture D, DPS, DPS+ and DPS+ and culture D milks after 9 d.

From these results it was concluded that there are microbes present in P and DP milks which survived pasteurisation and double pasteurisation but these were eliminated after sterilisation. Furthermore, these results indicated that the microbes present in inoculated P and DP milks have a strong effect on grain formation.

Study 3 - Isolation of 'milk isolates"

In this study microbes which survived the four different milk heat treatments (P, DP, PS, DPS) (Table 2), were isolated and identified. The results for the viable counts on MRS and NA media for all the heat treated milks on days 0 and 2 are given in Table 3. No growth was found on the plates of the PS, PS+, DPS, DPS+, UHT and UHT+ milks on either days 0 or 2.

Six rod-shaped isolates, four coccus-shaped isolates and two yeast strains were isolated from the plates (Table 4). The yeasts isolates were identified as members of the species *Candida lipolytica* (G1, API identification of 99.7%) and *C. guilliermondii* (G2, API identification of 88.6%). The results of the Rapid ID 32C are given in Table A4 of the appendix.

The general characteristics of the rod and coccus-shaped isolates are given in Table 5. All the isolates were found to be oxidase negative and no isolate produced gas from glucose. Four of the rod-shaped isolates, S1, S2, S3 and S4 were Gram, catalase and VP positive and produced acid from glucose and showed endospore formation. The other two rods, S5 and S6, were catalase and VP positive, and Gram negative, produced no acid from glucose and formed endospores. Three of the cocci, C1, C2 and C4, were Gram and VP positive, catalase negative, no endospore formation, but produced acid from glucose. Isolate C3 was found to be catalase positive, and Gram and VP negative, produced no acid from glucose and showed no endospore formation.

The identification status of the rod and coccus-shaped bacterial isolates is given in Table 6. Four of the rod-shaped isolates were identified as members of the *Bacillus cereus* group with the API 50 CHB system (Table A5 in appendix) and numerical clustering (Fig. 2) (S1 (API identification of 98.6%), S2 (API identification of 88.6%), S3 (API identification of 88.6%) and S4 (API identification of 87.9%)). Isolate S1 was confirmed to be a representative of the *Bacillus cereus* species using DNA sequence analysis of the 16 S ribosomal RNA gene (of the

Table 3. Average viable counts on MRS and NA media on day 0 and after 2 days of mass cultivation in milks with different heat treatments.

Heat treatment	Culture method	MRS (cfu.ml^{-1})		NA (cfu.ml^{-1})	
		Day 0	**Day 2**	**Day 0**	**Day 2**
P	A	1.3×10^{1}	2.24×10^{11}	4.0×10^{2}	1.6×10^{11}
	An	2.6×10^{4}	1.76×10^{11}	1.5×10^{4}	1.22×10^{11}
P+	A	2.2×10^{1}	2.8×10^{11}	5.0×10^{3}	8.8×10^{10}
	An	1.2×10^{1}	1.92×10^{10}	8.0×10^{1}	5.4×10^{10}
DP	A	8.4×10^{2}	6.2×10^{9}	1.6×10^{1}	$3. \times 10^{10}$
	An	1.24×10^{3}	1.29×10^{10}	1.3×10^{1}	4.4×10^{10}
DP+	A	3.4×10^{4}	8.4×10^{10}	3.6×10^{3}	7.4×10^{9}
	An	3.6×10^{4}	4.8×10^{10}	3.6×10^{3}	1.2×10^{10}
PS	A	-	-	-	-
	An	-	-	-	-
PS+	A	-	-	-	-
	An	-	-	-	-
DPS	A	-	-	-	-
	An	-	-	-	-
DPS+	A	-	-	-	-
	An	-	-	-	-
UHT	A	-	-	-	-
	An	-	-	-	-
UHT+	A	-	-	-	-
	An	-	-	-	-

Numbering:
A = Aerobic
An = Anaerobic
P = Pasteurised
P+ = Pasteurised with yeast extract and urea
DP = Double pasteurised
DP+ = Double pasteurised with yeast extract and urea
PS = Pasteurised, sterilised
PS+ = Pasteurised, sterilised with yeast extract and urea
DPS = Double pasteurised, sterilised
DPS+ = Double pasteurised, sterilised, with yeast extract and urea
UHT = Ultra high temperature milk
UHT+ = Ultra high temperature milk, with yeast extract and urea

Table 4. Microbial strains isolated from P and DP milks and identification methods used.

Isolated culture code	Characterisation method
Rod-shaped bacteria	
S1(DPM0)	API 50 CHB + API 20 E + NA + MI
S2(DPM2)	API 50 CHB + API 20 E + NA + MI
S3(DPN0)	API 50 CHB + API 20 E + NA
S4(DPN2)	API 50 CHB + API 20 E + NA
S5(DP+N2)	API 20 NE + API 20 E + MI
S6(DP+N2)	API 20 NE + API 20 E + MI
Coccus-shaped bacteria	
C1(DPM0)	API 50 CHL + API 20 E
C2(DP+M0)	API 50 CHL + API 20 E
C3(DP+N2)	API 20 NE + API 20 E + MI
C4(DP+M2)	API 50 CHL + API 20 E
Yeasts	
G1(DP+N2)	Rapid ID 32 C
G2(P+M2)	Rapid ID 32 C

Numbering: First digit - isolate morphology (S = Rod, C = Cocci, G = Yeast); second digit - isolate number; third digit - milk source (DP = Double pasteurised, DP+ = Double pasteurised with yeast extract and urea, PS = Pasteurised-sterilised, P+ = Pasteurised with yeast extract and urea. Fourth digit = Isolation medium (M - MRSL; N - NA); Fifth digit - Day number (0 and 2)
NA = Numerical clustering, MI = Molecular identification

Table 5. General characteristics of the rod and coccus-shaped "milk isolates".

Isolate	Gram stain	Catalase	Oxidase	Endospore and position	VP*	Acid from glucose	Gas from glucose
Rod-shaped							
S1	+	+	-	Central	+	+	-
S2	+	+	-	Central	+	+	-
S3	+	+	-	Central	+	+	-
S4	+	+	-	Central	+	+	-
S5	-	+	-	-	+	-	-
S6	-	+	-	-	+	-	-
Coccus-shaped							
C1	+	-	-	-	+	+	-
C2	+	-	-	-	+	+	-
C3	-	+	-	-	-	-	-
C4	+	-	-	-	+	+	-

*= Acetoin production

Table 6. Identification of "milk isolates".

Isolated culture code	ID
Rod-shaped bacteria	
S1	*Bacillus cereus* isolate-1
S2	*Bacillus cereus* isolate-2
S3	*Bacillus cereus* isolate-3
S4	*Bacillus cereus* isolate-4
S5	*Chryseobacterium meningosepticum*
S6	*Pseudomonas putida*
Coccus-shaped bacteria	
C1	*Pediococcus* sp.
C2	*Lactococcus lactis* ssp. *lactis*
C3	*Acinetobacter* sp.
C4	*Lactococcus lactis* ssp. *lactis*
Yeast	
G1	*Candida lipolytica*
G2	*Candida guilliermondii*

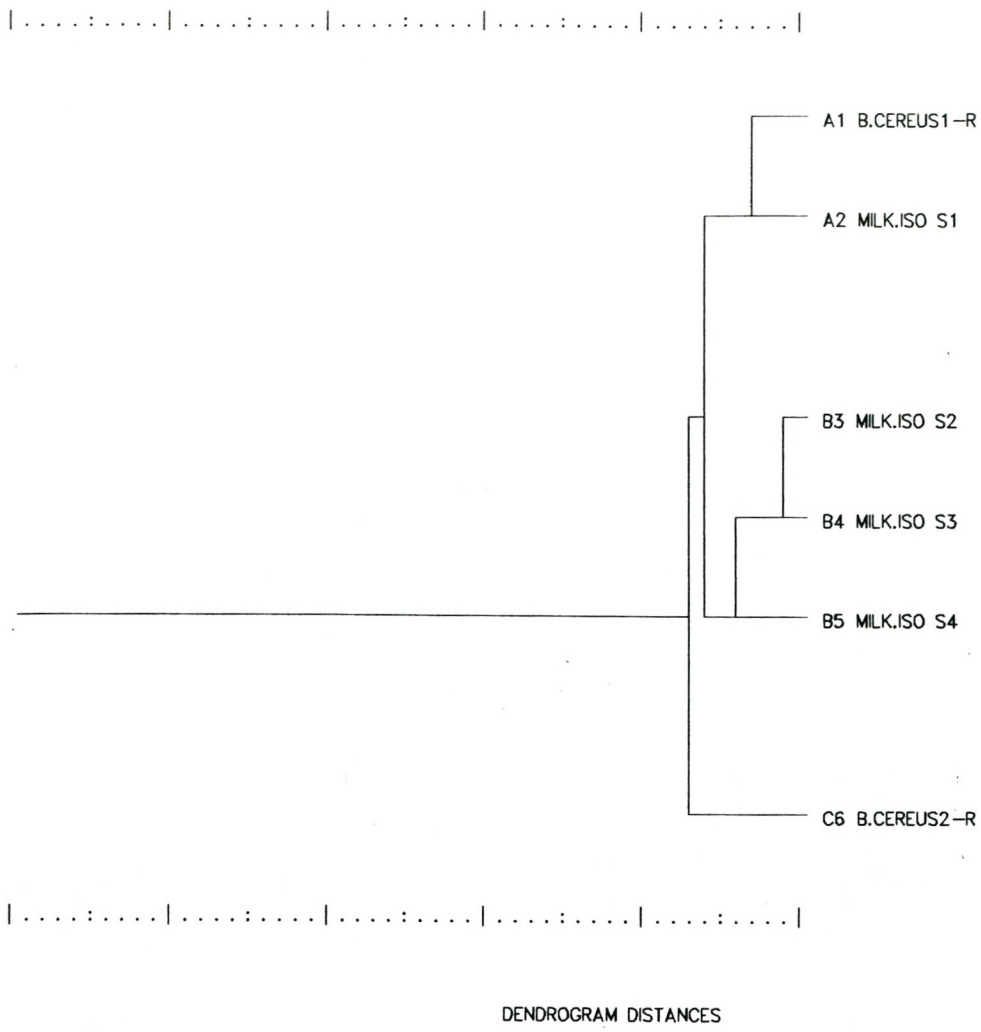


Figure 2. Numerical clustering of the four rod-shaped "milk isolates" and two *Bacillus cereus* reference strains (R).

600 bp aligned, 597 were homologous) and S2 was found to be related to *B. cereus* (of the 626 bp aligned, 614 were homologous). According to Baillie (1999), several *B. cereus* sub-groups are phenotypically related to the "*B. cereus* main-group". These sub-groups are comprised of *B. cereus*, *B. anthracis*, *B. thuringiensis* and *B. mycoides*.

Bacillus cereus endospores are known to be able to survive pasteurisation and they are also considered to be contaminants of milk from cows fed on grass and cows which are housed indoors (Griffiths, 1992). Furthermore, endospores of *B. cereus* are hydrophobic and readily adhere to the surface of steel, glass and even rubber (Griffiths, 1992). Stevensson *et al.* (2000) reported that the endospores of *B. cereus* mainly survive and propagate due to biofilm formation in pasteurisers. Their survival in South African pasteurised milk is probably due to the survival of endospores during the pasteurisation process or even as a result of the post-contamination of pasteurised milk.

Isolates S5 and S6 were identified as *Chryseobacterium meningosepticum* (API identification of 99.9%) and *Pseudomonas putida* (API identification of 99.6%), respectively, using the API 20 NE data base (Table A6 in appendix). The identifications were confirmed with molecular identification (of the 700 bp aligned, 693 were homologous and of the 597 bp aligned, 593 were homologous, respectively). Mackenzie (1973) reported that the lack of efficient cleaning and disinfecting of equipment may also lead to biofilm formation, which can subsequently result in a rapid increase of fast-growing lactococci, coliforms and other Gram-negatives such as *Pseudomonas*, *Alcaligenes*, "*Flavobacterium*" and *Chromobacterium.* Hugo *et al.* (1999) isolated 107 *Chryseobacterium* isolates from dairy, and two of them were found to be members of the species *C. meningosepticum.* Yamaguchi & Yokoe (2000) reported that strains belonging to *Chryseobacterium* were isolated from various ecosystems, such as water, soils and marine environments.

The coccus-shaped isolates were identified as a *Pediococcus* sp. (isolate C1, API identification of 80.3%) and *Lactococcus lactis* ssp. *lactis* strains (isolates C2 and C4, API identification of 85.8%) with the API 50 CHL database (Table A7 in appendix). Isolate C3 was identified as an *Acinetobacter* sp. (API identification of 99.9%) with the API 20 NE database. This identification as an *Acinetobacter* sp. was confirmed with molecular identification (of the 703 bp aligned, 700 were homologous). The results of the API 20 E are given in Table A8 of the appendix.

Study 4 – Grain formation using Kefiran cultures in UHT milk

In this study, the ability of the Kefiran cultures (Table 1) to form grains in UHT+ milk were evaluated and the results are given in Table A9 in the appendix and in Fig. 1C. It was

found that the grain mass of all the cultures on day 9 were less than 5.7 g. When the mass of the grains in UHT+ milk (Table A9) are compared to that of DP+ milk after 9 d (Table A1), it is clear that there are other factors which contribute to grain formation and that the eight Kefiran cultures used in the study are not alone responsible for grain formation.

Study 5 - Grain formation in UHT milk using the "milk isolates" as inoculum

In this study the ability of the "milk isolates" (S1, S2, S3, S4, S5, S6, C1, C2, C3, C4, G1 and G2) to form grains was evaluated. The results are given in Table A10 in the appendix and in Fig. 1D. It was found that the mass of grains produced using the rod-shaped isolates was higher than that produced by the coccus-shaped isolates. The maximum mass of the grains from the coccus-shaped isolates in UHT+ milk was 3.8 g (isolate C2), whereas the mass for the rod-shaped isolates varied between 16.4 g and 35.8 g, with the exception of isolate S2 which only produced 7.1 g. The yeast isolate, G2 gave 18.7 g grains. From the data it was concluded that the most of the cultures ("milk isolates") that survived the milk heat treatment do contribute to grain formation.

Study 6 – Grain formation in UHT milk with four combinations of the "milk isolates" and Kefiran cultures

In this study four combinations of specific "milk isolates" with all the Kefiran cultures were evaluated for grain formation. In **combination 1**, the "milk isolate" G2 was combined with the Kefiran cultures and the results are given in Table A11 of the appendix and in Fig. 3A. The control contained G2 but without the Kefiran cultures and resulted in a maximum grain mass of 13.5 g. The Kefiran yeast culture (6) plus the yeast "milk isolate" (G2) resulted in 10.5 g. A maximum of 8.0 g and a minimum of 0.7 g of grains were produced by the other Kefiran isolates in combination with G2. From the data obtained it appeared as if the Kefiran cultures were inhibited by the presence of the yeast (G2).

The results for **combination 2** with "milk isolate" S6 (*Pseudomonas putida*) are given in Table A12 of the appendix and in Fig. 3B. The presence of the "milk isolate" S6 also appeared to inhibit the grain forming ability of Kefiran cultures F, 3, 5 and 6. In contrast, the grain formation by Kefiran cultures A, B, D and H were stimulated by the presence of the "milk isolate" (S6). The control (S6 without the Kefiran isolates) gave 16.4 g of grains. A maximum of 22.1 g (H) and a minimum of 5.9 g (F) of grains were produced by the other Kefiran cultures in combination with S6.

The results for **combination 3** with "milk isolate" S3 (*Bacillus cereus* isolate) are given in Table A13 of the appendix and in Fig. 3C. The "milk isolate" S3 was found to inhibit the

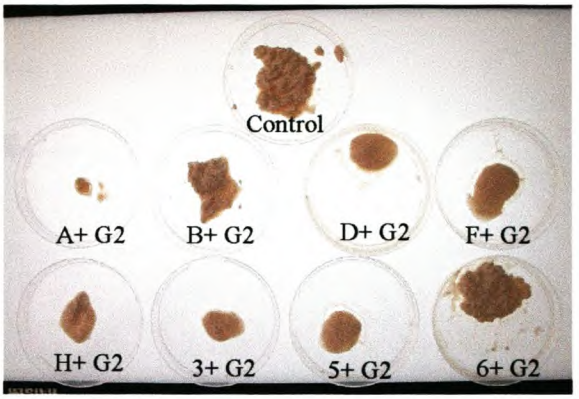


Figure 3A. Grain-like accumulation in UHT+ milk (combination 1) after inoculation with a combination of Kefiran cultures and "milk isolate G2".

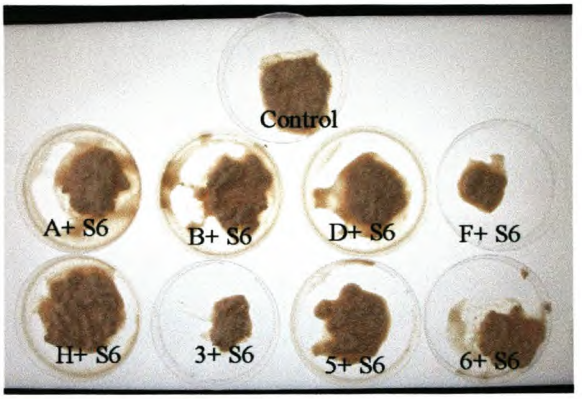


Figure 3B. Grain-like accumulation in UHT+ milk (combination 2) after inoculation with a combination of Kefiran cultures and "milk isolate S6".

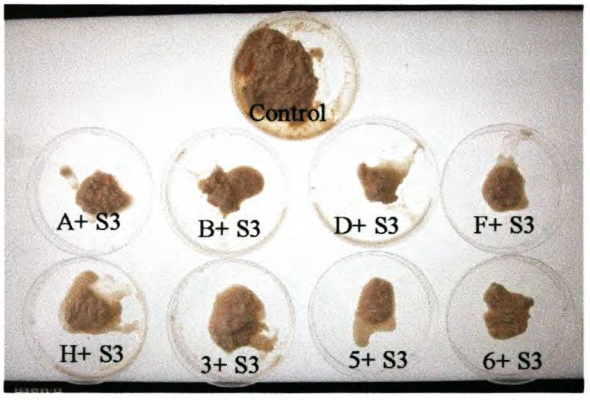


Figure 3C. Grain-like accumulation in UHT+ milk (combination 3) after inoculation with a combination of Kefiran cultures and "milk isolate S3".

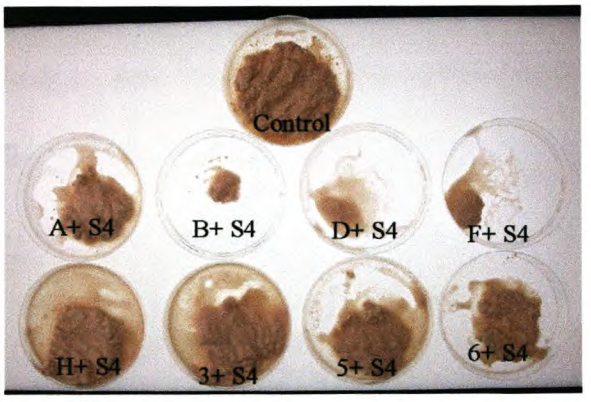


Figure 3D. Grain-like accumulation in UHT+ milk (combination 4) after inoculation with a combination of Kefiran cultures and "milk isolate S4".

grain formation of all the Kefiran cultures, with the maximum mass of grains produced (10 g) in combination with Kefiran culture 3. The control (S3 without any Kefiran cultures) gave 28.9 g of grains.

The results for **combination 4** with "milk isolate" S4 (*Bacillus cereus*) are given in Table A14 of the appendix and in Fig. 3D. The "milk isolate" S4 was also found to inhibit the Kefiran cultures B, D and F, with a maximum of grains produced (29.2 g) and a minimum of 2.7 g in combination with the Kefiran cultures. The control (S4 without any Kefiran cultures) resulted in the maximum grains produced (40.0 g).

Study 7 - Final combinations of Kefiran cultures and 'milk isolates"

The results of the four final combinations are given in Table 7. Culture D (**combination 1**) was found to produce 39.0 g of grains in DP+ milk (Fig. 4, plate 1). When the Kefiran cultures A, F, 3, 6 and D (**combination 2**) were all used together in combination in DP+ milk, only 8 g of grains were obtained (Fig. 4, plate 2). From the data it was concluded that Kefiran cultures A, F, 3 and 6 inhibited grain accumulation by Kefiran culture D and by any other organisms present in DP+ milk.

In **combination 3** (A, F, 3, 6, D and S4) in UHT+ milk, only 5.0 g of grains were obtained (Fig. 4, plate 3). From this it was concluded that the grain formation ability of "milk isolate" S4 was probably inhibited by the Kefiran culture combination (A, F, 3, 6 and D) in UHT+ milk. This conclusion was also based on a previous observation where "milk isolate" S4 (Study 6) alone produced 40.0 g grains in UHT+ milk.

In contrast with combination 3, **combination 4** (A, F, 3, 6, D and S6) produced 21.5 g grains in UHT+ milk (Fig. 4, plate 4). From this data it was concluded that "milk isolate" S6 was stimulated by this Kefiran culture combination (A, F, 3, 6 and D) in UHT+ milk. This confirms a previous observation in Study 6 where 16.4 g of grains were produced in UHT+ milk in the presence of "milk isolate" S6.

Study 8 - Beverage production using combinations of single strains

In this study the grains obtained from the four combinations in Study 7 were used to produce four beverages using the traditional Kepi production method. The results of the beverage production are given Table 8. The final pH of beverages 1 to 4 were 4.3, 5.3, 4.7 and 4.4, respectively. According to Marshall (1982), the pH of the final traditionally produced Kepi beverage usually ranges between 4.2 and 4.6 and it was thus concluded that the pHs of the four beverages were in line with that of the traditional Kepi beverage.

Beverage 1, using Kefiran culture D as starter culture, was found to be very sour with a

Table 7. Final grain mass, pH, TA and lactose content of the four final combinations of Kefiran cultures and "milk isolates".

Combination number	Isolate	Mass (g)	pH	TA	Lactose ($g.100g^{-1}$)
1	D	39.0	3.7	2.1	3.0
2	D, A, F, 3, 6	8.0	3.7	2.2	3.2
3	D, A, F, 3, 6, S4	5.0	4.0	1.6	3.3
4	D, A, F, 3, 6, S6	21.5	4.1	1.6	3.3

Table 8. Final grain mass, pH, TA and lactose content of the four beverages produced with the single strain combinations after 12 incubation steps in double pasteurised milk.

Beverage	Original isolates used to produce grains	Mass of grains added (g)	Mass of grains after 12 d of beverage production (g)	Final pH	Final TA	Final lactose ($g.100g^{-1}$)
1	D	5.0	10.9	4.3	0.5	3.3
2	D, A, F, 3, 6	5.0	6.2	5.3	0.4	3.7
3	D, A, F, 3, 6, S4	5.0	4.9	4.7	0.6	3.7
4	D, A, F, 3, 6, S6	5.1	8.6	4.4	0.6	3.5

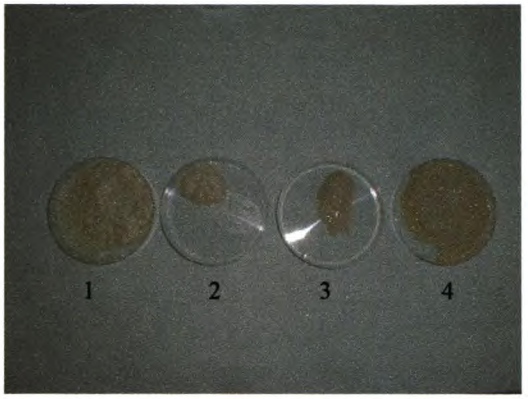


Figure 4. Grain accumulation obtained from the final combinations (1- 4) of specific Kefiran cultures with or without "milk isolates".

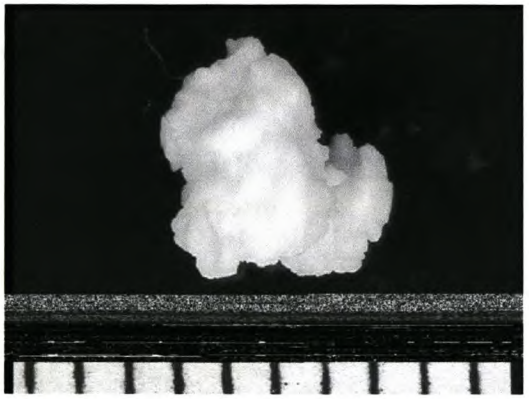


Figure 5A. Grain obtained from Kefiran culture D in DP milk before the beverage production (beverage 1).

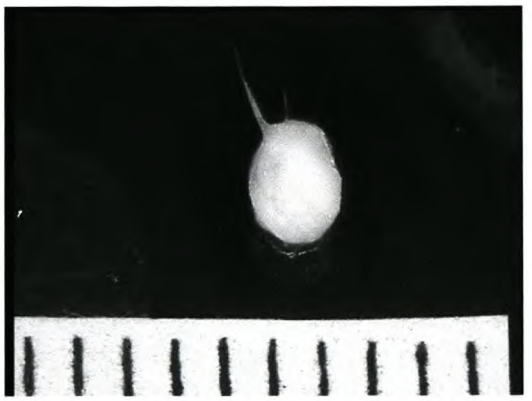


Figure 5B. Recovered grain obtained after the beverage production (beverage 1).

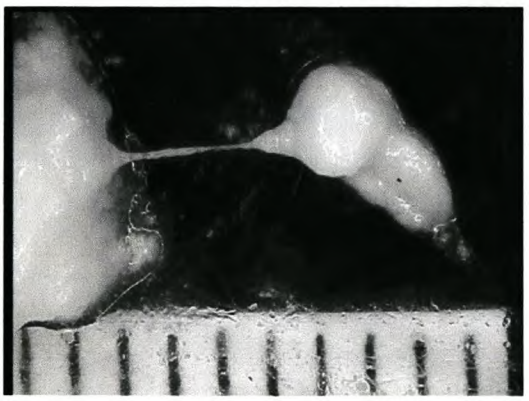


Figure 5C. Recovered grains with ECP production obtained after the beverage production (beverage 4).

Figure 5D. Recovered grains with ECP in distilled water after the beverage production (beverage 1).

strong fermentation odour; the product texture was gritty and viscose but not as viscose as traditional Kepi. The aroma and taste was light buttery, but again not as prevalent as found in traditional Kepi. The beverage had a creamy texture and a pleasant effervescent mouth feel.

Beverages 2 and **3** were found to be unacceptable. Beverage 2 showed no coagulation with no flavour aroma. Beverage 3 showed a slight coagulation and was not viscose, with an unacceptable sour odour.

Beverage 4 was less gritty than beverage 1; had a strong sweet taste with a viscosity similar to traditional Kepi. The beverage had a creamy texture, an effervescent mouth feel and the aroma was more buttery and stronger than found in beverage 1. Beverage 1 was more like normal Kepi, although the viscosity and aroma levels found in beverage 4 would be more like that found in traditional Kepi.

The grains obtained in this study (Fig. 5A) were found to be similar to traditional Kepi grains and were firm and elastic when pressed between the fingers. Furthermore, the grains did not dissolve in water but kept their structure and were retained when sieved. From these typically traditional grain characteristics it was concluded that, even though the microbial compositions were probably not the same, the general appearance was similar to traditional grains. The recovered grains from beverages 1 and 4 were examined under a stereo microscope and ECP production was observed between the grains after the 12 transplants (Fig. 5B and 5C). These grains also did not dissolve when washed with distilled water (Fig. 5D).

Conclusions

The aim of this study was to evaluate grain formation with purified cultures isolated from Kefiran strings using a mass cultivation process. In this study it was found that double pasteurised milk (DP) with and without Kefiran cultures resulted in grain accumulation. The possibility that there were microbes present in commercially pasteurised and double pasteurised milk which could contribute to grain formation was confirmed when pasteurised commercially purchased milk was subjected to different heat treatments (double pasteurisation, pasteurisation plus sterilisation and double pasteurisation plus sterilisation). No grain formation was found with pasteurised and sterilised (PS) and double pasteurised and sterilised (DPS) milk.

The above suspected presence of other microbes in commercially pasteurised milk was further confirmed when 12 different organisms were isolated. These "milk isolates" (S1, S2, S3, S4, S5, S6, C1, C2, C3, C4, G1 and G2) were identified using combinations of the API data system, numerical clustering and molecular identification. The coccus-shaped "milk isolates" were identified as a *Pediococcus* sp., *Acinetobacter* sp. and two strains of *Lactococcus lactis* ssp. *lactis*, while the two yeasts were identified as *Candida lipolytica* and *C. guilliermondii.* The rod-

shaped isolates were found to be members of the *Chryseobacterium meningosepticum* and *Pseudomonas putida* species. Four isolates were found to be related to the *Bacillus cereus* group. The addition of the rod-shaped "milk isolates" to UHT milk resulted in grain formation and from these results it was concluded that the "milk isolates" did contribute to grain formation.

The results obtained in this study, clearly indicates that a combination of Kefiran cultures and "milk isolates" can be used to produce grains which are similar to traditional Kepi grains, through the mass cultivation process. Furthermore, it was possible to produce an acceptable Kepi beverage from these grains.

However, future research needs to be done on the survival of the "milk isolates" during pasteurisation and double pasteurisation. A larger trained sensoric panel also needs to evaluate these single strain combinations grain beverages and compare it then with Kepi produced with traditional grains. It would also be interesting to compare these beverages produced with pure cultures combination with Kepi made from pure inoculated starter cultures. In this study only three multi-strain combinations were tested and it is thus essential that the other combinations of Kefiran cultures and "milk isolates" be tested to attempt to and produce a beverage with an aroma which is more characteristic of the traditional Kepi.

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APPENDIX A

To Chapter Four

Tables A1 – A14 are given in this appendix. The large amount of data generated was placed in a separate appendix to simplify the discussion section of this chapter.

Table A1. Final pH, TA and lactose content of the DP+ milk when Kefiran cultures were added and the mass of grains produced.

Isolate	Final pH	Final TA	Final Lactose ($g.100g^{-1}$)	Final Mass (g)
Control – day 0	6.7	0.3	4.6	-
Control – day 9	3.9	1.1	3.5	40.0
A	3.5	2.7	3.8	39.6
B	3.5	2.5	3.0	29.7
D	3.5	2.7	2.9	24.9
F	3.7	2.3	3.1	35.6
H	3.8	2.1	3.5	49.4
3	3.9	2.7	3.2	56.1
5	3.8	2.0	3.4	34.1
6	4.1	2.0	3.2	35.8

Table A2. Final pH, TA and lactose content of the four different heat treated milks before the mass cultivation process on day 0.

Milk source	pH	TA	Lactose (g.100g^{-1})	Mass (g)
P/P+	6.8	0.2	4.5	-
DP/DP+	6.7	0.3	4.6	-
PS/PS+	6.7	0.2	4.5	-
DPS/DPS+	6.9	0.3	4.4	-

P = Pasteurised
P+ = Pasteurised with yeast extract and urea
DP = Double pasteurised
DP+ = Double pasteurised with yeast extract and urea
PS = Pasteurised-sterilised
PS+ = Pasteurised-sterilised with yeast extract and urea
DPS = Double pasteurised-sterilised
DPS+ = Double pasteurised-sterilised, with yeast extract and urea

Table A3. Final pH, TA and lactose content of the four different heat treated milks and the mass of grains produced.

Milk source	Final pH	Final TA	Final Lactose (g.100g^{-1})	Mass (g)
Study 4a				
P	4.5	0.8	3.1	43.4
DP	4.5	1.0	3.4	40.0
PS	4.3	0.8	3.3	9.7
DPS	4.5	1.1	3.8	11.4
Study 4b				
P+	4.6	1.2	3.4	62.5
DP+	4.5	1.3	3.6	41.3
PS+	4.8	1.1	3.7	10.9
DPS+	4.7	1.1	3.6	9.8
Study 4c				
P+ and culture D	4.1	1.0	3.2	33.5
DP+ and culture D	4.0	1.7	2.9	57.1
PS+ and culture D	4.5	0.8	3.3	2.3
DPS+ and culture D	4.5	1.3	3.0	3.9

+= Yeast extract (2% (m/v)) and urea (0.5% (m/v)) were added.

Table A4. Characterisation of the yeast isolated from milk, using the Rapid ID 32C identification system.

Test	Isolate G1	Isolate G2
Galactose	-	+
ACT	+	?
Saccharose	-	+
N Acetyl glucosamine	+	+
LAT	?	-
Arabinose	-	+
Cellobiose	-	+
Raffinose	-	+
Maltose	-	+
Trehalose	-	+
2 ceto-gluconate	-	+
Methyl-D-glucoside	-	+
Sorbitol	-	+
Xylose	-	+
Ribose	-	-
Glycogene	+	+
RHA	-	-
PLE	-	+
Erythritol	+	-
Melibiose	-	-
GRT	-	-
Melezitose	-	+
Gentibiose	-	-
LVT	-	-
Mannitol	?	-
Lactose	-	-
Inositol	-	-
Glucose	+	+
SBE	-	?
Gluconate	-	+
Esculine	-	-

Table A5. Characterisation of the coccus-shaped "milk isolates" with endospores, using the API 50 CHB identification system.

Test	S1	S2	S3	S4
Glycerol	+	+	+	+
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	-	-	-	-
Ribose	+	+	+	+
D-Xylose	-	-	-	-
L-Xylose	-	-	-	-
Adonitol	-	-	-	-
B Methyl-xyloside	-	-	-	-
Galactose	-	-	-	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	-	-	-	-
L-Sorbose	-	-	-	-
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	?	?	?
Mannitol	-	-	-	-
Sorbitol	-	-	-	-
α Methyl-D-mannoside	-	-	-	-
α Methyl-D-glucoside	-	-	-	-
N Acetyl glucosamine	+	+	+	+
Amygdaline	+	-	-	-
Arbutine	+	+	+	+
Esculine	+	+	+	+
Salicine	+	+	+	+
Cellobiose	+	+	+	+
Maltose	+	+	+	+
Lactose	-	-	-	+
Melibiose	-	-	-	-
Saccharose	+	+	+	+
Trehalose	+	+	+	+
Inuline	-	-	-	-
Melezitose	-	-	-	-
D-Raffinose	-	-	-	-
Amidon	+	+	+	-
Glycogene	+	-	-	-
Xylitol	-	-	-	-
B Gentiobiose	-	-	-	-
D-Turanose	-	-	-	?
D-Lyxose	-	-	-	-
D-Tagatose	-	-	-	-
D-Fructose	-	-	-	-
L-Fructose	-	-	-	-
D-Arabitol	-	-	-	-
L-Arabitol	-	-	-	-
Gluconate	+	+	+	+
2 ceto-gluconate	-	-	-	-
5 ceto-gluconate	-	-	-	-

Table A6. Characterisation of the rod-shaped "milk isolates" S5 and S6 and coccus-shaped C3, using the API 20 NE identification systems.

Test	S5	S6	C3
NO_3	-	-	-
Tryptophane	-	-	-
Glucose	-	-	-
Arginine	-	+	-
Urea	+	-	-
Esculine	+	-	-
Gelatine	+	-	-
PNPG#	+	-	-
Glucose	+	+	+
Arabinose	-	?	+
Mannose	+	?	-
Mannitol	+	-	-
N Asetyl glucosamine	+	-	-
Maltose	+	-	-
β-gentiobiose	-	+	-
Caprate	-	+	+
Adipate	-	-	+
Malate	-	+	+
Citrate	+	+	+
Phenyl-acetate	-	+	?

= p-nitro-phenyl-βD-galactopyranoside

Table A7. Characterisation of the coccus-shaped "milk isolates", using the API 50 CHL identification system.

Test	C1	C2	C4
Glycerol	-	-	-
Erythritol	-	-	-
D-Arabinose	-	-	-
L-Arabinose	-	-	-
Ribose	+	+	+
D-Xylose	-	-	-
L-Xylose	-	-	-
Adonitol	-	-	-
B Methyl-xyloside	-	-	-
Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	-		
Rhamnose	-	-	-
Dulcitol	-	-	-
Inositol	-	-	-
Mannitol	-	-	-
Sorbitol	-	-	-
α Methyl-D-mannoside	-	-	-
α Methyl-D-glucoside	-	-	-
N Acetyl glucosamine	+	+	+
Amygdaline	-	-	-
Arbutine	+	+	+
Esculine	+	+	+
Salicine	+	+	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	+	+	+
Melibiose	-	-	-
Saccharose	-	+	+
Trehalose	+	+	+
Inuline	-	-	-
Melezitose	-	-	-
D-Raffinose	-	-	-
Amidon	-	?	?
Glycogene	-	-	-
Xylitol	-	-	-
B Gentiobiose	+	+	+
D-Turanose	-	-	-
D-Lyxose	-	-	-
D-Tagatose	+	-	-
D-Fructose	-	-	-
L-Fructose	-	-	-
D-Arabitol	-	-	-
L-Arabitol	-	-	-
Gluconate	-	?	?
2 ceto-gluconate	-	-	-
5 ceto-gluconate	-	-	-

Table A8. Results of API 20 E for the rod-shaped and cocci-shaped milk isolates.

	S1	S2	S3	S4	S5	S6	C1	C2	C3	C4
ONPG[#]	-	-	-	-	+	-	+	+	-	-
Arginine	-	+	-	-	-	+	+	+	-	+
Lysine	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	+	+	-	-	+	-
H_2S production	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	+	-	-	-	-	-
Tryptophane	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-
VP*	+	+	+	+	+	+	+	+	-	+
Gelatinase	+	+	+	+	+	-	-	-	-	-
NO_2 production	+	+	-	+	-	-	-	-	-	-

[#] = Ortho-nitro-β-D-galactopyranoside
* = Acetoin production

Table A9. Final pH, TA and lactose content of the UHT+ milk when Kefiran cultures were added and the mass of grains produced.

Isolate	Final pH	Final TA	Final Lactose ($g.100g^{-1}$)	Final Mass (g)
Control – day 0	6.6	0.2	4.9	0.0
Control – day 9	6.6	0.2	4.9	0.0
A	4.6	1.0	3.5	3.1
B	4.2	1.2	3.3	3.8
D	3.8	1.5	2.7	3.4
F	4.6	1.2	3.4	5.7
H	4.6	1.1	2.5	2.2
3	3.9	1.6	3.6	3.9
5	4.6	1.1	3.1	4.4
6	4.9	1.1	3.3	0.0

Table A10. Final pH, TA and lactose content of the UHT+ milk when "milk isolates" were added and the mass of grains produced.

Isolate	Final pH	Final TA	Final Lactose ($g.100g^{-1}$)	Final Mass (g)
Control	6.6	0.2	4.9	0.0
S1	4.4	1.1	3.8	30.1
S2	4.5	1.1	3.8	7.1
S3	4.5	1.1	3.9	29.1
S4	4.5	1.1	4.1	35.8
S5	4.0	1.4	3.7	16.4
S6	4.5	1.1	3.7	26.5
C1	4.5	1.0	4.3	1.6
C2	4.6	1.0	4.4	3.8
C3	4.6	1.0	4.1	1.8
C4	4.5	1.1	3.9	2.4
G1	4.5	1.1	4.0	6.2
G2	4.5	1.1	4.1	18.7

Table A11. Final pH, TA and lactose content of the UHT+ milk of combination 1 (the eight Kefiran cultures with "milk isolate" G2) and the mass of grains produced.

Isolate	Final pH	Final TA	Final Lactose ($g.100g^{-1}$)	Final Mass (g)
Control = G2	4.5	1.0	3.9	13.5
A + G2	4.7	1.0	3.4	0.7
B + G2	4.3	1.1	3.3	8.0
D + G2	3.7	1.8	2.3	3.1
F + G2	4.6	1.0	3.6	3.7
H + G2	4.8	0.9	2.3	3.0
3 + G2	3.9	1.3	3.3	1.7
5 + G2	4.6	1.1	3.2	2.1
6 + G2	4.5	1.1	3.1	10.5

Table A12. Final pH, TA and lactose content of the UHT+ milk of combination 2 (the eight Kefiran cultures with "milk isolate" S6) and the mass of grains produced.

Isolate	Final pH	Final TA	Final Lactose ($g.100g^{-1}$)	Final Mass (g)
Control = S6	4.6	1.3	3.9	16.4
A + S6	4.7	1.2	3.1	19.1
B + S6	4.4	1.2	3.2	19.7
D + S6	4.0	1.6	2.8	17.1
F + S6	3.7	2.1	2.2	5.9
H + S6	4.1	1.6	2.9	22.1
3 + S6	4.4	1.3	3.1	14.2
5 + S6	4.1	1.6	2.8	8.5
6 + S6	4.7	1.1	2.8	9.0

Table A13. Final pH, TA and lactose content of the UHT+ milk of combination 3 (the eight Kefiran cultures with "milk isolate" S3) and the mass of grains produced.

Isolate	Final pH	Final TA	Final Lactose ($g.100g^{-1}$)	Final Mass (g)
Control = S3	4.6	1.2	3.9	28.9
A + S3	4.5	1.3	3.5	6.4
B + S3	4.5	1.2	3.7	8.2
D + S3	3.7	2.0	2.9	5.9
F + S3	3.8	1.8	2.9	6.1
H + S3	3.8	1.8	2.8	8.7
3 + S3	4.5	1.2	3.3	10.0
5 + S3	3.8	1.8	2.9	5.7
6 + S3	4.4	1.1	3.1	6.7

Table A14. Final pH, TA and lactose content of the UHT+ milk of combination 4 (the eight Kefiran cultures with "milk isolate" S4) and the mass of grains produced.

Isolate	Final pH	Final TA	Final Lactose ($g.100g^{-1}$)	Final Mass (g)
Control = S4	4.6	1.2	3.9	39.7
A + S4	4.6	1.2	3.7	15.9
B + S4	4.6	1.3	3.8	2.7
D + S4	3.9	1.8	3.0	3.9
F + S4	3.8	1.9	3.0	4.6
H + S4	3.8	1.9	3.0	29.2
3 + S4	4.6	1.3	3.6	25.9
5 + S4	3.7	2.0	2.9	18.7
6 + S4	4.5	1.2	3.3	14.6

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

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Kepi is a fermented dairy product which is made by using an unique starter, called Kepi grains (Marshall *et al.,* 1984; Rea *et al.,* 1996). The lactic acid bacteria, yeasts and Kefiran that constitute the Kepi grains have been described as a symbiotic community that imparts unique properties to Kepi. Kefiran appears to be essential for grain formation (La Rivière *et al.,* 1967; Lin *et al.,* 1999), but the mechanism of grain formation is not fully understood and it remains undecided which organism is really responsible for the production of this protein-polysaccharide matrix (Özer & Özer, 2000).

The principal advantage provided by the Kepi grains is that they are re-usable and thus will minimise production costs and simplify processing. The major technological problem, however, is that Kepi grains are not easily available as their growth under normal conditions is extremely slow and they are known to only develop from pre-existing grains (Saloff-Coste, 2000). Apart from the traditional method of Kepi making with Kepi grains, the beverage can also be made with commercially prepared pure starter cultures (Libudzisz & Piatkiewicz, 1990). Unfortunately, the beverage produced with the commercial starters cannot be compared with the traditional Kepi and is an expensive method (Marshall & Cole, 1985). To produce Kepi grains from pure cultures has not yet been successful. The aim of this study was therefore to isolate and identify the microbes present in Kefiran during the mass culturing procedure (Schoevers, 1999) and then to use these single strain starters either singly or in combination to produce Kepi grains through the mass cultivation process.

The isolated cultures from Kefiran as found in this study were identified as *Lactobacillus parakefiri*, *Lb. kefiri*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. gallinarum, Lb. acidophilus*, *Lb. bavaricus, Candida lambica* and one isolate was found to be related to the genus *Lactobacillus*, but could not be positively identified to the species level. With the exception of *Lb. gallinarum* and *Lb. bavaricus*, the other Kefiran cultures have frequently been reported to be present in Kepi grains (Garrote *et al.*, 2001). Although *Lb. kefiranofaciens* has been reported to be the main Kefiran producer in Kepi grains (Fujisawa *et al.*, 1988), Özer & Özer (2000) concluded that the mechanism of grain formation is still not understood and there is still not consensus as to which organism is really responsible for the production of Kefiran. In this study, however, *Lb. kefiranofaciens* was not detected under the isolation conditions used. Possible reasons for this can be that it was not present or, if present, could not be identified as *Lb. kefiranofaciens* since a species description is not included in the API database or in GenBank for the DNA sequencing comparisons. Garrote *et al.* (2001) were also unable to isolate *Lb.*

kefiranofaciens from Argentinean Kepi grains. It is thus possible that *Lb. kefiranofaciens* was isolated in this study but could not be positively identified with fermentation profiles from literature or from the sequencing data currently available. *Lactobacillus kefiranofaciens* could thus be the main Kefiran producer which contributed to grain accumulation in this study.

The next step in this study was to determine whether the strains isolated from the Kefiran strings were able to play a role in the mechanism of grain formation during the mass cultivation of Kepi grains. It was found that culturing in double pasteurised milk with and without added Kefiran cultures resulted in grain accumulation and it was thus concluded that there were microbes present in pasteurised and double pasteurised milk which probably contributed to grain formation. These other microbes were then isolated from the pasteurised and double pasteurised milk. These "milk isolates" included strains of: *Pediococcus* sp.; *Acinetobacter* sp.; two strains of *Lactococcus lactis* ssp. *lactis; Candida lipolytica; C. guilliermondii; Chryseobacterium meningosepticum; Pseudomonas putida* and four isolates which are related to the *Bacillus cereus* group. *Chryseobacterium, Pseudomonas* and *Bacillus* species are often isolated from raw milk samples probably as a result of contamination or non-effective pasteurisation procedures (Mackenzie, 1973). Whether these isolates were present in the pasteurised milk used in this study as a result of contamination and then survived the commercial pasteurisation process, will still have to be determined.

The question as to whether the "milk isolates" played a role in grain formation was investigated and it was found that when the rod-shaped "milk isolates" were used as inoculum, strains grain accumulation in UHT milk was found. It was then concluded that the "milk isolates" did contribute to grain formation but it was not determined to what level or .

In a further study, these "milk isolates" were combined with the Kefiran cultures and grains which were very similar to the traditional Kepi grains produced. The grains were firm, elastic and did not dissolve in water but kept their structure and were retained when sieved. From these typically traditional grain characteristics it was concluded that, even though the microbial compositions were probably not the same, the general appearance was similar to traditional Kepi grains and that it is thus possible to produce grains using pure single strain Kefiran cultures and "milk isolates" in UHT milk.

An acceptable beverage was produced from these grains. The combinations of the grains included *Lb. gallinarum* in double pasteurised milk as well with a combination of *Lb. gallinarum, Lb. acidophilus, Lb. kefiri, Lb. delbrueckii* ssp. *bulgaricus, Candida lambica* and *Pseudomonas putida* in UHT milk. From the results obtained in this study, it was therefore concluded that a Kepi-like beverage with similar characteristics as the traditional Kepi beverage, can be produced from these single strain starter grains. As far as can be ascertained in the

literature this is the first report of the production of kepi grains from pure single strain combinations.

Since no studies have been reported on the success of the production of Kepi grains from single strain starters, there are still many unanswered questions. Thus, future research needs to be done on the survival of the "milk isolates" during pasteurisation and double pasteurisation. There is also a need to evaluate the effectiveness of pasteurisation, reconsider the pasteurisation parameters and post pasteurisation contamination possibilities of South African milk. It is also necessary to evaluate the beverage made from these single strain grains in this study as to whether these unwanted "contaminants" are present in the Kepi beverage, for example the *Pseudomonas* species with which the grains were made. A larger trained sensoric panel also needs to evaluate these "single strain combination" produced grain beverages and compare it with Kepi produced with traditional grains. It would also be interesting to compare these beverages produced with pure cultures combination grains with Kepi made with pure inoculated starter cultures.

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