PCR-BASED DGGE TYPIFICATION OF THE MICROBIAL COMMUNITY IN KEPI GRAINS

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DECLARATION

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

ABSTRACT

Kepi is a fermented milk beverage that originated in Eastern Europe. Traditional Kepi is a lightly acidic, carbonated beverage, with a slight yeasty taste. The starter used to produce this beverage is an irregularly shaped, yellowish-white grain-like structure similar in appearance to a cauliflower floret. The characteristic flavour of Kepi is produced by a complex spectrum of microbial species that include species of yeasts, lactic acid bacteria, acetic acid bacteria and mycelial fungi. At the end of the fermentation process the grainy starter can be recovered and re-used, since the microbes can easily be recovered as a solid matrix.

The microbes comprising Kepi grains have only been identified using classical identification techniques such as selective growth media, morphological, physiological and biochemical characteristics. In this study, polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) analysis was used to typify and identify the complex microbial consortium present in the Kepi grains. A part of the 16S ribosomal RNA (rRNA) gene from the microbial population in mass-cultured, traditionally cultured and Irish Kepi grains were amplified using *'Eubacterial'* specific primers and a part of the 26S rRNA gene was amplified using yeast specific primers. The PCR fragments were resolved by DGGE, resulting in unique fingerprints for the Eubacteria and yeasts present in the different Kepi grain types. The traditionally cultured Kepi grains were found to incorporate the most Eubacteria and yeast species, while the mass-cultured Kepi grains contained the lowest number of Eubacteria and yeast species.

The different Eubacteria and yeast species were identified by cloning the PCR products and sequencing the cloned inserts. The obtained DNA sequences were compared to sequences available on the NCBI website. Six lactobacilli were identified: *Lb. crispatus* (KC-4); three *Lb.* species (KC-36, KC-38 and KC-43); and two unculturable lactobacilli (KC-2 and KC-3). The yeasts were identified as *Saccharomyces cerevisiae* (KC-y18) and *Candida lambica* (KC-y1). Unidentified isolates from kefiran strings that could not be identified using traditional methods were also identified by cloning the PCR products and sequencing the cloned inserts. The four isolates were identified as *Lb. kefiri* (KGI-A), *Lb. parakefiri* (KGI-B), *Lb. gallinarum* (KGI-D) and an unculturable *Lactobacillus* (KGI-5).

The phylogenetic relationship between the identified lactobacilli and the lactobacilli commonly found in Kepi grains was determined. The identified lactobacilli were grouped together in a clade with a bootstrap support value of 84%. The clade also contained representatives of *Lb. delbrueckii* subsp. *lactis, Lb. acidophilus, Lb. gallinarum, Lb. helveticus, Lb. crispatus, Lb.* species and unculturable lactobacilli. The bands in the PCR-based DGGE fingerprints of the Eubacteria and the yeasts were identified, and a DGGE marker was subsequently constructed for the rapid identification of the Eubacteria present in mass-cultured Kepi grains.

The data obtained in this study clearly showed that Kepi grains that are cultured differently, as well as Kepi grains from different origins have unique PCR-based DGGE banding patterns for both the Eubacteria and yeasts present in the grains. The complex microbial consortium comprising Kepi grains could be typified and identified using PCR-based DGGE, DNA cloning and sequencing. The identification of the members of the microbial consortium is of importance for the future commercialisation of the mass-cultured Kepi grains.

UITTREKSEL

Kepi is 'n gefermenteerde melkdrankie wat sy oorsprong het in Oos Europa. Tradisionele Kepi is 'n effens suur, gekarboneerde drankie wat effens na gis smaak. Die beginkultuur wat gebruik word om dié drankie te maak is 'n oneweredige, geel-wit korrelagtige struktuur wat baie lyk soos 'n blomkoolkoppie. Die karakteristieke smaak van Kepi word geproduseer deur 'n komplekse spektrum mikrobiese spesies wat giste, melksuur- en asynsuurbakterieë en misillêre fungi insluit. Aan die einde van die fermentasieproses kan die korrelagtige beginkultuur herwin word en weer gebruik word, aangesien die mikrobes maklik herwin kan word as 'n soliede matriks.

Die mikrobes waaruit Kepikorrels bestaan, is nog slegs met behulp van klassieke identifikasiemetodes soos selektiewe groeimedia, morfologiese, fisiologiese and biochemiese eienskappe geïdentifiseer. In hierdie studie is polimerase kettingreaksie (PKR)-gebaseerde denaturerende gradiënt jelelektroforese (DGGE) analise gebruik om die komplekse mikrobiologiese konsortium in die Kepikorrels te tipeer en te identifiseer. 'n Gedeelte van die 16S ribosomale RNS (rRNS) geen van die mikrobiologiese populasie in massagekweekte, tradisioneel gekweekte en Ierse Kepikorrels is geamplifiseer met *'Eubakteriële'* spesifieke peilers en 'n gedeelte van die 26S rRNS geen is geamplifiseer met gis spesifieke peilers. Die PKR fragmente is onderskei deur DGGE, wat unieke vingerafdrukke vir die Eubakteriële- en gisspesies in die verskillende Kepikorrel tipes gelewer het. Die tradisioneel gekweekte Kepikorrels het die meeste Eubakteriële- en gisspesies geïnkorporeer, terwyl die Ierse Kepikorrels die minste Eubakteriële- en gisspesies geïnkorporeer het.

Die verskillende Eubakteriële- en gisspesies is geïdentifiseer deur klonering van die PKR produkte en deur die gekloneerde insetsels se volgordes te bepaal. Die DNS volgordes is dan vergelyk met volgordes wat op die NCBI webwerf beskikbaar is. Ses lactobacilli is geïdentifiseer: *Lb. crispatus* (KC-4); drie *Lb.* spesies (KC-36, KC-38 en KC-43); en twee onkultiveerbare lactobacilli (KC-2 en KC-3). Die giste is geïdentifiseer as *Saccharomyces cerevisiae* (KC-y18) en *Candida lambica* (KC-y1). Ongeïdentifiseerde isolate van kefiranstringe is ook geïdentifiseer deur klonering van die PKR produkte en deur die gekloneerde

insetsels se volgorde te bepaal. Dié vier isolate is geïdentifiseer as *Lb. kefiri* (KGI-A), *Lb. parakefiri* (KGI-B), *Lb. gallinarum* (KGI-D) en 'n onkultiveerbare *Lactobacillus* (KGI-5).

Die filogenetiese verwantskap is bepaal tussen die geïdentifiseerde lactobacilli en lactobacilli wat geredelik in Kepikorrels gevind word. Die geïdentifiseerde lactobacilli was saam in 'n groep gegroepeer met 'n bootstrap waarde van 84%. Die groep het ook verteenwoordigers van *Lb. delbrueckii* subsp. *lactis, Lb. acidophilus, Lb. gallinarum, Lb. helveticus, Lb. crispatus, Lb.* species en 'n onkultiveerbare laktobacilli ingesluit. Die bande in die PKR-gebaseerde DGGE vingerafdrukke van die Eubakterieë en die giste is geïdentifiseer, en 'n DGGE merker is gemaak vir die vinnige identifikasie van die Eubakterieë wat in die massagekweekte Kepikorrels teenwoordig is.

Die data wat in die studie verkry, is wys duidelik dat Kepikorrels wat op verskillende maniere gekweek is, en wat verskillende oorspronge het, unieke PKR-gebaseerde DGGE bandpatrone het vir beide die Eubakterieë en giste wat in die korrels teenwoordig is. Die komplekse mikrobiologiese konsortium waaruit Kepikorrels bestaan kon getipeer en geïdentifiseer word deur PKR-gebaseerde DGGE, klonering van DNS en volgordebepaling. Die identifikasie van lede van die mikrobiologiese konsortium is belangrik vir die toekomstige kommersialisasie van die massagekweekte Kepikorrels.

Dedicated to my parents and all my loved ones, with deep gratitude for their endless love and support

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

CHAPTER 1

INTRODUCTION

Kepi is a fermented milk beverage that is believed to have originated in Eastern Europe, and is also consumed in many other parts of the world (Garotte *et al*, 1998; Wouters *et al.*, 2002). The traditional Kepi beverage is variable in character, slightly carbonated and contains alcohol (Tamine *et al.*, 1999). The starter that is used to produce the Kepi beverage is an ill-defined, irregularly shaped white or yellow grain similar in appearance to a cauliflower floret (Wszolek *et al.*, 2001).

The Kepi grain contains a balance of specific microbes that co-exist in a complex symbiotic relationship (Tamine *et al.*, 1999). In the process of Kepi production the grains are grown in milk, the microbes are shed into the milk from the grains, and the organisms then continue to multiply, while producing the characteristic acid and flavours (Garotte *et al.*, 1998; Kwak *et al.,* 1996). In general, Kepi grains incorporate several species of yeasts, lactic acid bacteria (lactobacilli and lactococci), acetic acid bacteria and mycelial fungi (Tamine *et al.*, 1999). The different microbes are held together in a water-insoluble matrix composed of equal amounts of glucose and galactose, which give the grains a rubbery texture (Rea *et al.*, 1996). This polysaccharide matrix is called kefiran, mainly produced by *Lactobacillus kefiranofaciens* (La Rivière *et al.,* 1967; Micheli *et al.*, 1999; Mukai *et al.*, 1991).

A large part of the South African population is lactose intolerant, making them unable to consume milk due to the high concentration of lactose (Beukes *et al.*, 2001). Lactose is, however, broken down to lactate during the fermentation process (Gurr, 1987). A number of studies have shown that lactose malabsorbers can consume certain fermented dairy products without harmful effects (Roginski, 1988). Maas (Amasi, Inkomazi), a traditional fermented milk beverage made from unpasteurised milk, is an example of such a fermented milk beverage that has been produced for many generations by allowing unpasteurised milk to sour (Keller & Jordaan, 1990). However, legislation now stipulates that raw (unpasteurised) milk or raw cream may not be sold to the public unless it will undergo further processing (Anon. 1997). Due to this legislation, consumers will now not be able to make traditional Maas from unpasteurised milk, and there is

therefore an economical opportunity for an easily fermented milk product with a high nutritional value, similar in taste to traditional Maas. Kepi meets these requirements. Kepi production can be considered cheaper than commercial Maas, mainly due to the fact that the grains are re-usable and that only the milk has to be obtained to produce the beverage (van Wyk, 2000). Kepi has also been shown to inhibit certain pathogens (van Wyk, 2000). The nutrient composition of this fermented beverage is similar to that of milk, and can thus be seen as an excellent source of vitamins A, B_1 and B_2, calcium, phosphorus, thiamine, protein, fat and lactose (Buttriss, 1997; Gurr, 1987; Marshall, 1993). Kepi could thus play an important role in the nutrition of the low income black South African population.

Before the starter grains can be commercially distributed, it is of vital importance to ascertain the exact microbial composition of the Kepi grains. In the past, the microbes comprising Kepi grains were identified using growth media, morphological, physiological and biochemical characteristics (Kwak *et al.*, 1996; Pintado *et al.*, 1996). However, identification of complex microbial consortia is complicated by the fact that certain organisms are not able to grow on synthetic growth media (Kawai *et al.*, 2002). Isolating microbes for classical identification techniques is also difficult and time consuming (Short & Suttle, 1999; Wyder *et al.*, 1999). Molecular methods can, however, be used to identify complex microbial populations (Cody *et al.*, 2000; Muyzer *et al.*, 1993). The objective of this study is to typify the complex microbial consortium in different Kepi grains, by using PCR-based DGGE fingerprinting, DNA cloning and sequencing for identification of the different microbes comprising Kepi grains.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

The fermentation of milk is an ancient practice and these products have been popular in Europe for the past 4 000 years. The popularity of these beverages has spread world-wide and there is currently an extensive selection of fermented milks produced (Roginski, 1988). The nutrient composition of fermented milk is usually much the same as that of milk, which is an excellent source of vitamin A, vitamin B_1 and B_2, calcium, phosphorus, thiamine, protein, fat and lactose (Marshall, 1993). In fermented milks, however, the concentrations of lactic acid, free fatty acids, free amino acids and galactose are higher than in milk, whereas the vitamin and lactose content is on average lower than that of milk. Fermentation has little effect on the mineral content of the milk and not only is the concentration of the minerals the same, but the bioavailability of the minerals is also generally as good as that of unfermented milk (Buttriss, 1997; Gurr, 1987).

Fermentation is also a preservation process that is accomplished by the accumulation of lactic acid, as well as in some cases, a small amount of acetic acid, resulting in the lowering of the pH (Ayres *et al.*, 1980; Kurmann *et al.,* 1992). During fermentation, 20 - 30% of the lactose in milk is hydrolysed to its component sugars, glucose and galactose, by the starter bacteria. Consequently, the lactose content of fermented milks are lower than that of milk, which helps tolerance of the product by those with a reduced ability to digest lactose (Beukes *et al.*, 2001; Buttriss, 1997). Fermentation also extends the shelf-life of the product and contributes to the formation of a product with a higher viscosity (Roginski, 1988). Fermented milk products are therefore of great significance for their therapeutic value, for alleviating lactose intolerance, social value and also as a means of generating an income (Beukes *et al.*, 2001).

Bovine milk is mainly used in the production of fermented milks, but in some countries sheep, buffalo and horse milk is used (Roginski, 1988; Wszolek *et al.*, 2001). The aqueous extraction of whole soyabeans, referred to as soyamilk, has also been used for the production of fermented products (Mann, 1989).

Kepi differs from other fermented dairy products in that, at the end of the fermentation process the fermenting microbes can be recovered as a solid matrix that can be re-used. This is due to the fact that the microbes are built up in layers and embedded in protein, as well as polysaccharide material. This process forms the Kepi grain (Duitschaever *et al.,* 1987; Duitschaever, 1989; Rea *et al.,* 1996).

B. KEPI AND ITS ORIGIN

Kepi is the most famous of the truly alcoholic fermented milks and is manufactured under a variety of names such as kephir, kepi, kiapur, kefyr, képhir, kéfer, knapon, kefir, and kippe, which are all derived from the Turkish word *keif* meaning "pleasant taste" (Kemp, 1984; Kuo & Lin, 1999; Kurmann, 1992). This milk beverage is popular in Eastern European countries, especially in Russia, Poland, Czechoslovakia, Hungary and countries of Scandinavia (Libudzisz & Piatkiewicz, 1990; Rea *et al.,* 1996; Wouters *et al.*, 2002). Kepi became popular as a result of its nutritional value and favourable physiological effects (Libudzisz & Piatkiewicz, 1990). The history of this product is lost in time and it is considered that Kepi originated in Caucasian China. The origin of Kepi grains, the starter culture from which the Kepi beverage is made, is uncertain (Koroleva, 1988a; Koroleva, 1988b). The composition of the Kepi grains and production methods used in the making of the Kepi beverage vary greatly, which leads to different qualities and properties of the beverage (Libudzisz & Piatkiewicz, 1990).

C. PRODUCTION OF KEPI

For centuries Kepi has been made in goat or sheep skin bags by continuous fermentation that was both natural and uncontrolled, using raw bovine or caprine milk (Duitschaever *et al.,* 1987; Tamine *et al.,* 1999). In daytime the bags were subjected to sunlight and during the night they were taken into the house and hung near the door, where everyone who came in or went out had to push the sack with their foot so that the liquid was mixed. Clay pots and wooden buckets were also used as containers during the fermentation (Duitschaever *et al.,* 1987). When the fermented product was removed the sacks were refilled with fresh milk and the process was repeated (Kemp, 1984; Koroleva, 1988).

The Kepi beverage is characterized by a high acidity (*ca.* 0.8% acid) and depending on the holding time after fermentation, by either a lower or higher carbon dioxide (CO_2) and a relatively low ethanol content (Rea *et al.,* 1996). The sensory characteristics of Kepi include: pH of at least 4.0; clean, pleasant taste without any bitterness; prickling and sparkling; a slight taste of yeast; a smooth texture; and an alcohol content of 0.8 - 2.0% (Kemp, 1984; Marshall & Cole, 1985). Organic acids and volatile flavour compounds are also present in Kepi, and include orotic, citric, pyruvic, lactic, uric, acetic, propionic, butyric and hippuric acids, as well as acetoin, ethanol, acetaldehyde and diacetyl (Guzel-Seydim *et al.,* 2000). The fat content of Kepi depends on the type of milk used in the production of the product (Duitschaever, 1987; Kneifel & Mayer, 1991; Marshall & Cole, 1985; Pintado *et al.,* 1996).

In contrast to the traditional production, the commercial production of Kepi involves the recovery of the grains and re-use after they have been washed. During the production of Kepi care must be taken as excessive washing of the grains can upset the microbial balance and thereby reduce the microbial activity. The production of Kepi is estimated at about 24 h, and any kind of milk can be used for the production of this beverage (Duitschaever, 1987; Steinkraus, 1996).

Factors that affect the quality of commercially produced Kepi include: the grain:milk ratio; cultivation temperature; production time; conditions prior to separation of the grains from the fermented product; agitation conditions of the milk with the grains during fermentation; and washing of the grains after they have been removed from the fermented product (Garotte *et al.,* 1998). These factors also influence the microbiological content of the grains and the fermented milk, as well as the synergism between the different microbes in the grains (Abraham & de Antoni, 1999; Duitschaever *et al.,* 1987; Garotte *et al.,* 1998; Leroi & Pidoux, 1993). The microbiological content of the grains in turn affects the taste of the Kepi beverage. This is due to the fact that the CO_2 content, acid content and the amount of volatile flavour compounds in Kepi is determined by the diverse spectrum of microbes present in the grains and the beverage, as well as the interactions between these microbes (Kandler & Kunath, 1983; Leroi & Pidoux, 1993).

D. KEPI GRAINS

Grain morphology

Kepi grains are small, irregularly shaped, yellowish-white particles that resemble miniature cauliflower florets (Abraham & de Antoni, 1999; Guzel-Seydim *et al.,* 2000; Kemp, 1984; Koroleva, 1988a; Micheli *et al.,* 1999; Tamine *et al.,* 1999). The grains are gelatinous with an uneven surface and a diameter of about 8 - 10 mm (Garotte *et al.,* 1997; Kwak *et al.,* 1996; Rea *et al.,* 1996; Roginski, 1988). Kepi grains are not soluble in water, while in milk and soyamilk they swell and turn a yellowish-white colour (Garotte *et al.,* 1998; Liu & Lin, 2000). The total solid content of Kepi grains in $g.kg^{-1}$ is: 800 – 900 water; 60 sugar; 30 protein; 2 lipid; and 7 ash (Mann, 1985).

Different methods of preserving the grains play an important role in retaining the activity of the grain (Garotte *et al.,* 1997). Grains stored in water can only be kept for 8 – 10 days, while grains that have been air-dried for 12 – 18 months of storage remain active even though they take a considerable time to re-adapt their metabolic system to the production of biomass (Cilliers, 2001; Garotte *et al.*, 1997). If Kepi grains are frozen and kept at -80°C, the microbial composition of the grains changes less in comparison with grains that are stored at -20°C or -4°C (Garotte *et al.*, 1997; Steinkraus, 1996). Storage at 4°C (wet or dried) is an alternative method of preserving Kepi grains due to the inhibition of acid production, but these grains showed a negligible increase in weight during storage (Cilliers, 2001). In contrast the grains kept at -20°C and -80°C increased their weights during storage at a rate comparable to that found with non-stored grains (Garotte *et al.,* 1997). This could mean that the grains stored at 4°C lose their ability to produce some components for the production of the polysaccharide matrix in which the microbes are embedded. This may be due to the fact that some of the microbes die in the Kepi grain at 4°C. At -80°C the microbiological composition of the Kepi grains is altered to a lesser extent than at -20°C and 4°C, thus making -80°C the best preservation technique for Kepi grains (Garotte *et al.*, 1997). The Kepi made from these grains also have a microbial composition, level of acidity, viscosity and CO_2 content similar to fermented Kepi made from unfrozen grains (Garotte *et al.,* 1997). Freeze-drying of Kepi grains results in no major changes in the microbial composition, making it an attractive alternative method

for the preservation of the grains (Garotte *et al.,* 1997; Steinkraus, 1996). Cryoprotectants can also be used to prevent or reduce microbial inactivation during the freeze-drying process (Steinkraus, 1996).

Grain microbial composition

Kepi has a characteristic flavour that is produced by a diverse spectrum of microbial species (Duitschaever, 1987; Kwak *et al.,* 1996). This complex microbiological composition of the Kepi grains explains the difficulty in obtaining a starter culture with the optimal and constant microbial composition necessary for production of a quality Kepi beverage.

Microbes constituting the Kepi grains produce lactic acid, antimicrobial compounds and bactericidal compounds. The lactic acid lowers the pH during fermentation, contributing to both the taste and the preservation of the beverage, while the antimicrobial compounds and bactericidal compounds inhibit the growth of undesirable and pathogenic microbes in Kepi (Lin *et al.,* 1999). As the grains grow in the milk, the microbes present in the grain are shed into the milk, fermenting it while also multiplying (Mann, 1985).

Mesophilic, and thermophilic, and homofermentative and heterofermentative lactobacilli are present in the largest numbers in the Kepi grains, constituting 65 – 80% of the microbial population (Wood, 1998; Wouters *et al.*, 2002). Lactococci and both lactose and non-lactose-fermenting yeasts make up the remaining 20% of the microbes present in the grain (Wood, 1998). These values may differ if the grains come from different regions, e.g. Kepi grains that came from the Galician region in North-West Spain have been found to contain more heterofermentative lactobacilli (74%) than homofermentative lactobacilli (25%). The lactic acid bacteria also constitute between 30 and 60% of the grains, while the yeasts make up 33 – 60% of the grains (Angulo *et al.,* 1993). Taiwanese Kepi grains were examined and found that that the lactobacilli and streptococci populations were higher than the yeast population, and when Portuguese Kepi grains were microbiologically analyzed, acetic acid bacteria were not present, and the lactobacilli population dominated the total microbiological population (Kuo & Lin.,1999; Pintado *et al.,* 1996).

Homofermentative lactobacilli are the main component of the bacteria present in the Kepi grain. Homofermentative and heterofermentative lactic acid

streptococci (lactococci and leuconostocs), as well as acetic acid bacteria also form part of the Kepi grain, together with a distinct population of lactose-fermenting and non-fermenting yeasts (Neve, 1992). A symbiotic relationship exists between these microbes, validated by the fact that the lactic acid bacteria isolated from Kepi grains are unable to grow without the presence of yeasts in a simple sugar solution. Therefore, it has been postulated that the yeasts probably provide vitamins, amino acids and growth factors that are necessary for the growth of the lactic acid bacteria (LAB), while the LAB metabolic end-products could be utilized by the yeasts as an energy source (Leroi & Pidoux, 1993). The lactic acid bacteria, acetic acid bacteria and yeasts exist as clusters of symbiotic microbes held together by kefiran, a matrix consisting of fibrillar material (Garotte *et al.,* 1997; Kandler & Kunath, 1983; Micheli *et al.,* 1999; Rea *et al.,* 1996).

It is difficult to determine the precise number of microbes and species present in the grains due to the fact that it is difficult to separate the microbes from the polysaccharide matrix (Mukai *et al.*, 1990a; Mukai *et al.*, 1991; Neve, 1992). The heterogeneous distribution of the microbes on the surface of the grains also further complicates isolation, because no systematic method for the isolation of all the bacteria from the Kepi grain has been established (Kojima *et al.,* 1993). Extensive research has determined that there are a few specific species that always occur in the grain, whereas other microbes may either be present or absent (Pintado *et al.*, 1996). The reasons for the stability of certain species are not well established, though it appears that the polysaccharide plays an important role in maintaining the ecological niche (Pintado *et al.*, 1996). The different origins of the Kepi grains, different cultivation methods and storage conditions also contribute to the microbiological diversity in the grains. This in turn influences the taste of the Kepi beverage, as a variety of metabolic products are produced and consumed by different microbes (Garotte *et al.*, 1997; Pintado *et al.*, 1996; Leroi & Pidoux, 1993).

It has been found that bacteria are almost exclusively on the outside of the Kepi grain, yeasts in the center, whereas the intermediate areas contain a mixture of both bacteria and yeasts (Marshall *et al.*, 1984a; Wouters *et al.*, 2002). In Kepi grains that have been analyzed by scanning electron microscopy it was shown that the surface area of the grain is covered with a compact layer of rod-shaped bacteria, as well as actively budding yeast cells of oval and irregular shape (Lin *et*

al., 1999; Neve, 1992; Toba *et al.*, 1990). The concentration of the yeasts increased towards the center of the grains until dominating at the center (Lin *et al.*, 1999). Non-lactose fermenting yeasts were found in the deeper layers of the Kepi grain, with lactose fermenting species located in the peripheral regions (Marshall, 1993). Inside propagable grains rod-shaped bacteria and yeasts colonized separately in a spongy matrix, while in non-propagable grains the inner part was filled with fibrous materials in which some yeast cells were embedded (Toba *et al.*, 1990). The bacteria and yeasts were also observed in different ratios in the grains. In one sample the bacterial cells were arranged in clusters, entrapped by the dense and grainy matrix (Neve, 1992). In another sample a total different mode of colonization was observed, as bacterial growth in long chains resulted in a loose network in which yeast cells became occasionally entrapped (Neve, 1992). Streptococcal cells were, however, rarely detected (Duitschaever *et al.*, 1988; Neve, 1992). This may be due to the fact that the pH in the interior of the grains is very low, and lactococci do not grow well at such a low pH (Rea *et al.*, 1996; Garotte *et al.*, 1998).

Studies done on Kepi produced in Russia have shown that 1 ml of good quality Kepi had counts of 10^8 thermophilic lactobacilli, 10^9 homofermentative mesophilic lactococci, $10^7 - 10^8$ heterofermentative lactococci and $10^4 - 10^5$ acetic acid bacteria. The yeast cells numbered 10^4 - 10^5 (Koroleva, 1988b; Kuo & Lin, 1999).

Kepi grain fermentates have been shown to inhibit *Listeria innocua* DPC1770 and *Escherichia coli* O157:H45, indicating that the Kepi grains produce antimicrobial components. *Kluyveromyces marxianus*, a yeast present in Kepi grains also produces nisin that specifically inhibits the food-borne pathogens *Listeria monocytogenes, Staphylococcus aureus* and *Bacillus cereus* (Morgan *et al.*, 2000; Shimizu *et al.*, 1999; van Wyk, 2000).

Microbial populations in Kepi grains

Lactic acid bacteria

Lactic acid bacteria is a group of Gram-positive, non-sporing, catalase-negative, devoid of cytochromes, of non-aerobic habit, aerotolerant, fastidious, acid-tolerant and strictly fermentative cocci or rods, which produce lactic acid as the major end-product during the fermentation of carbohydrates (Axelsson, 1993;

Schlegel, 1995). There is a general agreement that the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* form the core of the LAB, while *Aerococcus, Carnobacterium, Enterococcus, Tetragenococcus* and *Vagococcus* have also been added (Axelsson, 1993; Kandler & Kunath, 1983; Schlegel, 1995). The characteristics as summarized in Table 1, are generally used to distinguish between obligately homofermentative, facultatively heterofermentative and obligately heterofermentative lactobacilli and include some of the well-known species in each group. The identification of species of lactobacilli have always been based on carbohydrate fermentation profiles, configuration of lactic acid produced, hydrolysis of arginine, growth requirements and growth at spesific temperatures. The identification of the LAB may also require analysis of the peptidoglycan, electrophoretic mobility of the lactate dehydrogenase (LDH), mol% G+C of the DNA, and even DNA-DNA homology studies (Axelsson, 1993).

Two main sugar fermentation pathways (Fig. 1) can be distinguished among LAB. Glycolysis (Embden-Meyerhof pathway) results in almost the exclusive production of lactic acid as the end-product of fermentation under standard conditions and this type of metabolism is referred to as homolactic fermentation. Members of the LAB are also able to metabolize substrates by way of the 6- phosphogluconate/phosphoketolase pathway, which results in the end-products ethanol, acetate and CO_2 in addition to lactic acid and is referred to as heterolactic fermentation (Axelsson, 1993; Schlegel, 1995). During the fermentation of sugars, different LAB species produce either L-lactic acid, D-lactic acid, equal amounts of both or predominantly one form, but measurable amounts of the other (Axelsson, 1993). Although LAB mainly produces lactic acid, many are also able to degrade lactic acid, especially if O_2 is available as electron acceptor. Some LAB are also able to degrade lactic acid under anoxic conditions in the presence of alternative electron acceptors, such as citrate and glycerol. *Lactobacillus brevis* is an example of a LAB which can degrade lactic acid by using glycerol as electron acceptor, while producing acetate, 1,3-propanediol and CO_2 (Oude-Elferink *et al.,* 2001).

<u>*Lactobacillus*</u>: The genus *Lactobacillus* is by far the largest of the genera included in the LAB group and encompasses species with a large variety of phenotypic, biochemical and physiological properties (Schlegel, 1995).

Table 1. The general distinguishing characteristics of the genus *Lactobacillus*.

Characteristic	Group I: Obligately Homofermentative	Group II: Facultatively Heterofermentative	Group III: Obligately Heterofermentative
Pentose fermentation	-	+	+
CO_2 from glucose	-	-	+
CO_2 from gluconate	-	+[a]	+[a]
FDP aldolase present	+	+	-
Phosphoketolase present	-	+[b]	+
Representative species	*Lb. acidophilus*	*Lb. casei*	*Lb. brevis*
	Lb. delbrueckii	*Lb. curvatus*	*Lb. buchneri*
	Lb. helveticus	*Lb. plantarum*	*Lb. fermentum*
	Lb. salivarius	*Lb. sake*	*Lb. reuteri*

[a]During fermentation

[b]Induced by pentoses (Axelsson, 1993)

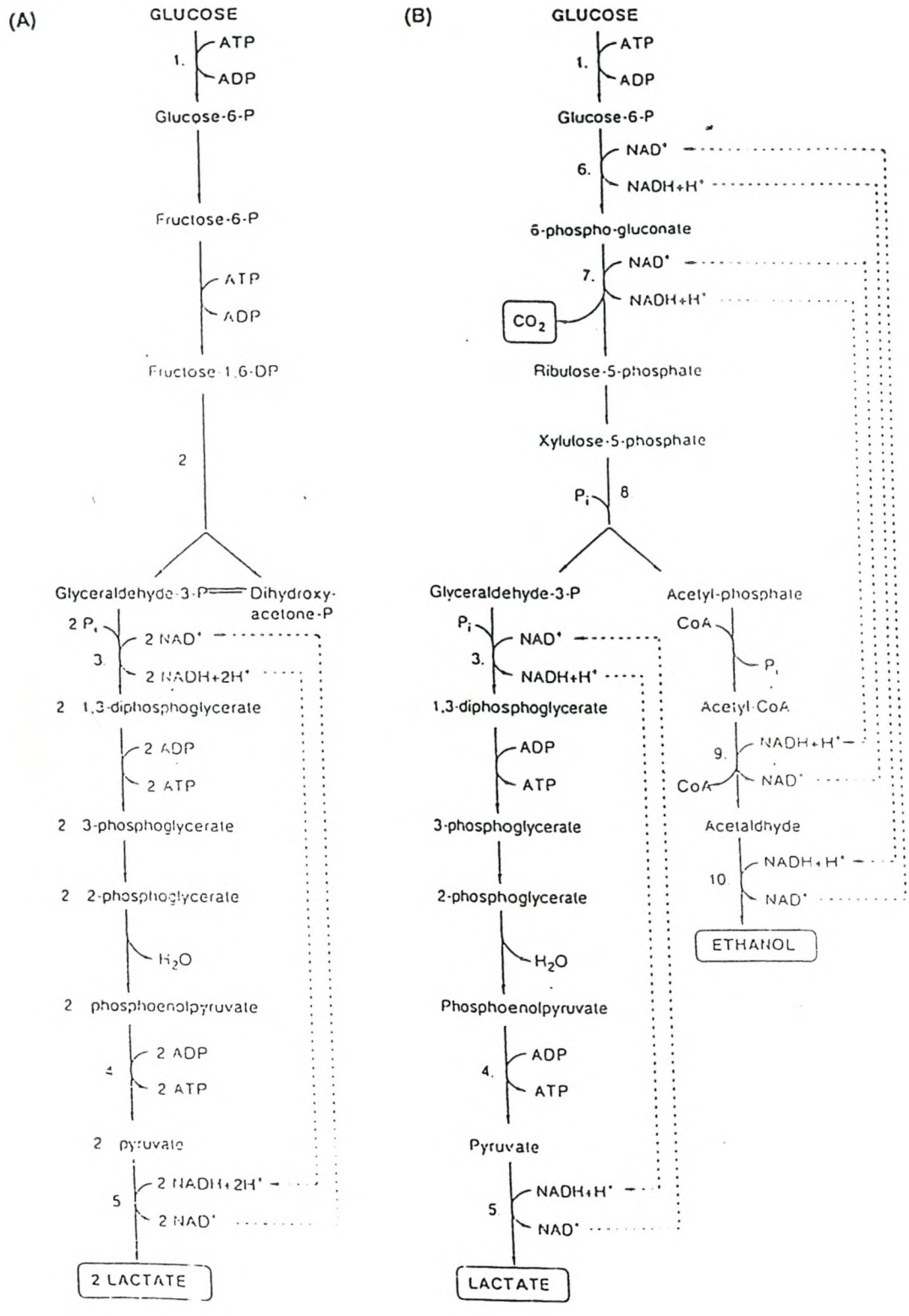

Figure 1. Major fermentation pathways of glucose by lactic acid bacteria (LAB). (A) Homolactic fermentation (Glycolysis); (B) heterolactic fermentation (6-phosphogluconate) (Axelsson, 1993).

Lactobacilli are rod-shaped, Gram-positive, non-spore forming, microaerophilic bacteria that are usually non-motile (Axelsson, 1993; Cogan *et al.*, 1997; Kandler & Kunath, 1983; Schlegel, 1995;).

The classification of the lactic acid rods which dominate in the Kepi grain is still taxonomically undecided (Assadi *et al.*, 2000; Kandler & Kunath, 1983; Lin *et al.*, 1999; Pintado *et al.*, 1996; Takizawa *et al.*, 1998). The National Collection of Dairy Organisms (NCDO) had a number of Kepi isolates which were deposited as *Lactobacillus brevis* and some which are similar to those described as *"Lactobacillus desidiosus"*, a name which no longer has taxonomic status (Kandler & Kunath, 1983). The heterofermentative Kepi lactobacilli were consequently re-investigated, and described as *Lactobacillus kefiri* (Kandler & Kunath, 1983). This specific *Lb. kefiri* isolate showed a 85 - 90% DNA/DNA homology to "*Lb. caucasicus*" ATCC 8007 (NCDO 190) (now *Lb. kefiri*) and was consequently considered to be a different species (Marshall *et al.,* 1984a).

Generally lactobacilli constitute 65 - 80% of the microbial population in the grain with respect to species diversity (Marshall, 1993). The main lactobacilli found in Kepi grains are homofermentative lactobacilli, namely *Lb. kefirgranum* and *Lb. kefiranofaciens*, while the heterofermentative lactobacilli *Lb. kefiri* and *Lb. parakefiri*, occur in low concentrations in the grain (Takizawa *et al.*, 1998). This corresponds with previously reported studies where it was found that 90% of the lactobacilli in Kepi grains were homofermentative lactobacilli, while 10% were heterofermentative lactobacilli (Takizawa *et al.*, 1998). However, about 80% of the lactobacilli present in the beverage itself were heterofermentative strains, while only 20% were homofermentative strains (Takizawa *et al.*, 1998). Thus, while *Lb. kefiri* is certainly the lactic acid bacterium present in the largest concentrations in the Kepi beverage, it is only a minor component in the Kepi grains (Kandler & Kunath, 1983).

Some of the LAB that have been isolated from Kepi are: *Lactobacillus acidophilus*, *Lb. brevis*, *Lb. casei*, *Lb. casei* ssp. *pseudoplantarum, Lb. casei* ssp. *tolerans, Lb. casei* ssp. *rhamnosus, Lb. casei* ssp. *alactosus, Lb. cellobiosus*, *Lb. delbrueckii* ssp. *bulgaricus, Lb. delbrueckii* ssp. *lactis, Lb. fermentum*, *Lb. grasseri, Lb. helveticus* ssp. *jugurti*, *Lb. helveticus* ssp *lactis, Lb. kefir, Lb. kefis, Lb. lactis* ssp. *lactis*, *Lb. plantarum* and *Lb. viridescens* (Angulo *et al.,* 1993; Assadi *et al.*, 2000; Duitschaever *et al.*, 1988; Fujisawa *et al.,* 1988; Kandler & Kunath, 1983;

Koroleva, 1988a; Kwak *et al.,* 1996; Marshall *et al.,* 1984a; Marshall *et al.*, 1984b; Micheli *et al.,* 1999; Pintado *et al.,* 1996; Schoeman, 2001; Takizawa *et al.*, 1994; Takizawa *et al.,* 1998).

Other lactobacilli that have specifically been isolated from Kepi include *Lactobacillus kefiranofaciens*, *Lb. kefirgranum* and *Lb. parakefiri* (Fujisawa *et al.,* 1988; Schoeman, 2001; Takizawa *et al.,* 1994). The morphological, biochemical and physiological characteristics of *Lb. kefiranofaciens, Lb. kefirgranum* and *Lb. parakefir* prove that these strains belong to the genus *Lactobacillus*, while DNA hybridization experiments showed that these strains exhibited relatively low levels of DNA relatedness to other *Lactobacillus* isolates on species level (Takizawa *et al.,* 1994). This consequently resulted in the proposal of placing *Lb. kefiranofaciens*, *Lb. kefirgranum* and *Lb. parakefiri* in three new species (Fujisawa *et al.,* 1988; Pintado *et al.,* 1996).

Lactococcus and *Enterococcus*: The genus *Lactococcus* comprises homofermentative, non-sporing, Gram-positive cocci, with lactic acid being the major metabolic end-product of the fermentation (Axelsson, 1993; Schlegel, 1995). *Lactococcus lactis* ssp. *diacetylactis* is also able to produce diacetyl (butter aroma) and acetoin by metabolizing citrate, which is present in milk in large amounts (Schlegel, 1995).

The lactococci species that have been isolated from Kepi include *L. filant, L. lactis* ssp. *lactis, L. lactis* ssp. *lactis* biovar *diacetylactis, L. lactis* ssp. *cremoris*, *L. lactis, Streptococcus durans* and *S. salivarius* ssp. *thermophilus* (Angulo *et al.,* 1993; Assadi *et al.,* 2000; Cogan *et al.*, 1997; Garotte *et al.,* 1997; Kwak *et al.,* 1996; Koroleva, 1988a; Marshall *et al.,* 1993; Rea *et al.,* 1996; Schoeman, 2001). One *Enterococcus* species has also been isolated from Kepi, namely *E. durans* (Marshall, 1993).

It has been ascertained that only small numbers of lactococci are found in Kepi grains, whereas large numbers are found in the Kepi beverage. This is probably due to the fact that the pH in the grain is very low due to high amounts of acid produced by the lactobacilli that inhibits the growth of lactococci (Schoeman, 2001). The lactococci are also not tightly bound to the grains and are easily washed out during the manufacturing of the beverage (Marshall *et al.,* 1984).

Leuconostoc: The leuconostocs can be separated from other lactic acid bacteria by their heterofermentative metabolism, with the end-products of the fermentation being CO_2, ethanol and lactate, and in some cases acetate (Axelsson, 1993; Schlegel 1995). They are also classified as Gram-positive, non-motile, non-sporing, facultative anaerobes (Schlegel, 1995). Leuconostocs are also able to ferment arginine, which lactobacilli are unable to do and they produce significant amounts of diacetyl from citrate in milk. Except for *Leuc. lactis*, this group of bacteria do not usually grow in milk, but their growth is stimulated by the presence of lactococci and yeasts. *Leuc. mesenteroides* ssp. *cremoris* is the species most often used in the dairy industry as a producer of diacetyl, which is the most important aroma-forming component in fermented milk products (Axelsson, 1993; Rea *et al.,* 1996; Wood, 1998). As leuconostocs are heterofermentative lactic acid bacteria, ethanol is an end-product of the fermentation, but it has been shown that the co-metabolism of lactose and citrate by leuconostocs result in no ethanol production. This would suggest that the ethanol present in Kepi is mainly produced by the yeasts in the grain (Rea *et al.,* 1996). The leuconostoc species that have been isolated from Kepi include *Leuconostoc. mesenteroides* ssp. *cremoris*, *Leuc. mesenteroides* ssp. *dextranicum*, *Leuc. mesenteroides* ssp. *mesenteroides* and *Leuc. kefir* (Koroleva, 1988a; Koroleva, 1988b; Kwak *et al.,* 1996; Schoeman, 2001).

Pediococcus: Pediococci are considered to be important constituents of the complex known as non-starter lactic acid bacteria, which is involved in the ripening of cheese. The main characteristics that are used to distinguish between species are the range of sugars that are fermented, hydrolysis of arginine, growth at different pH values and the configuration of the lactic acid produced during the fermentation process. Pediococci have only been found in one sample of Kepi grains from the Galician region, and was considered to be a contaminant of the Kepi beverage (Angulo *et al.,* 1993).

Propionibacteria

The genus *Propionibacterium* is described as Gram-positive, non-motile, micro-aerotolerant bacteria, with the cells being either branched or unbranched, rods or cocci. Club-shaped forms can also be found under unfavourable

conditions (Axelsson, 1993; Singleton, 1995). Propionibacteria can be regarded as micro-aerotolerant due to their tolerance to atmospheric oxygen and their ability to grow and regenerate ATP by anaerobic fermentation, and also due to the fact that they possess haem enzymes like cytochromes and catalase which enables them to grow aerobically (Schlegel, 1995). Under anaerobic conditions, members of the genus *Propionibacterium* ferment glucose, sucrose, lactose and pentoses as carbon and energy sources, as well as lactate, malate and glycerol to propionic acid. Catabolism of the hexoses proceeds via the fructose-bisphosphate pathway and propionic acid is the main volatile acid produced. Acetic acid and CO_2 can also be produced as end-products. The volatile organic acids produced do not only contribute to flavour, but can also inhibit the growth of Gram-negative bacteria, yeasts and mycelial fungi (Chaia *et al.*, 1995; Lyon & Glatz, 1995).

Another important characteristic of this genus is the ability to produce vitamin B_{12} (Lyon & Glatz, 1995). This vitamin is very important for normal functioning of the body, as it is an important co-factor for carbohydrate, lipid, nucleic acid and amino acid metabolism. It also plays an important role in the synthesis of DNA and RNA and is necessary for the normal metabolism of nerve tissue, as well as the formation of red blood cells (Buttriss, 1997; Marshall, 1984b). Vitamin B_{12} is also the only vitamin that contains essential mineral elements, cannot be made synthetically and must be produced either by bacteria or mycelial fungi (Lyon & Glatz, 1995).

In the past *Propionibacterium shermanii* was used as a member of the Kepi starter and was found to produce a quality product that was rich in vitamin B_{12} (Marshall, 1993). A 60 fold increase in the levels of vitamin B_{12} were achieved when 5% of a culture of *P. shermanii* was inoculated into milk along with 1 - 5% of ordinary Kepi grains (Marshall, 1993).

Acetic acid bacteria

The acetic acid bacteria are able to form volatile acids by the incomplete oxidation of sugars or alcohols and to excrete acetic acid, either transiently or into the medium as non-utilisable end-products. Acetic acid bacteria include Gram-negative rods with limited motility by using peritrichous (*Acetobacter*) or polar (*Gluconobacter*) flagella (Schlegel, 1995; Swings, 1992). These bacteria can be distinguished by their high acid tolerance, low peptolytic activity, lack of motility

and lack of coloured pigments (Schlegel, 1995). They are also found in association with yeasts in sugar-containing media (Schlegel, 1995). *Acetobacter* oxidizes both lactic acid and acetic acid to form CO_2 and H_2O, and prefers ethanol or lactate to glucose as substrates for growth. *Gluconobacter* however does not oxidize lactic acid and acetic acid to form CO_2, but produces acetic acid and uses glucose as substrate.

Though it is unusual to find acetic acid bacteria in Kepi due to the fact that they are strict aerobes, *A. aceti* and *A. rasens* have been found in Kepi grains (Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990, Marshall, 1993; Rea *et al.,* 1996; Wyder *et al.,*1999). It was reported that acetic acid bacteria probably improve the consistency of the Kepi by increasing the viscosity and thus plays an important role in maintaining the symbiosis among the Kepi grain microbial community (Koroleva, 1988a). Acetic acid bacteria are considered by some to be contaminants in the Kepi beverage, and are not thought to be part of the normal microbial population (Angulo *et al.,* 1993; Takizawa, 1998).

Yeasts

Yeasts belong to the protoascomycetes and can be described as spore-forming, non-motile, unicellular and aerobic microbes. The center of the Kepi grain is composed almost entirely of yeast species (Garotte *et al.,* 1997, Wouters *et al.*, 2002). It has been claimed that only lactose-fermenting yeasts should be considered to be part of the microbiological population present in Kepi grains, as these organisms play the leading role in the Kepi beverage alcoholic fermentation (Rea *et al.,* 1996; Wyder *et al.,* 1999). The main end-products of the alcoholic fermentation, ethanol and CO_2, are important for both the flavour and the refreshing taste of the Kepi beverage. A high percentage of the yeasts in the peripheral layers of the Kepi grain are lactose fermenters, with the non-lactose fermenting yeasts in the inner layers of the grain (Mann, 1989). Lactose fermenting yeasts also dominate in the Kepi beverage (Wyder *et al.,* 1999).

The yeasts, as part of the interactions during the fermentation process, contribute to the fermentation by supporting the starter, inhibiting undesired microbes and adding to the final product by means of desirable biochemical changes such as production of aromatic compounds. However, some interactions may be detrimental, causing spoilage (Viljoen, 2001). Some yeasts may continue

fermentation even after the product has been packed in containers, under favourable environmental conditions. This secondary alcoholic fermentation can occur when the Kepi beverage is distributed which results in substantial changes in the flavour and taste of the beverage. Excessive gas production leads to the formation of bulging containers and leaking contents due to the internal pressure created by the excess CO_2 (Kwak *et al.*, 1996). The biostabilization of Kepi by controlling ethanol and CO_2 production by using a non-lactose fermenting yeast during the production of the Kepi beverage has proved to be successful (Kwak *et al.*, 1996).

Yeasts that have been isolated from Kepi grains include *Kluyveromyces bulgaricus, K. fragilis, K. lactis, K. marxianus* ssp. *marxianus, Torula kefir, Torulaspora delbrueckii, Saccharomyces carlsbergensis, S. cerevisiae, S. exiguus; S. florentinus, S. fragilis, S. globosus, S. kefir, S. lactis, S. lypolitica, S. unisporus, S. turicensis, Candida collicolosa, C. friedicchii, C. holmii, C. kefir, C. pseudotropicalis, C. tenuis, Cryptococcus kefyr, Brettanomyces anomalus* and *Pichia fermentans* (Assadi *et al.*, 2000; Garotte *et al.*, 1997; Koroleva, 1988; Koroleva, 1988a; Kwak *et al.*, 1996; Leroi & Pidoux, 1993; Marshall, 1993; Pintado *et al.*, 1996; Wyder *et al.*, 1999).

Mycelial fungi

Geotrichium candidum, a mycelial fungus, has been isolated from Kepi grains. This mycelial fungus usually covers the surface of the Kepi grains if it is present (Cilliers, 2001; Garotte *et al.*, 1997; Pintado *et al.*, 1996; Prescott *et al.*, 1996). However, it does not affect the performance of the grains or influence the organoleptic properties of the Kepi beverage (Roginski, 1988; Schoeman, 2001).

Microbial interactions

Fermented products, such as dairy products, develop their nutritional and organoleptic qualities as a result of the metabolic activity of a succession of different microbes (Viljoen, 2001). The microbial interaction in mixed starters are very complex, as each species has its own substrates, metabolic pathway(s) and end-product(s) (Kwak *et al.*, 1996). Microbes can also use the end-products of one metabolic pathway as substrate for a different metabolic pathway (Koroleva, 1988a).

Kepi grains are especially complex as they consist of a mixture of lactic acid bacteria, acetic acid bacteria and yeasts (Koroleva, 1988a; Kwak *et al.,* 1996; Tamine *et al.,* 1999; Wouters *et al.*, 2002). During the fermentation process, lactococci is the first group of the lactic acid bacteria that starts to grow and produce lactic acid (Kwak *et al.*, 1996). Lactic acid production is slow at the beginning of the fermentation process, but as the lactococci are washed from the grains into the milk the rate of acid production greatly increases (Kwak *et al.*,1996). Acetic acid bacteria also increase the rate of acid production by the lactococci. Leuconostocs that are present in the grains grow in association with lactococci and produce aroma compounds such as diacetyl (Axelsson, 1993). The presence of large numbers of yeasts also favours the growth of the leuconostocs, and in turn produce CO_2 and ethanol in the milk. As the pH decreases, the lactobacilli start to grow and in turn produce lactic acid, as well as organoleptic compounds in the milk (Axelsson, 1993; Guzel-Seydim, 2000).

E. MASS CULTIVATION OF KEPI

It has been shown that if different Kepi grain samples are cultivated under the same conditions, their microbial population becomes rather similar. This proves the unique ability of the grain for self-regulating its microbial population (Koroleva, 1988a). By changing the environmental factors under which the Kepi grains are cultivated however, such as temperature, grain:milk ratio and frequency of milk renewal, the microbial population of the grains can be altered.

As the grains are used in the production of Kepi, the grains grow in size as the microbial population starts to multiply and kefiran starts to accumulate (Koroleva, 1988; Marshall & Cole, 1985). Grains can, however, only grow from pre-existing grains (Steinkraus, 1996). Kepi grain biomass increases from about 5 to 7% per day under normal incubation conditions, but this presents a problem when the grains are to be distributed on an industrial scale. Therefore, a method was developed to increase the biomass of the Kepi grains, with the potential of supplying the grains to customers (Schoevers, 2000). Grain cultivation at 25°C led to a biomass increase of 113% over 10 days compared to a 130% increase at 22°C (Schoevers, 2000). This is important if Kepi grains are to be commercialized.

F. MOLECULAR TYPIFICATION TECHNIQUES FOR COMPLEX MICROBIAL CONSORTIUMS

As only an estimated 20% of the natural occurring bacteria have been isolated and characterized, molecular techniques offer new opportunities for analyzing the structure and species composition of microbiological communities (Cody *et al.*, 2000). Selective media often fail to mimic the conditions that are required by particular microorganisms to proliferate in their natural habitat, and many microorganisms are bound to sediment particles and can thus not be detected by conventional microscopy (Muyzer *et al.*, 1993). It is also well known that phenotypic characteristics of microbes are often strain specific and inadequate for the recognition of species (Ferrari & Hollibaugh, 1999). An example of this is that traditional identification methods, which include biochemical tests, are not reliable for separating and distinguishing micrococci from staphylococci (Cocolin *et al.*, 2001).

Several molecular techniques have, however, been developed to study natural samples. These techniques identify microbes without prior isolation and reveal the enormous range of microbial diversity (Kawai *et al.*, 2002). Sequence variation in rRNA has been exploited in particular for inferring phylogenetic relationships among microbes and for designing specific nucleotide probes for the detection of individual microbial taxa in natural habitats. These techniques have also been applied to determine the genetic diversity of microbial communities and to identify previously uncultured microbes (Muyzer *et al.*, 1993). Molecular tools such as the polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization (FISH) are therefore more suitable for the identification and differentiation of species and strains than the classical microbiological method of culturing (Garret & Grisham, 1995; Muyzer *et al.*, 1993; Wyder *et al.*, 1999; Wagner *et al.*, 1998). PCR is especially used to examine the diversity of natural communities without the need to culture the microorganisms (Garret & Grisham, 1995; Short & Shuttle, 1999). An example is a rapid method for the identification of yeasts from Kepi at species level that has been developed. DNA was extracted from isolated yeasts, and PCR (Polymerase Chain Reaction) was performed using four universal primers corresponding to parts of 3 highly conserved rRNA genes. Restriction analysis of the PCR products

was done, and these fragments were visualized on an agarose gel. This resulted in reproducible species specific patterns (Wyder & Puhan, 1997).

PCR-based denaturing gradient gel electrophoresis (DGGE)

DGGE of PCR-amplified fragments coding for 16S rRNA has specifically emerged as a powerful diagnostic tool (Kawai *et al.*, 2002; Walter *et al.*, 2000). In DGGE, PCR-amplified 16S rRNA products with the same length but with different sequences can be separated on a polyacrylamide gel, resulting in unique fingerprints of DNA fragments. Separation in DGGE is based on the electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels, which is decreased as compared with that of the completely helical form of the molecule (Muyzer *et al.*, 1993). The fragments therefore separate according to differences in their melting behavior in the denaturing gel. The melting of the fragments proceeds in so-called melting domains: stretches of base pairs with identical melting temperatures. When the melting domain with the lowest melting temperature reaches its melting temperature at a certain position in the DGGE gel, the transition of helical to a partially melted molecule occurs. This then causes the molecule to halt in the gel. The sequence variation in the domains result in the different melting temperatures, and therefore particular fragments will stop migrating at different positions in the gel and can be effectively separated (Muyzer *et al.*, 1993). This method is sufficiently sensitive to separate duplexes that differ by only a single base pair and each band produced potentially represents a distinct bacterial species (Short & Shuttle, 1999). DGGE analysis does not require laboratory cultivation of bacteria and consequently enables assessment of the diversity of total bacterial populations, including nonculturable organisms (Cody *et al.*, 2000; Normander & Prosser, 2000; Walter *et al.*, 2000).

Another major advantage of the DGGE technique is that it allows the direct determination of bacterial genetic diversity (Cody *et al.*, 2000). This is done by transferring separation patterns to hybridization membranes by capillary blotting with modified gel media, or by electroblotting followed by analysis with DNA probes. DGGE can therefore be used for direct analysis of genomic DNA from genomes with millions of base pairs (Muyzer *et al.*, 1993).

Fluorescent *in situ* hybridization (FISH)

Fluorescent *in situ* hybridization is also a suitable tool for determinative and environmental microbial studies. This is specifically a molecular-genetic identification based on specific sequences of 16S and 23S rRNA genes that can be detected by *in situ* hybridization with fluorescently labeled oligonucleotides (Sekiguchi *et al.*, 1999; Wagner *et al.*, 1998). During the FISH analysis oligonucleotide probes, which are complementary to specific regions on the rRNA genes penetrate the bacterial cell wall. These probes are small, 16 - 20 bases long, single stranded deoxyribonucleic acids, targeted against a small region which is unique to a bacterial group, genus or species. When specific binding of these probes to the complementary DNA occurs, the cells can be instantaneously detected by epifluorescence microscopy (Sekiguchi *et al.*, 1999; Lee *et al.*, 1999).

There are several advantages in using this technique, especially as individual strains need not be isolated and cultured, and a more complete three-dimentional view of the diversity and dynamics of microbial consortiums can be obtained. These FISH probes yield a higher number of detectable cells than any other technique, and bacteria can be visualized at their site of action. The FISH method, therefore, allows a rapid, reliable and cultivation independent identification of complex microbial consortiums (Lee *et al.*, 1999; Manz *et al.*, 1999; Wagner *et al.,* 1998). Specific examples where this technique has been used include the investigation of the phylogenetic composition, spatial structure and dynamics of lotic bacterial biofilms, as well as structure-function analyses in microbial ecology (Lee *et al.*, 1999; Manz *et al.*, 1999).

G. CONCLUSION

The Kepi grains and the Kepi beverage have a microbiological consortium consisting of lactic acid bacteria, yeasts, and in certain cases acetic acid bacteria. The lactic acid bacteria are mainly responsible for the production of lactate and flavour components, while the yeasts mainly produce ethanol and CO_2. When acetic acid bacteria are present, acetic acid can also be produced. The microbial population and the ratio of the different microbes in the grain and the beverage vary, making it difficult to commercially produce Kepi of a consistent high quality.

Even though Kepi grains and the Kepi beverage have been studied intensively, the precise microbiological population in the grains and the beverage is not known. New species of bacteria and yeasts have been, and are still being isolated from the grains (Wyder *et al.*, 1999). As all microbes cannot grow on synthetic media in the laboratory, and thus be isolated, molecular techniques may succeed in typifying the microbial population in the Kepi grain, where traditional microbial techniques have failed.

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CHAPTER 3

PCR-BASED DGGE TYPIFICATION OF THE MICROBIAL CONSORTIUM PRESENT IN KEPI GRAINS

Abstract

Kepi grains have a complex microbiological composition which makes it difficult to obtain a starter culture with the optimal and constant microbial composition necessary for the production of a quality Kepi beverage. The microbes present in the grains have in the past been identified using traditional methods such as growth on selective media and morphological and physiological characteristics. The aim of this study was to typify the complex microbial community present in mass-cultured, traditionally cultured and Irish Kepi grains by the amplification of part of the variable 16S rRNA gene with primers which specifically amplify Eubacteria as well as part of the 26S ribosomal RNA (rRNA) gene with primers which specifically amplify yeasts by using the polymerase chain reaction (PCR), and resolving the PCR fragments by denaturing gradient gel electrophoresis (DGGE). Unique PCR-based DGGE fingerprints were obtained for the Eubacterial and yeast species present in the mass-cultured, traditionally cultured and Irish Kepi grains.

Introduction

Kepi is a fermented milk beverage that originated in Eastern Europe, and is enjoyed worldwide (Wouters *et al.*, 2002). The Kepi beverage is characterised by a high acidity (*ca.* 0.8%) and, depending upon the holding time after fermentation, by either a lower or higher carbon dioxide content (CO_2) and a relatively low alcohol content (Duitschaever *et al.*, 1987). The sensory characteristics of Kepi include: a pH of *ca.* 4.0; clean, pleasant taste without any bitterness; prickling and sparkling; a slight yeasty taste; a smooth texture; and an ethanol content of 0.5 - 2.0% (m/v) (Kemp, 1984; Marshall & Cole, 1985).

The starter culture used to produce this fermented beverage is an ill-defined, irregularly shaped, gelatinous white/yellow grain similar in appearance to

a cauliflower floret (Guzel-Seydim *et al.,* 2000). The grains contain a balance of specific microbes that co-exist in a complex symbiotic relationship and include species of yeasts, lactic acid bacteria (lactobacilli and lactococci), acetic acid bacteria and mycelial fungi. The characteristic flavour of Kepi is produced by this diverse spectrum of microbial species and at the end of the process the fermenting microbes can be recovered as a solid matrix, which can be re-used (Kwak *et al.,* 1996). Extensive research has identified a few specific species that are always present, whereas other microbes may either be present or absent from the grain (Pintado *et al.*, 1996).

The microbes occur in layers in the grain and are embedded in a polysaccharide material (kefiran) which forms the grain. According to Lin *et al.* (1999) there are almost exclusively bacteria on the outside of the grain and yeasts in the centre of the grain, while the intermediate areas contain a mixture of both bacteria and yeasts.

Due to the complex microbial composition of the grains, it is difficult to obtain a starter culture with the microbial composition necessary for the production of a quality Kepi beverage (Kojima *et al.,* 1993). It is also difficult and time-consuming to determine the precise microbial concentration and the number of different species present in the grains due to the difficulty in separating the microbes from the polysaccharide matrix (Neve, 1992). The microbes present in the grains have in the past been identified using selective growth media, and morphological and biochemical characteristics (Pintado *et al.*, 1996; Takizawa *et al.*, 1998; Wouters *et al.*, 2002).

The identification of the species of microbes present in complex microbial consortia is complicated by the fact that certain organisms are not able to grow on synthetic growth media (Muyzer *et al.*, 1993; Wyder *et al.,* 1999). As only an estimated 20% of the natural occurring bacteria have been isolated and characterised, molecular techniques offer new opportunities for determining and analysing the structure and species composition of microbiological communities (Cody *et al.*, 2000). Sequence variation in the 16S rRNA genes can be used to exploit phylogenetic relationships among microbes, which can be used to identify specific members of microbiological communities, as well as the presence of unculturable microbes (Muyzer *et al.*, 1993). The aim of this study was, therefore, to typify the complex microbial consortium present in Kepi grains by amplifying

part of the variable 16S rRNA gene using *'Eubacterial'* specific primers and the 26S rRNA gene using yeast specific primers, and resolving the PCR fragments by DGGE.

Material and methods

DNA isolation from Kepi grains

DNA was isolated according to the method of Van Elsas *et al.* (1997) from mass-cultured, traditionally cultured and Irish Kepi grains (Schoevers, 1999). The mass-cultured and traditionally cultured grains were obtained from the Department of Food Science, University of Stellenbosch and the Irish grains were obtained from Prof. Timothy Cogan (Teagasc, Moorepark Research Centre, Fermoy, Ireland).

A Kepi grain (approximately 2 mm in diameter), 0.6 g sterile glassbeads (0.2 - 0.3 mm diameter) (Sigma), 800 µl phosphate buffer (1 part 120 mM NaH_2PO_4 to 9 parts 120 mM Na_2HPO_4; pH 8), 700 µl phenol (Saarchem) and 100 µl 20% sodium dodecyl sulphate (Merck) were vortexed for 2 min and incubated for 20 min at 60°C. This step was repeated twice. The bead/cell mixture was centrifuged for 5 min at 1500 x g after which the aqueous phase was collected. The proteins were initially extracted with 600 µl phenol (pH 4.3) (Saarchem) and centrifuged for 5 min at 5900 x g and then extracted with a 600 µl phenol/chloroform/isoamylalcohol (25:24:1) mixture and centrifuged for 5 min at 5900 x g. This was repeated until the interphase was clean. The DNA was then precipitated with 0.1 x volume 3 M sodium acetate (NaAc) (pH 5.5) (Saarchem) and 0.6 x volume isopropanol (Saarchem) on ice for 60 min. The cell mixture was centrifuged for 10 min at 15 000 x g and the pellet was washed with 100 µl 70% (v/v) ethanol. The supernatant was removed, and the pellet was air-dried for 15 min. The pellet was re-dissolved in 100 µl TE (10 mM Tris, 1 mM EDTA; pH8) and the DNA was separated on a 1% (m/v) agarose gel containing ethidium bromide and visualized under UV light (Vilber Lourmat).

DNA amplification with 'Eubacterial' *specific primers*

Approximately 200 base pairs (bp) of the 5' end of the V3 variable region of the 16S rRNA gene were amplified using the *'Eubacterial'* specific primers F341

(5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') (the GC clamp sequence is underlined) and R534 (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al.*, 1993). The PCR reactions were performed in a total reaction volume of 25 µl containing 0.5 µM of each of the primer, 1 U of Expand *Taq* DNA Polymerase (Roche Diagnostics), the buffer supplied with the enzyme, 10 mg BSA (Promega), 0.5 mM dNTPs (Promega) and 1 µl of the isolated DNA. PCR reactions were performed in the Eppendorf Mastercycler Personal. An initial 4 min denaturation at 94°C was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 60 s and elongation at 72°C for 60 s and a final 5 min chain elongation at 72°C (Muyzer *et al.*, 1993). All the PCR products were separated on a 1.5% (m/v) agarose gel containing ethidium bromide using 0.5 x TBE electrophoresis buffer and the separated fragments were visualized under UV light (Vilber Lourmat).

DNA amplification with yeast specific primers

Approximately 250 base pairs (bp) of the 5' end of the 26S rRNA gene was amplified using the yeast specific primers NL1 (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3') (the GC clamp sequence is underlined) (O'Donnell, 1993) and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (Cocolin *et al.*, 2000). The PCR reactions were performed in a total reaction volume of 25 µl containing 0.5 µM of each of the primers, 1 U of Expand *Taq* DNA Polymerase (Roche Diagnostics), the buffer supplied with the enzyme, 10 mg BSA (Promega), 0.5 mM dNTPs (Promega) and 1 µl of the isolated DNA. PCR reactions were performed in the Eppendorf Mastercycler Personal.

The reactions were amplified over 30 cycles: denaturation was performed at 95°C for 60 s, annealing at 52°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 chain elongation at 72°C was done (Cocolin *et al.*, 2000). All the PCR products were separated on a 1.5% (m/v) agarose gel containing ethidium bromide using 0.5 x TBE electrophoresis buffer and the separated fragments were visualized under UV light (Vilber Lourmat).

Denaturing gradient gel electrophoresis (DGGE)

The PCR fragments were separated using DGGE, performed with the Biorad DCode Universal Mutation Detection System (Bio-Rad Laboratories, USA). PCR samples were directly applied onto 8% (m/v) polyacrylamide gels in 0.5 x TAE buffer with a gradient of between 45 and 70% for the Eubacterial PCR fragments and 40 and 60% for the yeast PCR fragments. The gradient was created by polyacrylamide, containing 1 to 100% denaturant (7 M urea and 40% (v/v) formamide). Electrophoresis was performed at a constant voltage of 130 mV for 7 h for the *'Eubacteria'* fragments, 5 h for the yeast fragments and a constant temperature of 60°C. The gel was stained with ethidium bromide for 30 min and the fragments were visualized under UV light (Vilber Lourmat).

Results and discussion

DNA amplification with 'Eubacterial' *and yeast specific primers*

The isolated DNA from the mass-cultured, traditionally cultured and the Irish Kepi grains were successfully amplified using the *'Eubacterial'* and yeast specific primers, respectively. The *'Eubacterial'* specific primers yielded a 200 bp PCR fragment (Fig. 1) of part of the 16S rRNA gene, while the yeast specific primers yielded a 250 bp PCR fragment of part of the 26S rRNA gene (Fig. 2).

DGGE fingerprinting

The PCR fragments were resolved on a DGGE gel and unique DNA banding patterns were obtained for the mass-cultured, traditionally cultured and Irish Kepi grains using the *'Eubacterial'* specific primers, as well as the yeast specific primers. The reproducibility of the DGGE fingerprints were confirmed by repeating PCR amplification reactions on the DNA isolated from the Kepi grains several times, as well as on DNA isolated from different grains in the same sample several times. All the obtained Eubacterial and yeast PCR-based DGGE fingerprints for the mass-cultured, traditionallly cultured and Irish Kepi grains had the same banding profiles unique to each different type of grain (Fig. 3 and Fig. 4).

The bands of the DGGE fingerprint are species specific, therefore, each band observed on the gel represents a different Eubacterial or yeast species in the Kepi grains (Cocolin *et al.*, 2000; Muyzer *et al.*, 1993).

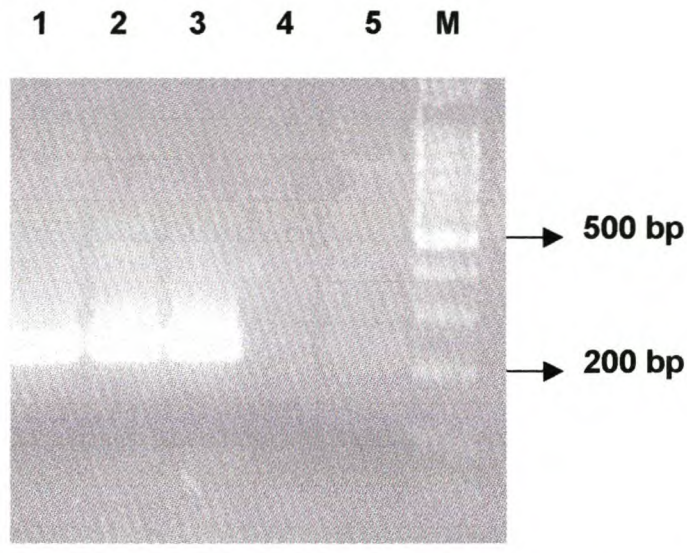

Figure 1. 1.5% (m/v) agarose gel showing the PCR amplification of 200 bp of part of the 16S rRNA gene using the *'Eubacterial'* specific primers F341 and R534 (Muyzer *et al.*, 1993). Lane 1: mass-cultured Kepi grains; Lane 2: traditionally cultured Kepi grains; Lane 3: Irish Kepi grains; Lane 4: PCR negative control; Lane 5: PCR negative control; and Lane M: 100 bp ladder (Promega).

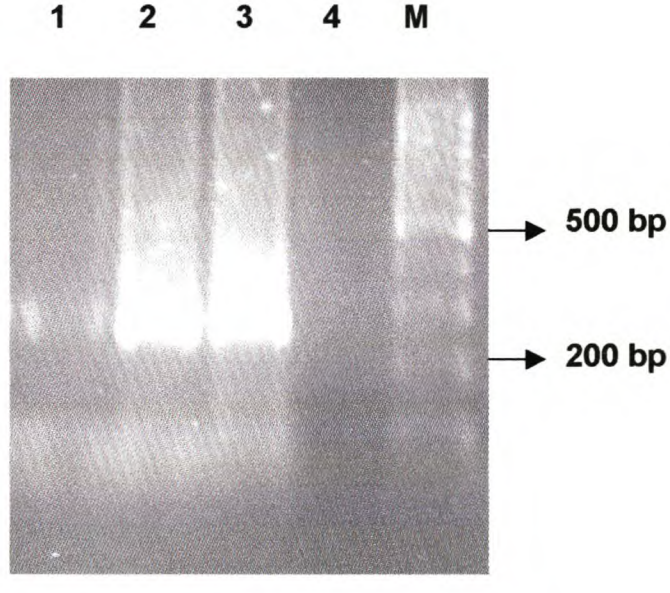

Figure 2. 1.5% (m/v) agarose gel showing the PCR amplification of 250 bp of part of the 26S rRNA gene using the yeast specific primers NL1 and LS2. Lane 1: mass-cultured Kepi grain; Lane 2: normally cultured Kepi grain; Lane 3: Irish Kepi grain; Lane 4: PCR negative control; and Lane M: 100 bp ladder (Promega).

Bands in the same position in the gel confirm that the same Eubacteria or yeast species are present in all these Kepi grains. PCR-based DGGE fingerprints of the mass-cultured, traditionally cultured and Irish Kepi grains amplified with *'Eubacterial'* primers yielded 7, 9 and 8, bands respectively for the different grains (Fig. 3). The DGGE fingerprints of the Irish Kepi grains contained a band that was unique (Fig. 3, band a), as it was not present in either the mass-cultured or traditionally cultured Kepi grains. The traditionally cultured and the Irish Kepi grains both also contained a band (Fig. 3, band b) that was not seen in the mass-cultured grains. The remaining bands were present in all the different Kepi grains.

The PCR-based DGGE fingerprints of the mass-cultured, traditionally cultured and Irish Kepi grains amplified with yeast specific primers were also unique to each different type of grain, confirming that the three different types of grains have a distinct yeast species composition (Fig. 4). Only one yeast species was present in all the different grains (Fig. 4, band c). A specific DGGE band (Fig. 4, band d) was present in the traditionally cultured and the Irish Kepi grains, but was absent from the mass-cultured grains. The traditionally cultured grains contained a yeast species which is not present in either the mass-cultured or the Irish Kepi grains (Fig. 4, band e), while the mass-cultured grains included a yeast species which was not present in either the traditionally cultured or the Irish Kepi grains (Fig. 4, band f).

Extensive research, as given in the literature, has shown that there are a few specific microbial species and genera that always occur in the Kepi grain, whereas other species and genera may either be present or absent from the grain (Kuo & Lin, 1999; Pintado *et al.*, 1996). This was also found in this study, as it can be seen in both the Eubacterial and yeast PCR-based DGGE fingerprints of the different types of grains, because none of the Eubacterial or yeast profiles of the mass-cultured, traditionally cultured or Irish Kepi grains are identical. This can be ascribed to the fact that the microbiological diversity of the Kepi grain is influenced by the origin of the grain, different cultivation methods and storage conditions (Pintado *et al.*, 1996).

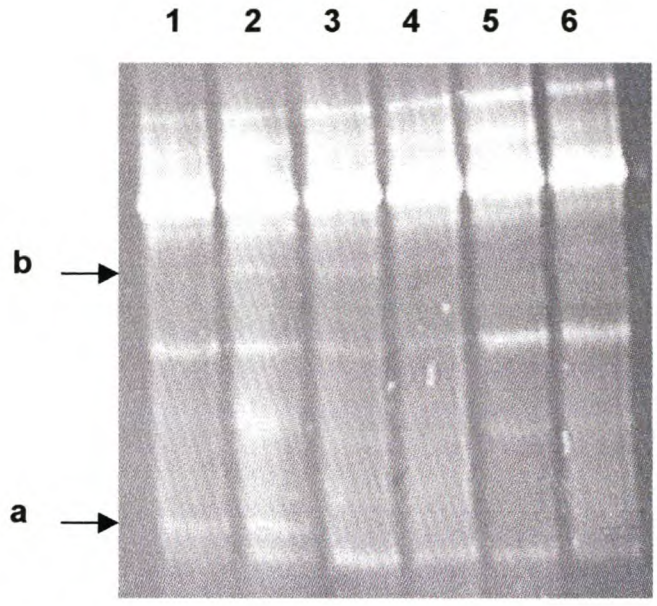

Figure 3. PCR-based DGGE fingerprints using the *'Eubacterial'* specific primers. Lanes 1 and 2: Irish Kepi grains; Lanes 3 and 4: Traditionally cultured Kepi grains; and Lanes 5 and 6: Mass-cultured Kepi grains.

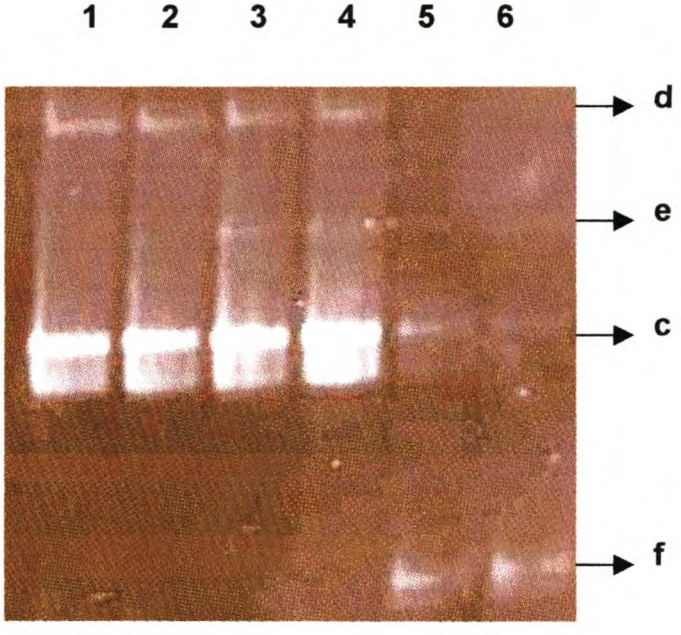

Figure 4. PCR-based DGGE fingerprints of the yeast species present in Kepi grains. Lanes 1 and 2: Irish Kepi grains; Lanes 3 and 4: Traditionally cultured Kepi grains; Lanes 5 and 6: Mass-cultured Kepi grains.

Conclusion

The data in this study shows that PCR-based DGGE fingerprinting can be successfully used to typify the microbial consortium present in Kepi grains, as well as to distinguish between Kepi grains cultured using different methods, or Kepi grains that have different origins. The grains can be compared with respect to both the Eubacterial and the yeast species present. It is, therefore, of great importance to identify the PCR fragments in the DGGE gels to determine which species represents which band, and then this method can be used to establish which Eubacterial species or yeast species are present in Kepi grains.

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CHAPTER 4

MOLECULAR IDENTIFICATION OF EUBACTERIAL AND YEAST SPECIES PRESENT IN KEPI GRAINS

Abstract

Kepi grains contain a balance of specific microbes that co-exist in a symbiotic relationship. The microbial population includes lactic acid bacteria (LAB), acetic acid bacteria, mycelial fungi and yeasts. In the past, the microbial population comprising Kepi grains have been identified using classical identification techniques. The aim of this study was to identify the complex microbial population in Kepi grains using molecular identification techniques. A part of the 16S ribosomal RNA (rRNA) gene from the *'Eubacterial'* and yeast consortium members was amplified, cloned, sequenced and compared to sequences available on GenBank. The phylogenetic relatedness of the amplified and sequenced lactobacilli was determined. From the data obtained, the different bands in both the *'Eubacterial'* denaturing gradient gel electrophoresis (DGGE) profile and the yeast DGGE profile were identified to species level, by resolving the PCR fragments on DGGE gels. A marker was constructed to identify the bands in the *'Eubacterial'* DGGE profile, providing a quick method to identify the members of the microbial population in mass-cultured Kepi grains.

Introduction

Kepi is a fermented milk beverage that originated in Eastern Europe. The starter culture used to produce this beverage is an ill-defined, irregularly shaped, gelatinous white/yellow grain (Guzel-Seydim *et al.,* 2000; Wouters *et al.*, 2002). The Kepi grains have a complex microbial composition that includes species of yeasts, lactic acid (LAB) and acetic acid bacteria, and mycelial fungi (Duitschaever *et al.*, 1988; Kwak *et al.*, 1996; Lin *et al.*, 1999).

Kepi has a characteristic flavour that is produced by the diverse spectrum of microbial species present (Duitschaever, 1989; Kwak *et al.,* 1996). Lactobacilli are present in the largest numbers (65 – 80%) of the microbial population (Wood, 1998; Wouters *et al.*, 2002). Lactococci and yeasts make up the remaining

percentage of the microbes present in the Kepi grain (Wood, 1998). These values may differ if the grains have different origins, or if the grains are cultured using different methods and substrates (Angulo *et al.*, 1993; Pintado *et al.*, 1996). A symbiotic relationship exists between the microbes present in the Kepi grains, and it has been determined that there are specific species that always occur in the grain, whereas other microbes may either be present or absent from the grain depending on the origin of the grains, as well as method of culturing and substrates added (Pintado *et al.*, 1996).

Some lactic acid bacteria that have been isolated from Kepi include: *Lactobacillus acidophilus*, *Lb. brevis*, *Lb. casei*, *Lb. fermentum, Lb. helveticus, Lb. kefiri, Lb. parakefiri, Lactococcus lactis* and *Leuconostoc mesenteroides* (Assadi *et al.*, 2000; Cogan *et al.*, 1997; Fujisawa *et al.*, 1988; Kandler & Kunath, 1983; Kwak *et al.*, 1996; Micheli *et al.*, 1999; Pintado *et al.*, 1996; Schoeman, 2001; Takizawa *et al.*, 1994; Takizawa *et al.*, 1998). Yeasts isolated from Kepi grains include *Kluyveromyces bulgaricus, K. fragilis, Torula kefir, Saccharomyces exiguus; S. fragilis, Candida kefir,* and *C. lambica* (Assadi *et al.*, 2000; Garotte *et al.*, 1997; Kwak *et al.*, 1996; Pintado *et al.*, 1996; Wyder *et al.*, 1999; Wyder & Puhan, 1997). *Acetobacter aceti* and *A. rasens,* as well as a mycelial fungus, *Geotrichium candidum* have also been isolated from Kepi grains (Garotte *et al.*, 1997; Marshall, 1993; Pintado *et al.*, 1996).

It is difficult to determine the precise number of microbes and species present in the grains, due to difficulty in separating the microbes from the polysaccharide matrix (Neve, 1992). The heterogeneous distribution of the microbes on the surface of the grains also further complicates the isolation procedure (Neve, 1992). The aim of this study was to identify members of the Eubacterial and yeast community present in the Kepi grains using molecular techniques. A PCR-based DGGE method for identifying specific members in the complex *'Eubacterial'* microbial community comprising Kepi grains was developed.

Material and methods

DNA amplification with 'Eubacterial' and yeast specific primers

Mass-cultured Kepi grain DNA (as isolated in Chapter 3), as well as DNA from four unidentified isolates from strings of kefiran (Cronjé, 2003) (KGI-A, KGI-B,

KGI,-D and KGI-5) were used for PCR amplification. Approximately 1.5 kilobase pairs (kb) of part of the 5' end 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). For the yeasts, 250 base pairs (bp) of the 5' end of the 26S rRNA gene were amplified using the yeast specific primers NLc1 (5'-GCC ATA TCA ATA AGC GGA GGA AAA G-3') and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (Cocolin *et al.*, 2000; Cocolin *et al.*, 2002; O'Donnell, 1993).

The PCR reactions were performed in a total reaction volume of 25 µl containing 0.5 µM of each of the primers, 1 U *Taq* DNA Polymerase (Roche Diagnostics), the buffer supplied with the enzyme, Dimethyl Sulfoxide (DMSO) (Merck), 0.5 mM dNTPs (Promega) and 1 µl of the isolated DNA. PCR reactions were performed in the Eppendorf Mastercycler Personal. An initial 3 min denaturation at 92°C was followed by 35 cycles of denaturation at 92°C for 30 s, annealing at 54°C for 30 s and elongation at 68°C for 60 s and a final 7 min chain elongation at 72°C.

A nested PCR amplification of part of the 5' end of the 16S rRNA gene, amplifying 200 bp, was also performed on the amplified 1.5 kb Eubacterial PCR fragments using the primers F341 (5'-<u>CGC CCG CCG CGC GCG GCG GGC GGG</u> GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') (the GC clamp sequence is underlined) and R534 (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al.*, 1993). This was done to confirm that each 200 bp band in the DGGE fingerprints was represented by a 1.5 kb PCR product, and therefore the complete population would be sequenced and identified. The PCR reaction was performed in a total reaction volume of 25 µl containing 0.5 µM of each of the primers, 1 U of Expand *Taq* DNA Polymerase (Roche Diagnostics), the buffer supplied with the enzyme, Dimethyl Sulfoxide (DMSO) (Merck), 0.5 mM dNTPs (Promega) and either 1 µl of the isolated DNA, or transformed cells. PCR reactions were performed using the Eppendorf Mastercycler Personal.

The Eubacterial DNA was amplified using an initial 4 min denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 60 s and elongation at 72°C for 60 s and a final 5 min chain elongation at 72°C (Muyzer *et al.*, 1993). All the PCR products were analyzed on a 1.5% (m/v)

agarose gel containing ethidium bromide using 0.5 x TBE electrophoresis buffer and the separated fragments were visualised under UV light (Vilber Lourmat).

PCR fragments were resolved using DGGE, performed with the Biorad DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, USA). PCR samples were directly applied onto 8% (m/v) polyacrylamide gels in 0.5 x TAE buffer with a gradient of between 45 and 70%. The gradient was created by polyacrylamide, containing 1 to 100% denaturant (7 M urea and 40% (v/v) formamide). Electrophoresis was performed at a constant voltage of 130 mV for 7 h at a constant temperature of 60°C. The gel was stained with ethidium bromide for 30 min and the fragments were visualised under UV light (Vilber Lourmat).

Cloning and DNA sequencing

The amplified 1.5 kb Eubacteria and 250 bp yeast PCR fragments were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) according to the manufacturer's instructions. These amplified fragments were cloned into the pGemT-Easy Vector System II (Promega). Transformed cells 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 50 µl containing 0.5 µM of each of the primers, 0.5 mM dNTPs (Promega), 1 U *Taq* DNA Polymerase (Roche Diagnostics), the buffer supplied with the enzyme and the transformed cells.

The reaction conditions consisted of an initial denaturation cycle at 92°C for 3 min followed by 35 cycles of denaturation at 92°C for 30 s, annealing at 54°C for 30 s, elongation at 68°C for 60 s and a final 7 min elongation at 72°C. Products were analysed on a 1.5% (m/v) agarose gel containing ethidium bromide using 0.5 x TBE electrophoresis buffer and the separated fragments were visualized under UV light (Vilber Lourmat).

Both the amplification products of the transformed cells and the amplification products of the unidentified isolates from kefiran (Cronjé, 2003) were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) according to the manufacturer's instructions. The amplification products of the transformed cells were cut with the restriction enzymes *Msp*I and *Alu*I (Promega) to ascertain that the cloned inserts were different prior to sequencing.

All the purified PCR fragments were sequenced using the ABI PRISM 377 DNA Sequencer (PerkinElmer) at the DNA Sequencing Facility at the University of Stellenbosch. The sequences obtained from the cloned PCR fragments and the unidentified isolates from kefiran were compared to sequences available in Genbank using the BLAST algorithm (Altschul *et al.*, 1997). The sequences were aligned to closely related sequences and phylogenetically analysed using the Phylogenetic Analysis Using Parsimony (PAUP) program (Swofford, 2000).

The partial nucleotide sequences of the small-subunit rRNA gene of this study and that of the outgroup, *Leuconostoc gasicomitatum* (GenBank accession number AF231132) were manually aligned with other small subunit rRNA sequences obtained from Genbank by inserting gaps using Sequence Alignment Editor v2.0 (Rambaut, 2002). Phylogenetic analyses were undertaken using PAUP version 4.0b10 (Swofford, 2000). Alignment gaps were treated as a new state and all characters were unordered and of equal weight. Heuristic searches were conducted using 1 000 random taxa additions and tree bisection and reconstruction (TBR) as the branch swapping algorithm to find maximum parsimony trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees was evaluated with 1 000 bootstrap replications (Hillis & Bull, 1993). Other measures calculated included tree length, consistency index (CI), retention index (RI) and rescaled consistency index (RC). Resulting trees were printed with TreeView Version 1.6.6 (Page, 1996).

Identification of the DGGE banding patterns

Approximately 200 bp of the 5' end of the 16S rRNA gene of all the transformed PCR fragments, isolates from Kepi grains obtained from the University of Stellenbosch Food Science Culture Collection (USFSCC) (Schoeman, 2001) (Table 1), Stellenbosch, South-Africa, and mass-cultured Kepi grains were amplified using the *'Eubacterial'* specific primers F341 and R534 in order to identify specific bands in the DGGE fingerprints (Muyzer *et al.*, 1993). A 250 bp fragment of the 5' end of the 26S rRNA gene of the cloned PCR fragments and mass-cultured Kepi grain DNA was amplified using the yeast specific primers NL1 and LS2 (Cocolin *et al.*, 2000; Cocolin *et al.*, 2002). The PCR reactions, as

Table 1. Isolates from Kepi grains obtained from the University of Stellenbosch Food Science Culture Collection (USFSCC), Stellenbosch, South Africa (Schoeman, 2001).

Name of isolate	Culture Collection number
Lactobacillus delbrueckii subsp. *delbrueckii*	1278
Lb. fermentum	1281
Lb. plantarum	1288
Lb. brevis	1289
Lb. delbrueckii subsp. *lactis*	1291
Lb. curvatus	1299
Lb. acidophilus	1306
Leuconostoc lactis	1279
Leuc. lactis subsp. *lactis*	1286
Leuc. mesenteroides subsp. *mesenteroides*	1303
Pediococcus sp.	1305

well as the DGGE analyses were performed as described previously.

Construction of the Eubacterial DGGE marker

Two µl of each of the different transformed cells containing the 1.5 kb PCR product (dissolved in 50 µl double distilled water and lysed for 5 min at 90°C) and 2 µl of the DNA from the isolates taken from the USFSCC was mixed, and 1 µl of the DNA mixture was used as template for the PCR reaction. Approximately 200 bp of the 5' end of the 16S rRNA gene were amplified using the *'Eubacterial'* specific primers F341 and R534 (Muyzer *et al.*, 1993). The PCR reaction, as well as the DGGE were performed as described previously.

Results and discussion

DNA amplification with 'Eubacterial' and yeast specific primers

The isolated DNA from both the mass-cultured Kepi grains and the unidentified isolates from kefiran (Cronjé, 2003) were successfully amplified using the *'Eubacterial'* and yeast specific primers. The *'Eubacterial'* specific primers yielded a 1.5 kb PCR fragment, while the yeast specific primers yielded a 250 bp PCR fragment. A 200 bp PCR fragment of part of the 1.5 kb PCR fragment that was used as the template was amplified, and resolved using DGGE. The DGGE profile of the 1.5 kb PCR fragments was compared to the DGGE profiles in Chapter 3, and it was established that all the 200 bp bands on the DGGE fingerprint were represented by a 1.5 kb PCR product (data not shown).

DNA sequencing and phylogenetic analysis

The sequences of the cloned PCR products from these strains were analysed using blast searches are presented in Table 2. Sequence comparison on NCBI of the '*Eubacteria*' revealed that they are closely related to sequences belonging to the genus *Lactobacillus*. The cloned Eubacteria (Table 2) were identified as: unculturable *Lactobacillus* clone 1340 (KC-2) (954 bp of the 961 bp sequenced were homologous to unculturable *Lactobacillus* clone 1340 AF371470.1); unculturable *Lactobacillus* clone 3443 (KC-3) (503 bp of the 508 bp sequenced were homologous to unculturable

Tabel 2. Percentage (%) similarity of the partial 16S rRNA sequences of the '*Eubacterial*' clones to their closest bacterial relatives available in the NCBI nucleotide sequence database.

Name	Phylogenetic affiliation and accession number	% Similarity
KC-2	Unculturable *Lactobacillus* clone 1340 (AF371470.1)	99.2%
KC-3	Unculturable *Lactobacillus* clone 3443 (AF371472.1)	99.0%
KC-4	*Lb. crispatus* (AF257097)	99.3%
KC-38	*Lb.* species (AF094065)	99.3%
KC-36	*Lb.* species (homologous to *Lb. helveticus* AF213704)	97.8%
KC-43	*Lb.* species (homologous to unculturable swine feces bacterium AF261776)	97.8%

Table 3. Percentage (%) similarity of the partial 16S rRNA sequences of the unidentified isolates from kefiran to their closest bacterial relatives available in the NCBI nucleotide sequence database.

Name	Phylogenetic affiliation and accession number	% Similarity
KGI-A	*Lb. kefiri* (AB024300)	99.0%
KGI-B	*Lb. parakefiri* (AY026750)	99.2%
KGI-D	*Lb. gallinarum* (AJ417737)	98.6%
KGI-5	Unculturable *Lactobacillus* clone 1340 (AF371470.1)	99.2%

Table 4. Percentage (%) similarity of the partial 26S rRNA sequences of the yeast clones to their closest yeast relatives available in the NCBI nucleotide sequence database.

Name	Phylogenetic affiliation and accession number	% Similarity
KC-y1	*Candida lambica* (SCE437312)	99.0%
KC-y18	*Saccharomyces cerevisiae* (PFY75726)	100%

Table 5. Sequence alignment of part of the 16S rRNA gene of members of the genus *Lactobacillus*

```
[                                                                    1          11         21         31         ]
[                                                                    |          |          |          |          ]
Leuconostoc gasicomitatum AF231132                                   GA-TGGATCCGCGGTGCATTAGTTAGTTGGTGGGGTAAAG
Lactobacillus fermentum AF378372                                     GA-TGGTCCCGCGGTGCATTAGTTAGTTGGTGAGGTAATG
Lactobacillus delbrueckii subsp. lactis AY050173                     GA-TGAGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAAAG
Lactobacillus acidophilus AF375937                                   TTGTAGAGCGACCGAGAAGAGAATTCTTGGTAAGGTAACG
Lactobacillus species AY094065                                       GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
KGI-D                                                                GA-TGGACCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
KC-38                                                                GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
Lactobacillus gallinarum AJ417737                                    GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
Lactobacillus helveticus strain CRL 1062 AF213705                    GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
KC-4                                                                 GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAAAG
Lactobacillus crispatus strain ATCC33820 AF257097                    GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAAAG
UBC1340 AF371470.1                                                   GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
USFB AF261776                                                        GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
KC-2                                                                 GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
UBC3443 AF371472.1                                                   GA-TGGACCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
KC-3                                                                 GA-TGGACCCGCGGTGGATTAGCTAGTTGGGAAGGTAACG
KC-36                                                                GA-TGGACCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
KC-43                                                                GA-TGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACG
Lactobacillus curvatus AJ270951                                      GA-TGGACCCGCGGTGCATTAGTTAGTTGGTGAGGTAAAG
Lactobacillus plantarum AF404710 .                                   GA-TGATCCCGCGGCGTATTAGTTAGTTGGTGAGGTAAAG
Lactobacillus brevis AF404709                                        GA-TGATCCCGCGGCGTATTAGTTAGTTGGTGAGGTAAAG
Lactobacillus casei AJ507644                                         GA-TGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAACG
Lactobacillus kefiri AB024300                                        GA-TGGACCCGCGGCGTATTAGCTTGTTGGTAAGGTAATG
KGI-A                                                                GA-TGGACCCGCGGGGTATTACCTTGTTGGTAAGGNGATG
Lactobacillus parakefiri AY026750                                    GA-TGGACCCGCGGCGTATTAGCTTGTTGGTAAGGTAACG
KGI-B                                                                GA-TGGACCCGCGGCGTATTAGCTTGTTGGTAAGGTAACG
```

Table 5. (continued)

[	41 51 61 71]
[	| | | |]
Leuconostoc gasicomitatum AF231132	GCTTAC-CAAGACAATGATGCATAGCCGAGTTGAGAGACT
Lactobacillus fermentum AF378372	GCTCAC-CAAGACGATGATGCATAGCCGAGTTGAGAGACT
Lactobacillus delbrueckii subsp. *lactis* AY050173	GCCTAC-CAAGGCAATGATGCGTAGCCGAGTTGAGAGACT
Lactobacillus acidophilus AF375937	GCTTAC-CAAGGCAATGATGCATAGCCGAGTTGAGAGACT
Lactobacillus species AY094065	GCTTAC-CAAGGCAATGATGCATAGCCGAGTTGAGAGACT
KGI-D	GCCTAC-CAAGGCAGTGATGCATAGCCGAGTTGAGAGACT
KC-38	GCCTAC-CAAGGCAGTGATGCATAGCCGAGTTGAGAGACT
Lactobacillus gallinarum AJ417737	GCTTAC-CAAGGCAATGATGCATAGCCGAGTTGAGAGACT
Lactobacillus helveticus strain CRL 1062 AF213705	GCTTAC-CAAGGCAATGATGCATAGCCGAGTTGAGAGACT
KC-4	GCTTAC-CAAGGCGATGATGCATAGCCGAGTTGAGAGACT
Lactobacillus crispatus strain ATCC33820 AF257097	GCTTAC-CAAGGCGATGATGCATAGCCGAGTTGAGAGACT
UBC1340 AF371470.1	GCTTAC-CAAGGCGACGATGCATAGCCGAGTTGAGAGACT
USFB AF261776	GCTTAC-CAAGGCGACGATGCATAGCCGAGTTGAGAGACT
KC-2	GCTTAC-CAAGGCGACGATGCATAGCCGAGTTGAGAGACT
UBC3443 AF371472.1	GCCTAC-CAAGGCAGNGATGCATAGCCGAGTTGAGAGACT
KC-3	GCCTAC-CAAGGNAGTGATGCATAGCCGAGTTGAGAGACT
KC-36	GCTTAC-CAAGGCAGTGATGCATAGCCGAGTTGAGAGACT
KC-43	GCTTAC-CAAGGCAGTGATGCATAGCCGAGTTGAGAGACT
Lactobacillus curvatus AJ270951	GCTCAC-CAAGACCGTGATGCATAGCCGACCTGAGAGGGT
Lactobacillus plantarum AF404710	GCCCAC-CAAGACGATGATACGTAGCCGACCTGAGAGGGT
Lactobacillus brevis AF404709	GCCCAC-CAAGACGATGATACGTAGCCGACCTGAGAGGGT
Lactobacillus casei AJ507644	GCTCAC-CAAGGCAATGATACGTAGCCGAACTGAGAGGTT
Lactobacillus kefiri AB024300	GCCTAC-CAAGGCAATGATACGTAGCCGACCTGAGAGGGT
KGI-A	GCCTAC-CCAGGCAATGATACGTAGCCGACCTGAGAGGCN
Lactobacillus parakefiri AY026750	GCCTAC-CAAGGCAATGATACGTAGCCGACCTGAGAGGGT
KGI-B	GCCTAC-CAAGGCAATGATACGTAGCCGACCTGAGAGGGT

Table 5. (continued)

[	81 91 101 111]
[	| | | |]
Leuconostoc gasicomitatum AF231132	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
Lactobacillus fermentum AF378372	GATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTA
Lactobacillus delbrueckii subsp. *lactis* AY050173	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
Lactobacillus acidophilus AF375937	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
Lactobacillus species AY094065	GAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
KGI-D	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
KC-38	GAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
Lactobacillus gallinarum AJ417737	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
Lactobacillus helveticus strain CRL 1062 AF213705	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
KC-4	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
Lactobacillus crispatus strain ATCC33820 AF257097	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
UBC1340 AF371470.1	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
USFB AF261776	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
KC-2	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
UBC3443 AF371472.1	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
KC-3	GATCGGNCACATTGGGACTGANACACGGNCCAAACTCCTA
KC-36	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
KC-43	GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
Lactobacillus curvatus AJ270951	AATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
Lactobacillus plantarum AF404710	AATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
Lactobacillus brevis AF404709	AATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
Lactobacillus casei AJ507644	GATCGGCCACATTGGGACTGAGACACGGCCNNNACTCCTA
Lactobacillus kefiri AB024300	AATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
KGI-A	AATCTGCCACATTGGGACTGAGACACGGCCCAAACTCCTC
Lactobacillus parakefiri AY026750	AATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
KGI-B	AATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA

Table 5. (continued)

	121 131 141 151
	[| | | |]
Leuconostoc gasicomitatum AF231132	CGGGAGGCTGCAGTAGGGAATCTTCCACAATGGGCGAAAG
Lactobacillus fermentum AF378372	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAG
Lactobacillus delbrueckii subsp. *lactis* AY050173	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
Lactobacillus acidophilus AF375937	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
Lactobacillus species AY094065	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
KGI-D	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
KC-38	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
Lactobacillus gallinarum AJ417737	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
Lactobacillus helveticus strain CRL 1062 AF213705	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
KC-4	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
Lactobacillus crispatus strain ATCC33820 AF257097	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
UBC1340 AF371470.1	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
USFB AF261776	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
KC-2	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
UBC3443 AF371472.1	CGGGAGGCANCAGTAGGGAATCTTCCACAATGGACGCAAG
KC-3	CGGGAGGGAGCAGTAGGGAATNTTCCACAATGGACGCAAG
KC-36	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
KC-43	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
Lactobacillus curvatus AJ270951	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG
Lactobacillus plantarum AF404710	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG
Lactobacillus brevis AF404709	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG
Lactobacillus casei AJ507644	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
Lactobacillus kefiri AB024300	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG
KGI-A	CGGGAGGGAGGGGGTGGGAATCTTCCACCATGGGCGAAAG
Lactobacillus parakefiri AY026750	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG
KGI-B	CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG

Table 5. (continued)

[	161 171 181 191]
[	| | | |]
Leuconostoc gasicomitatum AF231132	CCTGATGGAGCAACGCCGCGTGTGTGATGAAGG-CTTTCG
Lactobacillus fermentum AF378372	CCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTT-CG
Lactobacillus delbrueckii subsp. *lactis* AY050173	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TCTTCG
Lactobacillus acidophilus AF375937	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
Lactobacillus species AY094065	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
KGI-D	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
KC-38	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
Lactobacillus gallinarum AJ417737	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
Lactobacillus helveticus strain CRL 1062 AF213705	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
KC-4	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TCTTCG
Lactobacillus crispatus strain ATCC33820 AF257097	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
UBC1340 AF371470.1	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
USFB AF261776	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
KC-2	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
UBC3443 AF371472.1	TCTGATGGAGCAACGCCGCTCGAGTGAAGAAGG-TTTTCG
KC-3	TTTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
KC-36	TCTGATGGAGCA-CGCCGCGTGAGTGAAGAAGG-TTT-CG
KC-43	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTTCG
Lactobacillus curvatus AJ270951	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-TTTTCG
Lactobacillus plantarum AF404710	TCTGATGGAGCAT-GCCGCGTGAGTGAAGAAGGGTTT-CG
Lactobacillus brevis AF404709	TCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGGTTT-CG
Lactobacillus casei AJ507644	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG-CTTTCG
Lactobacillus kefiri AB024300	TCTGATGGAGCAACGCCGCGTGAGTGATGAAGGGTTT-CG
KGI-A	TCTGNTGGAGCAACCCCCCGTGAGTGATGAAGGGGCT-TG
Lactobacillus parakefiri AY026750	TCTGATGGAGCAACGCCGCGTGAGTGATGAAGGGTTT-CG
KGI-B	TCTGATGGAGCAACGCCGCGTGAGTGATGAAGGGTTT-CG

Table 5. (continued)

[	201 211 221 231]
[	| | | |]
Leuconostoc gasicomitatum AF231132	GGTCGTAAAGCACTGTTGTATGGGAAGAACAGCTAGAG-T
Lactobacillus fermentum AF378372	GCTCGTAAAACACTGTTGTAAGAGAAGAAT-GACATTGAG
Lactobacillus delbrueckii subsp. *lactis* AY050173	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGC
Lactobacillus acidophilus AF375937	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
Lactobacillus species AY094065	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
KGI-D	GACCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
KC-38	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
Lactobacillus gallinarum AJ417737	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
Lactobacillus helveticus strain CRL 1062 AF213705	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
KC-4	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
Lactobacillus crispatus strain ATCC33820 AF257097	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
UBC1340 AF371470.1	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
USFB AF261776	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGCAGT
KC-2	GATCGTAA-GCTCTGTTGTTGGTGAAGAAG-GATAGAGGT
UBC3443 AF371472.1	GATCGTAAAGCTCTGTTGTTGGTGAAGAAG-GATAGAGGC
KC-3	GACAAAGTCACGGNTAACTACGTGCCAGCAGTCGGGGNAA
KC-36	GATCGTAA-GCTCTGTTGTTGGTGAAAAAG--ATAAAG-T
KC-43	GACCGTAAAGCTCTGTTGTTGGTAAAAAAG--ATAAAGGT
Lactobacillus curvatus AJ270951	GATCGTAAAACTCTGTTGTTGGAGAAGAAC-GTATTTGAT
Lactobacillus plantarum AF404710	GCTCGTAAAACTCTGTTGTTAAAGAAGAAC-ACCTTTGAG
Lactobacillus brevis AF404709	GCTCGTAAAACTCTGTTGTTAAAGAAGAAC-ACCTTTGAG
Lactobacillus casei AJ507644	GGTCGTAAAACTCTGTTGTTGGAGAAGAAT-GGTCGGCAG
Lactobacillus kefiri AB024300	GCTCGTAAAACTCTGTTGTTGGAGAAGAAC-AGGTGTCAG
KGI-A	GCTCCNAAAACTCTGTTGTTGGAGAAGAAC-CGGTG-GGG
Lactobacillus parakefiri AY026750	GCTCGTAAAACTCTGTTGTTGGAGAAGAAC-CGTTGTCAG
KGI-B	GCTCGTAAAACTCTGTTGTTGGAGAAGAAC-CGTTGTCAG

Table 5. (continued)

```
[                                                                241        251        261        271        ]
[                                                                |          |          |          |          ]
Leuconostoc gasicomitatum AF231132                               AGGGAATGAC----------------TTTAGTTTGACGGT
Lactobacillus fermentum AF378372                                 AGTAACTGTTC---------------AATGTGT-GACGGT
Lactobacillus delbrueckii subsp. lactis AY050173                 AGTAACTGGTC---------------TTTATTT-GACGGT
Lactobacillus acidophilus AF375937                               AGTAACTGGCC---------------TTTATTT-GACGGT
Lactobacillus species AY094065                                   AGTAACTGGCC---------------TTTATTT-GACGGT
KGI-D                                                            AGTAACTGGCC---------------TTTATTT-GACGGT
KC-38                                                            AGTAACTGGCC---------------TTTATTT-GACGGT
Lactobacillus gallinarum AJ417737                                AGTAACTGGCC---------------TTTATTT-GACGGT
Lactobacillus helveticus strain CRL 1062 AF213705                AGTAACTGGCC---------------CTTTATTT-GACGGT
KC-4                                                             AGTAACTGGCC---------------TTTATTT-G--GGT
Lactobacillus crispatus strain ATCC33820 AF257097                AGTAACTGGCC---------------TTTATTT-GACGGT
UBC1340 AF371470.1                                               AGTAACTGGCC---------------TTTATTT-GACGGT
USFB AF261776                                                    AGTAACTGGCC---------------TTTATTT-GACGGT
KC-2                                                             AGTAACTGGCC---------------TTTATTT-GACGGT
UBC3443 AF371472.1                                               AGCAACTGGCCC--------------TTTATTT-GGCGGT
KC-3                                                             TACGTAGGTGGCAAGGGTTGTCCGGATTTATTG-GGCGTA
KC-36                                                            AGT-ACTGGCC----------------TTAT---GACGGA
KC-43                                                            AATAACTGGCC---------------TTTTTTT-GACGGN
Lactobacillus curvatus AJ270951                                  AGTAACTGATC---------------AGGTAGT-GACGGT
Lactobacillus plantarum AF404710                                 AGTAACTGTTC---------------AAGGGTT-GACGGT
Lactobacillus brevis AF404709                                    AGTAACTGTTC---------------AAGGGTT-GACGGT
Lactobacillus casei AJ507644                                     AGTAACTGTTG---------------TCGGCGT-GACGGT
Lactobacillus kefiri AB024300                                    AGTAACTGTTG---------------ACATCTT-GACGGT
KGI-A                                                            AGTAACTGTTT---------------TTTTCTT-GGCGGG
Lactobacillus parakefiri AY026750                                AGCAACTGTTG---------------ACAGCTT-GACGGT
KGI-B                                                            AGCAACTGNTG---------------ACAGCTT-GACGGT
```

Table 5. (continued)

[	281 291]
[	| |]
Leuconostoc gasicomitatum AF231132	ACCATA-C-CAG
Lactobacillus fermentum AF378372	ATCTTA-C-CAG
Lactobacillus delbrueckii subsp. *lactis* AY050173	AATCAA-C-CAG
Lactobacillus acidophilus AF375937	AATCAA-C-CAG
Lactobacillus species AY094065	AATCAA-C-CAG
KGI-D	AATCAA-C-CAG
KC-38	AATCA--C-CAG
Lactobacillus gallinarum AJ417737	AATCAA-C-CAG
Lactobacillus helveticus strain CRL 1062 AF213705	AATCGA-C-CAA
KC-4	AATCAA-C-CAG
Lactobacillus crispatus strain ATCC33820 AF257097	AATCAA-C-CAG
UBC1340 AF371470.1	AATCAA-C-CAG
USFB AF261776	AATCAA-C-CAG
KC-2	AATCAA-C-CAG
UBC3443 AF371472.1	AATCAA-C-CAG
KC-3	ANGCGAGCGCAG
KC-36	AT-CAC-C-ACA
KC-43	AATCAC-C-CAA
Lactobacillus curvatus AJ270951	ATCCAA-C-CAG
Lactobacillus plantarum AF404710	ATTTAA-C-CAG
Lactobacillus brevis AF404709	ATTTAA-C-CAG
Lactobacillus casei AJ507644	ATCCAA-C-CAG
Lactobacillus kefiri AB024300	ATCCAA-C-CAG
KGI-A	ATCCAA-C-CAC
Lactobacillus parakefiri AY026750	ATCCAA-C-CAG
KGI-B	ATCCNA-C-CAG

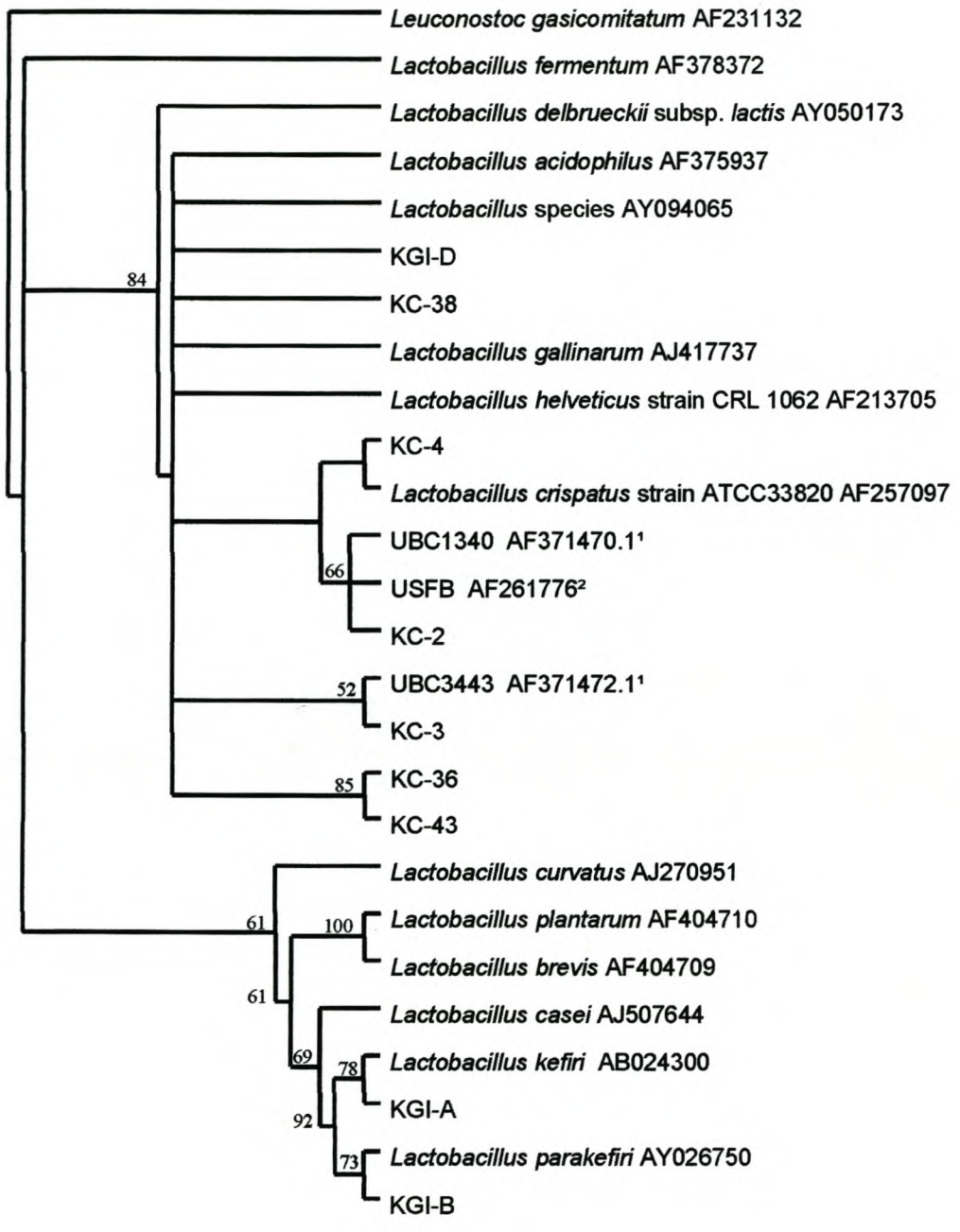

Figure 1. Neighbour-joining tree of partial 16S rRNA sequences (293 bp) of cloned inserts, unidentified isolates from kefiran and lactobacilli commonly isolated from Kepi grains. The numbers on the branches refer to the bootstrap values for a 1 000 repeats (Hillis & Bull, 1993). (UBC¹-unculturable bacterial clone; USFB²-unculturable swine feces bacterium).

Lactobacillus clone 3443 AF371472.1); *Lb. crispatus* (KC-4) (600 bp of the 604 bp sequenced were homologous to *Lb. crispatus* AF257097); and *Lb.* species (KC-38) (homologous to species within the *Lb. acidophilus* group and 467 bp of the 470 sequenced were most homologous to *Lb.* species AY094065).

The cloned lactobacilli KC-36 and KC-43 were found to be homologous and both were homologous to species within the *Lb. acidophilus* group, but were respectively found to be most homologous to *Lb. helveticus* (KC-36) (269 bp of the 275 bp sequenced were homologous to *Lb. helveticus* AF213704) and to an unculturable swine feces bacterium (KC-43) (447 bp of the 457 bp sequenced were homologous to unculturable swine feces bacterium AF261776). These two cloned sequences could however not be conclusively identified to the species level, as the homology (% similarity) of the cloned sequences to the sequences obtained from Genbank were only 97.8%, respectively.

The unidentified isolates from kefiran (Cronjé, 2003) (Table 3) were identified as: *Lb. kefiri* (KGI-A) (517 bp of 522 bp sequenced were homologous to *Lb. kefiri* AB024300); *Lb. parakefiri* (KGI-B) (534 bp of the 538 bp sequenced were homologous to *Lb. parakefiri* AY026750); *Lb. gallinarum* (KGI-D) (homologous to species within the *Lb. acidophilus* group and 509 bp of the 516 bp sequenced were homologous to *Lb. gallinarum* AJ417737); and unculturable *Lactobacillus* clone 1340 (KGI-5) (951 bp of the 958 bp sequenced were homologous to unculturable lactobacillus clone AF371470.1).

The DNA sequences of the cloned PCR fragments, as well as DNA sequences belonging to lactobacilli commonly found in Kepi grains obtained from Genbank were aligned (Table 5) and the phylogenetic data analysed and given in Fig. 1. The sequence data set contained 26 sequences, including the outgroup (*Leuconostoc gasicomitatum* AF231132). After the introduction of gaps, the alignment included 293 nucleotide positions of which 142 characters were constant, 75 were parsimony-uninformative and 76 characters were parsimony-informative. Parsimony analysis of the small subunit alignment yielded thirty-two equally parsimonious trees (TL = 313 steps, CI = 0.706, RI = 0.733, RC = 0.517).

All the KC-isolates, as well as UBC (uncultured bacterial clone) 1340 and 3443, USFB (uncultured swine feces bacterium) and KGI-D were grouped in a clade (bootstrap support value of 84%) that also contained representative isolates of *Lb. delbrueckii* subsp. *lactis, Lb. acidophilus*, *Lb. gallinarum*, *Lb. helveticus*, *Lb.*

crispatus and a *Lb.* species. Three sequences, UBC1340, USFB and KC-2 formed a group with a 66% bootstrap support value, UBC3443 and KC-3 with 52% and isolates KC-36 and KC-43 with a bootstrap support value of 85%. Isolate KGI-A grouped with *Lb. kefiri* (78% bootstrap support) and isolate KGI-B with *Lb. parakefiri* (73% bootstrap support).

It is widely acknowledged that the taxonomy of the genus *Lactobacillus* is still unsatisfactory due the fact that the genus is phenotypically heterogenous (Andrighetto *et al.*, 1998; Corsetti *et al.*, 2001; Giraffa *et al.*, 1998; Kandler & Weiss, 1986; Walter *et al.*, 2000). Physiological and biochemical criteria used for strain identification are often ambiguous because most of the *Lactobacillus* species have very similar nutritional requirements and grow under very similar environmental conditions (Andrighetto *et al.*, 1998; Giraffa *et al.*, 1998). Furthermore, the common dairy species *Lb. delbrueckii*, *Lb. helveticus* and *Lb. acidophilus* belong to the same phylogenetic group (the *Lb. delbrueckii* group) with 16S rRNA percentage homology ranging from 90.8 to 99.3% (Andrighetto *et al.*, 1998; Giraffa *et al.*, 1998).

Based on the literature, the *Lb. acidophilus* species, one of the most commonly isolated Kepi grain microbes, exhibits distinct genomic heterogeneity, encompassing a large number of strains that are difficult to differentiate by physiological and biochemical tests (du Plessis & Dicks, 1995; Kandler & Weiss, 1986). It has also been shown that *Lb. acidophilus* cannot be reliably differentiated from a number of species, including *Lb. crispatus* and *Lb. gallinarum* by any simple phenotypic test (du Plessis & Dicks, 1995; Kandler & Weiss, 1986). Even the lactic dehydrogenase (LDH) electrophoretic profiles (which are often used to differentiate between species) of *Lb. crispatus* and certain strains of *Lb. acidophilus* are identical (du Plessis & Dicks, 1995). It could, therefore, be concluded that the cloned *Lb. crispatus* and *Lb. gallinarum*, both species which have not previously been isolated from Kepi grains using traditional identification techniques, may have been present in Kepi grains, but may have been isolated and identified as *Lb. acidophilus* due to the taxonomic confusion that exists.

From the phylogenetic analysis it is clear that the cloned inserts KC-2, KC-3, KC-4, KC-36, KC-38 and KC-43 are homologous to species within the *Lb. acidophilus* group, and are closely related (du Plessis & Dicks, 1995). The DNA sequence of pure culture isolate KGI-5 (100% homolgous to the DNA sequence of

KC-2) has been shown to be closely related to *Lb. kefiranofaciens* and *Lb. kefirgranum* by numerical clustering, based on phenotypic and biochemical analyses (Cronje, 2003). It could be concluded, that KGI-5 (KC-2) could be either *Lb. kefiranofaciens* or *Lb. kefirgranum*, as neither of the sequences of these two isolates have up to now been deposited in Genbank.

Sequence analyses of the yeasts showed that they are closely related to the genera *Candida* and *Saccharomyces*. The two cloned PCR products obtained from the yeasts (Table 4) were identified as *Candida lambica* (KC-y1) (208 bp of the 210 bp sequenced were homologous to *Candida lambica* SCE437312) and *Saccharomyces cerevisiae* (KC-y18) (267 bp of the 267 bp sequenced were homologous to *Saccharomyces cerevisiae* PFY75726).

Identification of DGGE banding patterns

The cloned inserts, isolates from kefiran and isolates from Kepi grains obtained from the University of Stellenbosch Food Science Culture Collection (USFSCC), Stellenbosch, South-Africa (Table 1), were used to identify the banding patterns in the mass-cultured Kepi grain DNA profile. The DNA from the cloned inserts, isolates from kefiran, isolates from Kepi grains and the mass-cultured Kepi grains was successfully amplified using the *'Eubacterial'* and yeast specific primers, respectively. The *'Eubacterial'* specific primers yielded a 200 bp PCR fragment of part of the 16S rRNA gene, while the yeast specific primers yielded a 250 bp PCR fragment of part of the 26S rRNA gene. The PCR fragments were resolved on a DGGE gel with a denaturing gradient of between 45 and 70% for the Eubacteria, and a denaturing gradient of between 40 and 60% for the yeasts.

Unique DNA banding patterns were obtained for the mass-cultured Kepi grain DNA, while single bands were obtained for the cloned insert DNA, DNA of the isolates from kefiran and DNA of the isolates from Kepi grains. Identification of the Eubacteria and yeast species present in the mass-cultured Kepi grains was possible, as single bands were correlated to bands in the same position in the mass-cultured Kepi grain DGGE profile (Fig. 3) (Cocolin *et al*, 2002; Gurtner *et al.*, 2000; Kawai *et al.*, 2002).

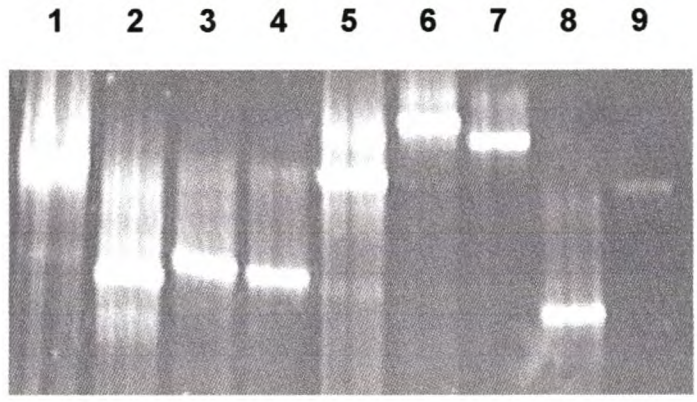

Figure 3. Correlation of single bands to Eubacterial PCR-based DGGE fingerprints using the *'Eubacterial'* specific primers F341 and R534. Lane 1: KC-4 (*Lb. crispatus)*; Lane 2: *Lb. fermentum*; Lane 3: *Leuc. lactis*; Lane 4: *Leuc. lactis* ssp. *lactis*; Lane 5: mass-cultured Kepi grain; Lane 6: *Lb. plantarum;* Lane 7: *Lb. brevis*; Lane 8: *Lb. delbrueckii* ssp. *lactis*; Lane 9: KC-36 (*Lb. helveticus).*

The cloned Eubacteria inserts and cultures that could be visualized as single bands, and that could be correlated to bands in the same position in the mass-cultured Kepi grain profile were then used to construct a DGGE marker for the rapid identification of the *Lactobacillus* species present in Kepi grains. The 200 bp PCR fragments were consequently used to construct the DGGE marker (Fig. 4).

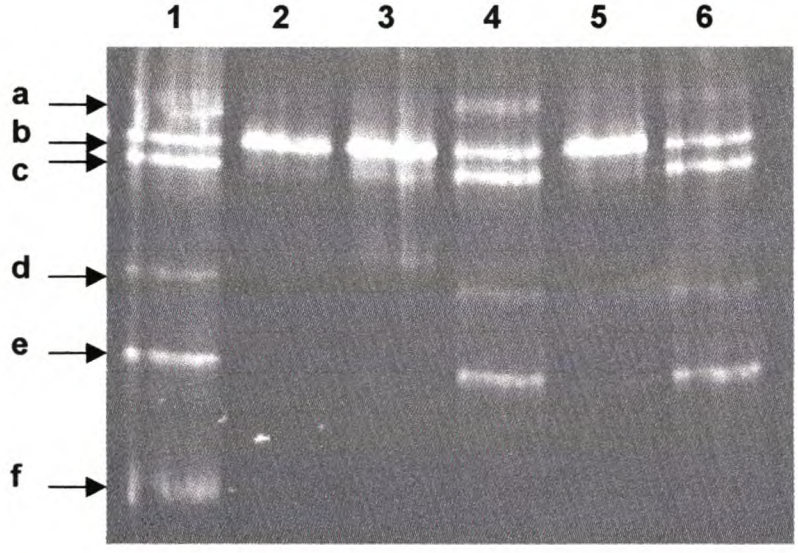

Figure 4. Identification of bands in the PCR-based DGGE fingerprints using the *'Eubacterial'* specific primers F341 and R534. Lane 1: Newly developed DGGE marker; Lane 2, 3 and 5: KC-4 (*Lb. crispatus)*; Lane 4 and 6: mass-cultured Kepi grain.

The bands in the *'Eubacterial'* DGGE profile were identified as *Lb. brevis* (Fig. 4, band a), KC-4 (closely related to *Lb. crispatus*) (Fig. 4, band b), KC-36 (homologous to species within the *Lb. acidophilus* group, and homologous to *Lb. helveticus*) (Fig. 4, band c), *Lb. fermentum* (Fig. 4, band d), *Lb. curvatus* (Fig. 4, band e) and KGI-B (closely related to *Lb. parakefiri*) (Fig. 4, band f). Of all these species, only *Lb. crispatus* (KC-4) has not previously been isolated from Kepi grains by using traditional identification techniques (Kwak *et al.*, 1996; Pintado *et al.*, 1996; Schoeman, 2001).

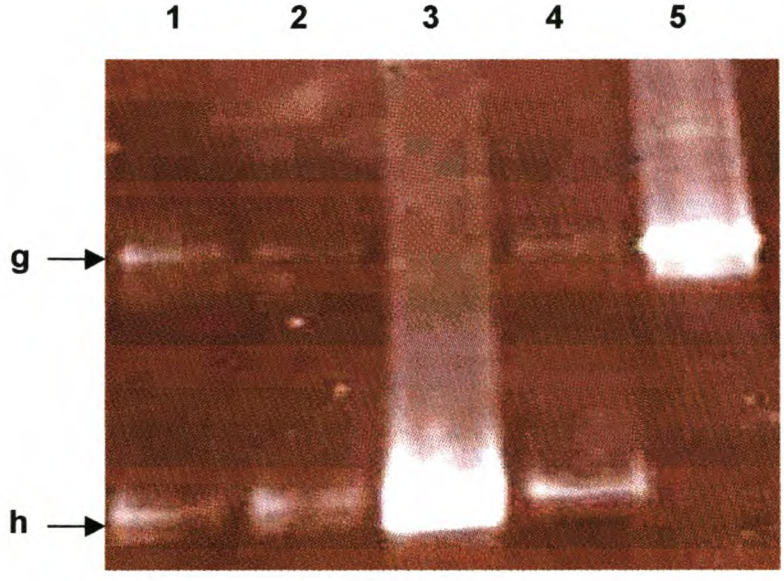

Figure 5. Identification of PCR-based DGGE fingerprints using the yeast specific primers NL1 and LS2. Lane 1, 2 and 4: mass-cultured Kepi grain; Lane 3: KC-y1 (*C. lambica)* and Lane 5: KC-y18 (*S. cerevisiae*).

Based on the DGGE fingerprints of the yeast species present in Kepi grains, the bands in the mass-cultured yeast DGGE fingerprint could also be correlated to single bands in the same position. These bands were then identified as KC-y1 (closely related to *Candida lambica*) (Fig 5, band g) and KC-y18 (closely related to *Saccharomyces cerevisiae*) (Fig 5, band h). Both these yeasts have previously been isolated from Kepi grains (Assadi *et al.*, 2000; Kwak *et al.*, 1996; Pintado *et al.*, 1996).

Conclusion

The complex microbial population in Kepi grains can be identified by PCR-based DGGE analyses. The bands in both the Eubacterial and the yeast DGGE fingerprints were identified to species level. The phylogenetic relatedness of the lactobacilli present in the grains was determined, and the cloned fragments were confirmed to be closely related. Both *Lb. crispatus* and *Lb. gallinarum* have not previously been isolated from Kepi grains, showing that the molecular identification method of PCR-based DGGE analysis is able to identify previously unculturable microbes. No acetic acid bacteria were detected using PCR-based DGGE analysis.

DGGE is a powerful diagnostic tool, and is very successfull in the typification and identification of complex microbial consortia. In the case of mass-cultured Kepi grains, DGGE yields fingerprints of all the Eubacterial and yeast species present in the grains, which makes it possible to identify the microbial composition of the mass-cultured Kepi grains. A DGGE marker was consequently developed for the rapid identification of the lactobacilli present in the mass-cultured Kepi grains. This could be an important tool to accurately identify the different lactobacilli present in the mass-cultured Kepi grains, as it is imperative to know the microbial composition of the mass-cultured Kepi grains before they are commercialized. PCR-based DGGE analysis is also much faster than the classical method of culturing on selective growth media, and relying on physiological and biochemical characteristics.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Kepi is the most famous of the alcoholic fermented milk beverages and is characterized by a high acidity, the presence of carbon dioxide and a low ethanol content (Kuo & Lin, 1999). The nutrient composition of fermented milk is much the same as that of milk, which is an excellent source of vitamins A, B_1 and B_2, calcium, phosphorus, thiamine, protein, fat and lactose. During fermentation, 20 to 30% of the lactose in the milk is hydrolyzed, giving a product that can be tolerated by lactose intolerant individuals (Beukes *et al.*, 2001).

The Kepi beverage is produced from a starter that closely resembles small cauliflower florets. These Kepi grains contain a balance of specific microbes that include species of yeasts, lactic and acetic acid bacteria, and mycelial fungi (Kwak *et al.*, 1996; Pintado *et al.*, 1996). It is, however, difficult to determine the species present in the grains due to the complex composition of the grains, and the heterogeneous distribution of the microbes on the surface of the grains (Kojima *et al.*, 1993; Neve, 1992; Pintado *et al.*, 1996). These factors make it difficult to identify the members of the grain microbial consortium and subsequently to obtain a starter culture with the optimal and constant microbial consortium necessary for the production of a quality and characteristic Kepi beverage. Thus the aim of this study was to typify the complex microbial consortium present in different Kepi grains by using polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) fingerprinting, as well as DNA cloning and sequencing of the 16S ribosomal RNA (rRNA) gene for the identification of the bands present in the DGGE fingerprints.

Mass-cultured Kepi grains, traditionally cultured Kepi grains and Irish Kepi grains were typified with regards to Eubacteria and yeast content using PCR-based DGGE fingerprinting. DNA was extracted from the different Kepi grains, and part of the variable 16S rRNA gene (Eubacteria*)* and 26S rRNA gene (yeasts) were amplified using *'Eubacterial'* and yeast specific primers (Cocolin *et al.*, 2000; Cocolin *et al.*, 2002; Muyzer *et al.*, 1993). The PCR fragments were resolved using DGGE and unique PCR-based DGGE fingerprints were observed for each different grain, with respect to both Eubacterial and yeast content. The

traditionally cultured Kepi grains were found to have the most complex microbial consortium, while the mass-cultured Kepi grains were found to have the least complex microbial population of the three different types of grains. These differences could be caused by substances added during the mass-culturing procedure, and differences between the traditional and mass-culturing process.

The Eubacterial DGGE fingerprints for the three different Kepi grains showed a high level of similarity, as most of the bands observed for each type were present in the same positions in the profiles of the other grains. The Irish Kepi grains contained an Eubacterial species not present in either the mass-cultured or the traditionally cultured Kepi grains, while the mass-cultured and traditionally cultured grains in turn contained an Eubacterial species not present in the Irish Kepi grains. The Irish Kepi grains and traditionally cultured grains also contained an Eubacterial species that was not present in the mass-cultured grains.

The method of mass-cultivation had an impact on the composition of the yeast population present in the Kepi grains, as the mass-cultured Kepi grain DGGE fingerprints of the yeasts were different to those from the traditionally cultured and Irish Kepi grains. The mass-cultured grains included a yeast species that was not present in either the traditionally cultured or Irish Kepi grains. This could be ascribed to the fact that during the mass-culturing process, growth medium is added that may cause environmental variations that subsequently leads to changes in the yeast population. The traditionally cultured Kepi grains also contained a yeast species that was not present in the mass-cultured or the Irish Kepi grains.

In this study the banding patterns of the DGGE fingerprints of both the Eubacteria and yeasts were further correlated to which microbial species gave which band in the DGGE gels. This was done by amplifying and sequencing part of the ribosomal RNA (rRNA) genes from mass-cultured Kepi grains, isolates from kefiran strings (Cronje, 2003), as well as isolates from Kepi grains obtained from the USFSCC (Schoeman, 2001). Six lactobacilli were identified as *Lb. crispatus* (KC-4); three lactobacilli just as *Lb.* species (KC-36, KC-38 and KC-43); and two as uncultured lactobacilli (KC-2 and KC-3). The unidentified isolates from kefiran strings that could not be identified using traditional methods were identified as *Lb. kefiri* (KGI-A), *Lb. parakefiri* (KGI-B), *Lb. gallinarum* (KGI-D) and an unculturable *Lactobacillus* (KGI-5). The bands in the Eubacterial DGGE gel were found to be

representative of the species *Lb. crispatus* (KC-4), *Lb.* species (KC-36), *Lb. parakefiri* (KGI-B), *Lb fermentum, Lb. curvatus* and *Lb. brevis.* Based on the above data a DGGE marker was consequently constructed to facilitate the rapid identification of the Eubacteria present in mass-cultured Kepi grains.

The fact that *Lb. crispatus* has not previously been isolated from Kepi grains could be explained by the fact that it is very difficult to distinguish *Lb. crispatus* and *Lb. acidophilus* with respect to phenotypic and biochemical characteristics (Kandler & Weiss, 1986, du Plessis & Dicks, 1995). It was, therefore, concluded that the cloned *Lb. crispatus* may have been present in Kepi grains, but may have been isolated and identified as *Lb. acidophilus* due to the taxonomic confusion that exists in the *Lactobacillus* genus.

The phylogenetic analysis showed that the six cloned lactobacilli were closely related to *Lactobacillus delbrueckii* subsp. *lactis, Lb. acidophilus*, *Lb. gallinarum*, *Lb. helveticus*, *Lb. crispatus* and a *Lb.* species, and that the lactobacilli are very closely related as they form a distinct clade with a bootstrap support value of 84% (Hillis & Bull, 1993). The DNA sequence of the isolate KGI-5 (KC-2) has also been shown to be closely related to *Lb. kefiranofaciens* and *Lb. kefirgranum* by numerical clustering based on phenotypic and biochemical analyses (Cronje, 2003). It was thus concluded, that KGI-5 (KC-2) could be either *Lb. kefiranofaciens* or *Lb. kefirgranum*, as neither of the sequences of these two isolates has previously been deposited in Genbank.

The two bands in the yeast DGGE gel were identified as *Candida lambica* (KC-y1) and *Saccharomyces cerevisiae* (KC-y18). Both these yeast species have previously been isolated from Kepi grains, of which the yeast *S. cerevisiae* has been reported to be one of the most commonly isolated microbes from Kepi grains (Assadi *et al.*, 2000; Marshall *et al.*, 1984b).

Concluding Remarks

From the data obtained in this study it is clear that the microbial population comprising Kepi grains can be typified and identified using PCR-based DGGE analysis. Kepi grains from different origins, and Kepi grains cultured using different methods can as a result of the new DGGE marker also be compared with respect to which species are present or absent in the grains. The newly developed DGGE marker is also rapid and useful in establishing which species are

present or absent in mass-cultured Kepi grains. This will especially be useful when the mass-cultured Kepi grains are commercialized, as the starter grains cannot be distributed if the exact microbial composition of the Kepi grains are not known.

Kepi grains can also be typified during the different stages of mass-culturing and/or traditional culturing, so as to typify and identify the species present at different periods of the mass-culturing process. Shifts and changes in the microbial community can also be studied using the PCR-based DGGE. Furthermore the typification and identification of undesirable microbes that may eventually cause spoilage, or off-flavours in the Kepi beverage, will also be of value during the commercialization of the Kepi beverage.

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