

**IMPACT OF ENVIRONMENTAL FACTORS ON THE METABOLIC
PROFILES OF KEFIR PRODUCED USING DIFFERENT KEFIR
GRAINS AND SUBSEQUENT ENRICHMENT OF KEFIR
PREPARED WITH MASS CULTURED GRAINS**

by

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DECLARATION

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ABSTRACT

The fermentation of milk has been known for millennia and leads to nutritious and prolonged shelf-life dairy products. In Southern Africa, traditional fermented dairy products have the same value as local staple foods and are consumed as a part of or as a whole meal. However, the retail price and the technology make many commercialised fermented dairy products unaffordable to the majority of the population. There is thus a need for a healthy nutritious low-cost easily prepared fermented dairy product. A product that could be the answer to the above need, is Kefir. The principle advantage is that the Kefir beverage is made from reusable Kefir grains, which unfortunately are not easily available and grow slowly. Kefir grains can only be obtained from pre-existing grains, which presents a problem in the marketing of the grains. A mass culturing technique was developed to produce large masses of grains but preparation of Kefir using these grains results in a product (MG Kefir) lacking in the sensory attributes of Traditional Kefir.

Thus, the overall objective of this research was to determine the impact of environmental factors on the metabolic profiles of Kefir produced using different Kefir grains and this was then followed by the subsequent enrichment of Kefir prepared with mass cultured grains so as to obtain a Kefir beverage that has improved organoleptic qualities.

To determine the impact of environmental factors Traditional and MG Kefir were prepared under controlled and uncontrolled conditions. Traditional Kefir was found to give the best beverage and was thus considered as the control. Under controlled conditions the optimum incubation temperature for the production of Kefir was 22°C as over-acidification was observed at 25°C. The metabolic profiles of both Traditional and MG Kefir showed that both contained acetaldehyde, ethanol, acetone, diacetyl and acetic acid. In addition, the metabolic profiles revealed that an inadequate ratio of diacetyl to acetaldehyde as well as the lack of ethyl acetate was responsible for the flavour defect in MG Kefir.

In order to overcome this defect, citrate and ascorbate (0.015 % w.v⁻¹) were added during Kefir fermentation to enhance the diacetyl and ethyl acetate production. This addition showed a positive impact on diacetyl but not on ethyl acetate production. Improvement of the overall flavour of Kefir was observed as the ratios of diacetyl to acetaldehyde were higher (0.21 – 0.5) in the samples with added citrate and ascorbate than in the samples without (0.12 – 0.17).

The production of ethyl acetate in MG Kefir was enhanced by combining the effects of longer incubation (24 h + 18 h at 22°C), addition of ethanol and acetic acid at 0.79% (m.v⁻¹) and the addition of either *Lactococcus lactis* ssp. *diacetylactis* biovar *diacetylactis* 318 or *Candida kefir* 1283. The best yields were obtained in samples containing *C. kefir* 1283 and only added ethanol (9.22 mg.L⁻¹), indicating that ethanol is an important factor in ethyl acetate production by Kefir starter strains and

suggesting that the absence of ethyl acetate is an indication that the grains do not contain a yeast capable of producing sufficient ethyl acetate. During this investigation, the impact of ethyl acetate on the organoleptic quality of Kefir during storage at refrigerated and room temperatures were also studied. The results indicated that refrigerated Kefir contained up to 40 mg.L⁻¹ of ethyl acetate and was not found defective and thus ethyl acetate was considered a positive contributor to Kefir flavour. This is of particular interest as ethyl acetate is a potent flavour compound at concentrations below 5 mg.L⁻¹.

Improvements of MG Kefir's flavour were successful and will be of value for commercial Kefir production where the main aim is to optimise the flavour of Kefir. However, stabilising the grain microbial consortium was found to be important as it is responsible, over time, for both stable and acceptable Kefir. Acceptability of Traditional, MG and other Kefirs (Candi-Kefir and Lacto-Kefir) prepared with microbially stabilised MG was evaluated by 85 consumers. Results indicated that pH ($r = 0.978$; $p < 0.05$) was a significant driver of liking for flavour, especially for female consumers ($r = 0.982$; $p < 0.05$). In addition, three clusters, each characterised by different liking attributes were identified. Cluster I generally disliked all the products whereas slight acidic Kefir such as Candi-Kefir (7.63) and Lacto-Kefir (7.09) were preferred by Cluster III. Cluster II showed preference to Kefir with moderate acidity and high ethanol content. In that regard, Traditional Kefir obtained the best score (7.50) and MG Kefir the lowest score (4.87). The sensory study is of value as it led to the identification of the drivers of consumers liking by the different types of consumers.

In the course of this project, near infrared reflectance spectroscopy was developed as a rapid method to estimate lactic and acetic acids, which are the organic acids responsible for acidity in Kefir, as well as pH and titratable acidity (TA). The results showed that the calibration models for lactic acid (RPD = 2.57 – 3.16), pH (RPD = 2.90) and TA (RPD = 2.60) were good for screening purposes ($2 < \text{RPD} < 3$); indicating that these models would show if the concentrations of lactic acid, the pH or the TA varied from the normal range.

This study has demonstrated that the flavour of MG Kefir, prepared with enriched grains, was successfully improved and has provided some understanding on the preference liking of Kefir, an unknown fermented dairy product to South African consumers.

UITTREKSEL

Die fermentering van melk is al vir millennia bekend en lei tot voedsame suiwelprodukte met 'n verlengde raklewe. In Suidelike Afrika het tradisioneel gefermenteerde suiwelprodukte dieselfde waarde as plaaslike stapelvoedsels en word dit as 'n maaltyd of as deel van 'n maaltyd geëet. Die kleinhandelsprys en tegnologie van kommersieel gefermenteerde suiwelprodukte maak hierdie produkte egter onbekostigbaar vir die grootste deel van die populasie. Daar is dus 'n behoefte aan 'n gesonde, voedsame, goedkoop, maklik-om-te-berei gefermenteerde suiwelprodukt. 'n Moontlike produk om aan die bogenoemde te voldoen is Kefir. Die hoof voordeel is dat die Kefir drankie van herbruikbare Kefirkorrels gemaak word, maar ongelukkig is hierdie korrels nie vrylik beskikbaar nie, en vermeerder dit stadig. Kefirkorrels kan net van reeds bestaande korrels verkry word wat problematies is vir die bemaking van hierdie korrels. 'n Massakwekingstegniek is ontwikkel vir die produksie van groot hoeveelhede korrels maar die voorbereiding van Kefir met hierdie korrels lei tot 'n produk (MG Kefir) wat sensories minder aanvaarbaar is as tradisionele Kefir.

Die hoofdoel van hierdie navorsing was dus om die invloed van omgewingsfaktore op die metaboliese profiele van Kefir, berei deur gebruik te maak van verskillende Kefirkorrels, te bepaal. Dit is gevolg deur die verryking van Kefir berei van massagekweekte korrels om 'n Kefir drankie met verbeterde organoleptiese kwaliteite te verkry.

Tradisionele en MG Kefir is voorberei onder gekontroleerde en ongekontroleerde toestande om die impak van omgewingsfaktore te bepaal. Die beste drankie is van tradisionele Kefir verkry en is dus beskou as die kontrole. Die optimum temperatuur vir die produksie van Kefir onder gekontroleerde toestande is 22°C aangesien oor-versuring by 25°C waargeneem is. Die metaboliese profiele van beide tradisionele en MG Kefir het gewys dat beide produkte asetaldehyd, etanol, aseton, diasetiel en asynsuur bevat. Die metaboliese profiele het verder gewys dat 'n onvoldoende diasetiel tot asetaldehyd verhouding sowel as 'n tekort aan etielasetaat verantwoordelik was vir 'n geur defek in MG Kefir.

Om hierdie defek te voorkom is sitraat en askorbaat (0.015% m.v⁻¹) tydens Kefir fermentasie bygevoeg om diasetiel en etielasetaat produksie te verhoog. Hierdie byvoeging het 'n positiewe effek gehad op diasetiel produksie, maar nie op die produksie etielasetaat nie. 'n Verbetering in die algehele geur van Kefir is waargeneem aangesien die diasetiel tot asetaldehyd verhoudings hoër (0.21 – 0.5) was in die monsters met bygevoegde sitraat en askorbaat as in die monsters daarsonder (0.12 – 0.17).

Die produksie van etielasetaat in MG Kefir is verhoog deur die effekte van 'n verlengde inkubasie tydperk (24 h + 18 h by 22°C), die byvoeging van etanol en asynsuur teen 0.79% (m.v⁻¹) en die byvoeging van óf *Lactococcus lactis* ssp. *diacetylactis* biovar *diacetylactis* 318 óf *Candida kefyr*

1283 te kombineer. Die beste opbrengs is verkry in monsters wat *C. kefir* 1283 en slegs etanol (9.22 mg.L⁻¹) bevat het. Dit dui daarop dat etanol 'n belangrike faktor is vir etielasetaat produksie in Kefir beginstamme en wys moontlik op die afwesigheid van etielasetaat wat daarop dui dat die korrels nie 'n gis bevat wat bevoeg is om genoegsame hoeveelhede etielasetaat te produseer nie. Tydens hierdie ondersoek is die impak van etielasetaat op die organoleptiese kwaliteit van Kefir gedurende opberging by verkoelde- en kamertemperatuur ook bestudeer. Die resultate het gewys dat verkoelde Kefir tot 40 mg.L⁻¹ etielasetaat bevat het sonder dat dit defektief was. Etielasetaat is dus beskou as 'n positiewe bydraer in terme van Kefir geur. Dit is van besondere belang aangesien etielasetaat 'n sterk geurkomponent teen konsentrasies laer as 5 mg.L⁻¹ is.

Verbeteringe in MG Kefir se geur was suksesvol en sal van waarde wees vir kommersiële Kefir produksie waar die hoofdoel die optimisering van Kefir geur is. Stabilisering van die korrels se mikrobiologiese konsortium is ook belangrik aangesien daar gevind is dat dit oor tyd verantwoordelik is vir stabiele en aanvaarbare Kefir. Die aanvaarbaarheid van tradisioneel, MG en ander Kefirs (Candi-Kefir en Lacto-Kefir), voorberei van mikrobiologies gestabiliseerde MG, is deur 85 verbruikers geëvalueer. Die resultate het aangedui dat pH ($r = 0.978$; $p < 0.05$) 'n belangrike faktor is in die bepaling van verbruikers se voorkeur van geur is, veral by vroulike verbruikers ($r = 0.978$; $p < 0.05$). Drie groepe, elk gekenmerk deur verskillende voorkeur en aanvaarbaarheid eienskappe, is verder geïdentifiseer. Groep I het oor die algemeen van geen van die produkte gehou nie en Groep III het die effense suur Kefirs soos Candi-Kefir (7.63) en Lacto-Kefir (7.09) verkies. Groep II het die Kefir met 'n matige suurheid en hoë etanolinhoud verkies. Tradisionele Kefir het die hoogste telling (7.50) en MG Kefir die laagste telling (4.78) behaal. Die sensoriese studie is van waarde aangesien dit geleidelik het tot die identifisering van die drywers van verbruikersvoorkeure deur die verskillende tipes verbruikers.

Tydens hierdie projek is 'n naby-infrarooi reflektansie spektroskopiese metode ontwikkel vir die vinnige skatting van melk- en asynsuur, die organiseerders wat verantwoordelik is vir die suurheid van Kefir, asook die pH en titreerbare suurheid (TS). Die resultate het getoon dat die kalibrasiemodelle vir melksuur (RPD = 2.57 – 3.16), pH (RPD = 2.90) en TS (RPD = 2.60) voldoende was vir siftingsdoeleindes ($2 < \text{RPD} < 3$). Dit dui daarop dat hierdie modelle sal aandui wanneer die konsentrasie van melksuur, pH of TS afwissel van die normale reeks.

Hierdie studie het gewys dat die geur van MG Kefir, berei van verrykte korrels, suksesvol verbeter is en het ook geleidelik tot insigte in die voorkeur van aanvaarbaarheid van Kefir, 'n onbekende gefermenteerde suiwelprodukt vir Suid-Afrikaanse verbruikers.

Romans 8: 28

“All things work for good for those who are called according to His purpose”

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The language and style used in this dissertation are in concordance 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 I

INTRODUCTION

Fermentation is an ancient process of transformation of raw food compounds into value-added products or foods. Almost every region or country of the world is characterised by a certain type of fermented food (Oyewole, 1997; Jespersen, 2003).

The fermentation of milk has been known for millennia and leads to nutritious and prolonged shelf-life dairy products. In Africa, where refrigeration facilities are often not available in rural and many urban households, consumption of fermented “yoghurt-like” products is very popular. In the case of Southern Africa, traditional fermented dairy products such as Maas or Sethemi have the same value as many local staple foods and are consumed as a part of or as a whole meal (Van Wyk *et al.*, 2002; Kebede *et al.*, 2007). The importance of milk and particularly derivatives such as Maas or yoghurt in the diet, is that they are more complete foods and can be consumed ‘*on the go*’; whereas other products need cooking or refrigeration facilities.

However, the retail price makes many commercialised fermented dairy products unaffordable for the majority of the population, compared to local staple foods (maize meal, bread, cereals and beans) (Schönfeldt *et al.*, 2010). The worldwide financial crisis that started in 2006 has worsened the situation leading to job losses and creating inflation, which led to the increase of the retail prices of the most commonly eaten food (Schönfeldt *et al.*, 2010). A clear illustration is the price of the 2L bottle of full cream fresh milk, which steadily increased from 10.1 ZAR (before June 2006) to 16.1 ZAR by June 2009 (Schönfeldt *et al.*, 2010). Therefore, in this actual unstable economical era, where the prices of basic food commodities unexpectedly increase, there is a need among the South African population, for a healthy nutritious low-cost easily prepared fermented dairy product. This is particularly true since recent figures have shown that there has been an increase in the demand for Maas (3.2%) and yoghurt (8.5%) by South African consumers (Coetzee, 2011). Again, this is a clear indication that the need for products similar to Maas or yoghurt is present in the South African market. Kefir can be the answer to the above problem.

Originally, an ‘*ethnic product*’, Kefir is nowadays a popular drink in the Northern hemisphere, whereas it is generally unknown in Africa, where other types of fermented dairy products (e.g. Maas or Amasi, Sethemi, Mbanik, Rob, Nono, Yoghurt) are popular (Jespersen, 2003; Kebede *et al.*, 2007). Kefir as a fermented dairy product presents numerous advantages. Similarly to Maas or yoghurt, Kefir is made from processed milk but can also be made from raw milk, which is advantageous for herd owners. Kefir is prepared using a starter called Kefir grains, which are a consortium of microorganisms, essentially composed of lactic acid bacteria and yeasts (Witthuhn *et al.*, 2005; Sarkar, 2008). One of the advantages of Kefir grains is that as they grow, the grains can be subdivided and thereby used to ferment other batches of milk. Kefir is also known to have probiotic attributes, due to the presence of probiotic species in the product (Ötles &

Cagindi, 2003). Many commercial probiotic dairy products do not comply with the requirements of a probiotic product (e.g. number of viable cells, absence of the specified probiotic microorganism) (Brink *et al.*, 2005). Indeed, many South African probiotic dairy products have been shown to be mislabelled (Theunissen *et al.*, 2005).

The major problem with Kefir grains is that they grow very slowly (Libduzisz & Piatkiewicz, 1990; Van Wyk *et al.*, 2002). Thus, a process to enhance growth was developed by Schoevers & Britz (2003). This process consisted in culturing Kefir grains in full cream pasteurised milk containing yeast extract and urea. Unfortunately, the mass cultured Kefir grains obtained from this process result in a product (MG Kefir) lacking in the characteristic sensory attributes of Kefir (Latsky, 2004; Prof. T.J. Britz, Food Science Department, Stellenbosch, South Africa, Personal Communication, 2004). Traditional Kefir has a pronounced buttery and subtle fruity flavour, balanced with a blend of other flavour compounds (e.g. acetaldehyde, ethanol and 2-butanone) and a distinctive acidity essentially originating from lactic acid as well as acetic acid to a minor extent (Bakhshandeh *et al.*, 2011). Since metabolites are synthesised by the symbiotic activity of the Kefir grain consortium, the lack of taste in Kefir prepared with mass cultured Kefir grains could only be ascribed to an intrinsic microbial imbalance within the mass cultured Kefir grains or the absence of a significant number of flavour forming microbes (Witthuhn *et al.*, 2005). The lack of taste observed in Kefir prepared with mass cultured Kefir grains could possibly be overcome by enhancing the flavour of the product or by promoting the synthesis of a particular flavour compound. However, knowing which flavour contributing compounds are formed and in what concentrations they can be found is important for assessing the quality, preventing flavour defects and enhancing the flavour profile.

The overall objective of this research is to determine the impact of environmental factors on the metabolic profiles of Kefir produced using different Kefir grains and this will be then followed by the subsequent enrichment and evaluation of Kefir prepared with mass cultured grains so as to obtain a Kefir beverage that has acceptable organoleptic qualities.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

Milk has a high nutritive value, not only for the new-born mammal and the human consumer (Wouters *et al.*, 2002), but also for the microorganisms which find it a suitable medium for growth (Schuttle, 1999). It is a complete food, almost unique as a balanced source for most human dietary needs (Wouters *et al.*, 2002) since it contains both energy supplying nutrients (fat and carbohydrates) and body-building nutrients (proteins and minerals). Milk also contains adequate amounts of almost all vitamins necessary for the proper functioning of the biochemical processes that are carried out in human bodies and are essential for life (Schuttle, 1999).

The observation that milk turns sour when kept at room temperature was exploited to minimise spoilage and then led to the production of countless forms of fermented products (Wouters *et al.*, 2002). Fermentation is still an ingenious way of preserving food when facilities such as refrigeration are not accessible (Tamime, 2002). Fermentations, especially those produced by lactic acid bacteria (LAB), will contribute to furnish in a single step, value-added products (Tamime, 2002; Prajapati & Nair, 2003) with:

- an extended shelf-life due to the acidity from lactic acid and other acids such as acetic and formic acid;
- enhanced sensory attributes (formation of flavour compounds);
- improved nutritional value; and
- antimicrobial properties due to the presence of antimicrobial agents.

The worldwide growing interest in fermented milks is based mainly on the understanding by consumers that regular consumption of fermented milks has health benefits caused by the ingestion of live probiotics (Prajapati & Nair, 2003; Van de Water, 2004). Moreover, lactose intolerant people find in fermented dairy products, a way to maintain their nutritional requirements without being upset by lactose indigestion symptoms (Gilliland, 1986).

Kefir is an example of a fermented product which embraces many benefits including those mentioned above. It is especially adapted to low-income households because:

- Kefir can be kept at room temperature for hours without undergoing huge variations in flavour and taste (Van Wyk *et al.*, 2002);
- Kefir is a safer product than traditional dairy beverages such as Maas made from raw milk (Van Wyk *et al.*, 2002);
- no special skills are needed to prepare Kefir; and
- Kefir provides a well-balanced and economical package of nutrients (Table 1) (Ötles & Cagindi, 2003; Sarkar, 2007).

Table 1. The nutritional composition of Kefir (Renner & Renz-Schaven, 1986; Hallé *et al.*, 1994; Kevicius & Sarkinas, 2004; Irigoyen *et al.*, 2005).

Components	Per 100g	Components	Per 100g
Energy	234.9 kJ	<i>Mineral content (mg)</i>	
Fat (%)	3.50	Calcium	120-
Protein (%)	3.30	Phosphor	100
Lactose (%)	2.90 – 4.00	Magnesium	1200
Water (%)	87.5	Potassium	150
		Sodium	100
Ethyl alcohol (mg)	900.00	Chloride	50
Lactic acid (mg)	800 – 1000		
Cholesterol (mg)	13.0	<i>Trace elements (mg)</i>	
		Iron	0.050
<i>Essential amino acids (mg)</i>		Copper	0.012
Tryptophan	50 – 70	Molybdenum	0.005
Phenylalanine + tyrosine	350	Manganese	0.005
Leucine	340	Zinc	0.360
Isoleucine	210		
Threonine	170	<i>Aromatic compounds</i>	
Methionine + cysteine	120	Acetaldehyde	NA
Lysine	270 – 376	Diacetyl	NA
Valine	220	Acetoin	NA
<i>Vitamins (mg)</i>			
A	0.06		
Carotene	0.02		
B ₁	0.04		
B ₂	0.17		
B ₆	0.05		
B ₁₂	0.50		
Niacin	0.09		
C	1.00		
D	0.08		
E	0.11		

NA = Data not available

B. KEFIR PRODUCTION

Origin of Kefir

The origin of Kefir is considered to be the northern slopes of Caucasian China (Koroleva, 1988a). But in fact, nobody really knows how and where Kefir grains first appeared. A well known legend states that the Prophet Mohammed gave Kefir grains to the Orthodox people living in the Caucasian Mountains in Eastern Europe. He also taught them how to use the grains and made them promise that they would keep the existence of the Kefir grains secret, otherwise the grains would lose their strength and healing power (Koroleva, 1988a). Their existence was kept secret for a long time and it was only in 1908 that "Dairy Moscow" owned by a man called Blandov started to produce Kefir (Koroleva, 1988a). Immigration of the Eastern peoples also contributed to popularising Kefir across the world (Garrote *et al.*, 2001). According to Roginski (1988), a second legend stated that Kefir grains originated from the containers used in the manufacture of Kefir. Originally, Kefir was prepared in bags made from animals' hides (Duitschaever, 1989). It is well known that during the ongoing fermentation whitish colonies resembling boiled grains were formed and recovered from the container walls and were subsequently called Kefir grains.

Preparation of Kefir

The traditional, home-made and industrial methods employed in the manufacture of Kefir are discussed below and summarised as flow-diagrams in Fig. 1.

Traditional and home-made Kefir

In ancient times, people prepared Kefir with raw milk in sacks made with animal hides. They then exposed the sacks to the sunlight, enhancing the fermentation process. In contrast, during the night, the sacks were hung near the door so that anyone who entered the room could mix the bag content (Koroleva, 1988a). As the Kefir was consumed, more milk was added.

Home-made Kefir is now prepared by adding Kefir grains ($2 - 10\% \text{ m.v}^{-1}$) to fresh pasteurised or UHT milk and incubating at room temperature for 24 h (Saloff-Coste, 1996). If the milk is "home pasteurised", it is allowed to cool to between 20° and 25°C before adding the Kefir grains (Saloff-Coste, 1996). At the end of the fermentation the grains are removed using a sieve and re-used as inoculum. Kefir beverage can also be incubated at a lower temperature which will optimise yeast activity resulting in ethanol and carbon dioxide (CO_2) production (Koroleva, 1988a) or it may be kept either in a fridge or at room temperature (if no refrigeration is available) for long and short term preservation, respectively.

Industrial processes

The "Russian method" is a two step process which consists of preparing the "mother culture" (Starter I) by adding 2 to 3% (m.v^{-1}) Kefir grains to fresh pasteurised milk (Fig. 1). At the end of the

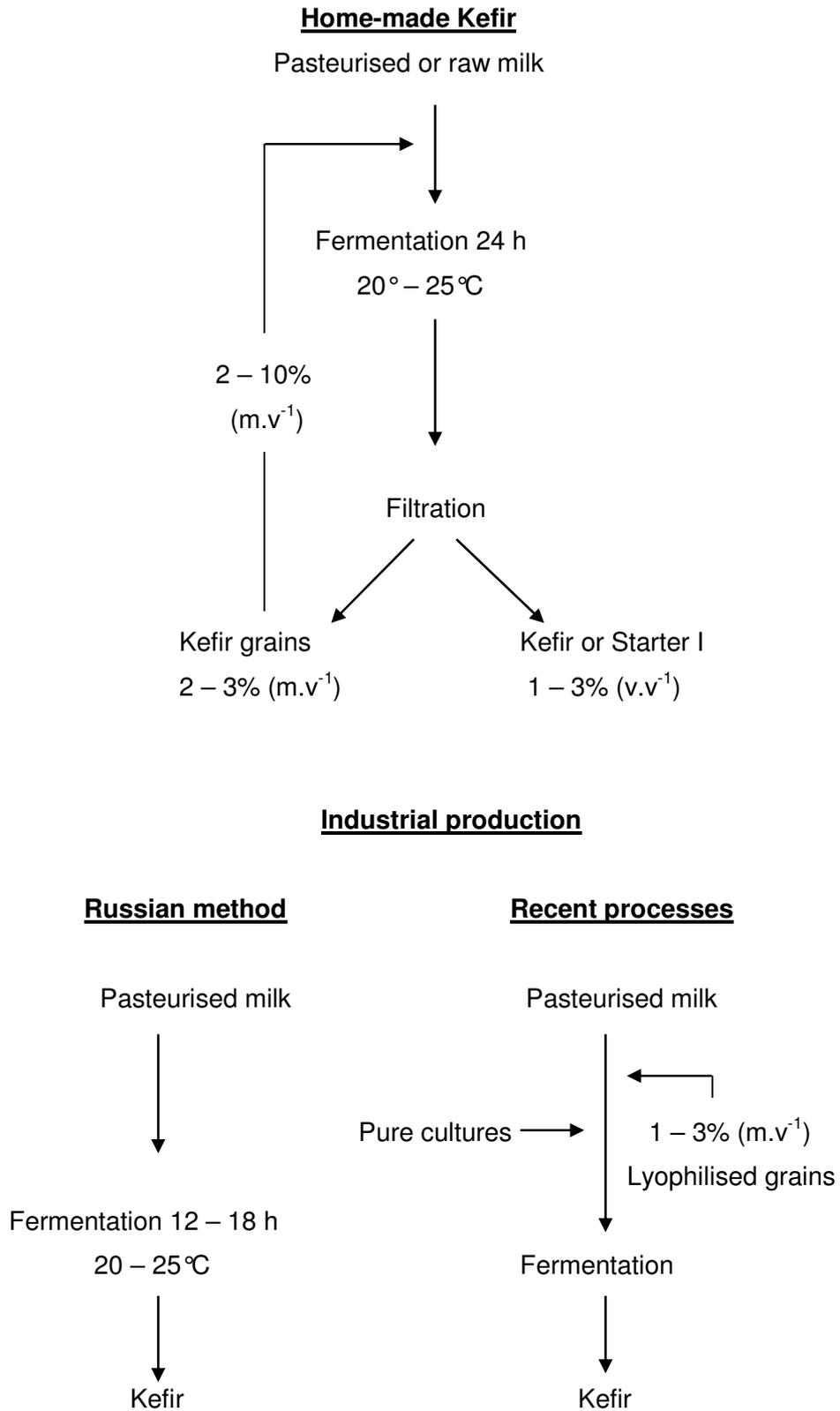


Figure 1. Kefir manufacture (Saloff-Coste, 1996; Ötles & Cagindi, 2003).

fermentation (24 h) the grains are removed by filtration and the beverage obtained is added (1 – 3% v.v⁻¹) to milk which is then fermented for 12 to 18 h (Roginski, 1988; Hallé *et al.*, 1994). The negative aspects of the “Russian method” are the inconsistency of the end-product due to changes in starter activity, the use of two steps that increase the chance of contamination and the blowing of the final containers after bottling due to the production of CO₂ by the microbial population (Saloff-Coste, 1996; Wszolek *et al.*, 2006).

In other processes, lyophilised grains (1 – 3% m.v⁻¹) which are directly inoculated into the milk, are used (Saloff-Coste, 1996). This method leads to a more consistent product. Some researchers have attempted to prepare Kefir using pure cultures representative of Kefir grains’ microbial population. Pure cultures were used as inoculum, either all together or one after the other (Duitschaever *et al.*, 1987; Assadi *et al.*, 2000; Beshkova *et al.*, 2002). Duitschaever *et al.* (1987) formulated a mixed culture containing a yoghurt starter (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*), buttermilk starter (*Streptococcus lactis* and *Leuconostoc*), *Lactobacillus acidophilus* and a non-lactose fermenting yeast (*Saccharomyces cerevisiae*) in proportions of 3%, 1%, 5% and 1% (v.v⁻¹), respectively. Beshkova *et al.* (2002) did not use the same starters but rather a yoghurt culture (*Lactobacillus bulgaricus* HP1, *Streptococcus thermophilus* T15), *Lactobacillus helveticus* MP12, *Lactococcus lactis* C15 and *Saccharomyces cerevisiae* A13 in proportions of 2%, 2%, 1% and 0.5% (v.v⁻¹), respectively. They concluded that traditional Kefir was better than Kefir prepared from pure cultures (Assadi *et al.*, 2000; Beshkova *et al.*, 2002).

C. KEFIR GRAINS AS A NATURAL STARTER

“Starter cultures are preparations containing high numbers of viable microorganisms which may be added to a substrate to bring about desirable changes” (Holzapfel, 1997). Thus, Kefir grains may be considered a natural starter for use in the preparation of Kefir. In appearance the grains look like small clumps of cauliflower, gelatinous to touch and whitish to yellowish in colour. Even though scarce, Kefir grains may also look like flat sheets that can be big enough to cover one’s hand (Gates & Schatz, 1996). They vary in size, ranging from a few millimetres to 4 cm in diameter (Kuo & Lin, 1999; Garrote *et al.*, 2001). Their growth by sub-culturing is associated with the synthesis of a polysaccharide called kefiran and biomass increase (Garrote *et al.*, 2001). Kefir grains grow slowly: 5 to 7% daily mass increase (Libduzisz & Piatkiewicz, 1990). But their growth rate can be accelerated (500% in 20 days) by culturing them in full cream pasteurised milk containing yeast extract and urea (Schoevers & Britz, 2003). The most effective increase was obtained in media containing fructose or glucose and sucrose with ammonium sulphate, potassium phosphate and yeast extract (Harta *et al.*, 2004).

Besides fermenting milk, Kefir grains can be used for other purposes such as making bread. It was found that sourdough bread made by using Kefir grains instead of baker’s yeast had

a firmer texture, a lower acidity and retained its freshness for longer than that made with baker's yeast (Plessas *et al.*, 2005; Plessas *et al.*, 2011). In 2008, Plessas *et al.* evaluated the potential of immobilised Kefir on orange pulp as yeast for bread making and found that bread has an improved aromatic profile compared to baker's yeast bread.

When Kefir grains are not being used, they can be frozen, freeze-dried or dried as a means of preservation (Garrote *et al.*, 1997; Oberman & Libudsisz, 1998; Witthuhn *et al.*, 2005).

D. MICROBIAL COMPOSITION

Microbial composition of Kefir grains

Kefir grains are microbially-rich, cauliflower-like structures normally consisting of three groups of microorganisms living as part of a symbiotic association. These include lactic acid bacteria (LAB), yeasts and acetic acid bacteria (AAB) (Garrote *et al.*, 2001; Lin *et al.*, 1999; Loretan *et al.*, 2003; Chen *et al.*, 2008; Magalhães *et al.*, 2011a). The data in Tables 2, 3 and 4 show the different microbes isolated from Kefir grains and from the Kefir beverage.

Lactic acid bacteria

They include lactobacilli, lactococci (Garrote *et al.*, 2001; Witthuhn *et al.*, 2005; Chen *et al.*, 2008; Pedrozo Miguel *et al.*, 2010; Magalhães *et al.*, 2011a) and leuconostocs (Lin *et al.*, 1999; Garrote *et al.*, 2001; Jianzhong *et al.*, 2009). The major LAB population may be either homofermentative or heterofermentative (Kuo & Lin, 1999; Lin *et al.*, 1999) comprising 65 – 80% of the total microbial population (Wouters *et al.*, 2002). In a study by Angulo *et al.* (1993), the heterofermentative lactobacilli counts were found to be higher than the homofermentative counts (74.5 vs. 25.7%). The same distribution pattern was reported by Garrote *et al.* (2001) where 20 isolates of heterofermentative lactobacilli were found versus 16 homofermentative isolates.

Acetic acid bacteria

They were reported to represent only 20% of the total microbial population and are usually present in lower counts ($<10^5$ cfu.g⁻¹) (Garrote *et al.*, 2001; Angulo *et al.*, 1993; Pedrozo Miguel *et al.*, 2010). However, counts as high as 10^8 cfu.g⁻¹ were found by Abraham & De Antoni (1999). In other studies AAB were not found (Pintado *et al.*, 1996; Witthuhn *et al.*, 2005) and sometimes they were just considered to be contaminants (Angulo *et al.*, 1993). According to Rea *et al.* (1996) AAB may stimulate the growth of other organisms since they are vitamin B₁₂ producers (Zourari & Anifantakis, 1988). Koroleva (1988a) reported that the consistency of Kefir can be improved by using a starter containing AAB, but not at a level higher than 10^6 cfu.g⁻¹. This fact implies that the presence of AAB may be important for a good Kefir consistency and therefore a quality product.

Table 2. Lactobacilli present in Kefir beverage and Kefir grains.

Microorganisms	Reference
<i>Lactobacilli acidophilus</i>	Kwak <i>et al.</i> , 1996; Santos <i>et al.</i> , 2003; Kesmen & Kacmaz, 2011
<i>Lactobacillus brevis</i>	Angulo <i>et al.</i> , 1993; Simova <i>et al.</i> , 2002; Witthuhn <i>et al.</i> , 2005
<i>Lactobacillus bulgaricus</i>	Frengova <i>et al.</i> , 2002
<i>Lactobacillus casei</i>	
<i>ssp. pseudopantarum</i>	Simova <i>et al.</i> , 2002
<i>ssp. rhamnosus</i>	Angulo <i>et al.</i> , 1993; Marshall, 1993
<i>ssp. tolerans</i>	Angulo <i>et al.</i> , 1993
<i>Lactobacillus delbrueckii</i>	
<i>ssp. bulgaricus</i>	Kwak <i>et al.</i> , 1996; Simova <i>et al.</i> , 2002
<i>ssp. lactis</i>	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
<i>Lactobacillus fermentum</i>	Angulo <i>et al.</i> , 1993; Witthuhn <i>et al.</i> , 2005
<i>Lactobacillus gasseri</i>	Angulo <i>et al.</i> , 1993
<i>Lactobacillus helveticus</i>	Lin <i>et al.</i> , 1999; Frengova <i>et al.</i> , 2002; Jianzhong <i>et al.</i> , 2009
<i>Lactobacillus kefir</i> ^(*)	Pintado <i>et al.</i> , 1996; Takisawa <i>et al.</i> , 1998; Garrote <i>et al.</i> , 2001; Chen <i>et al.</i> , 2009; Jianzhong <i>et al.</i> , 2009; Pedrozo Miguel <i>et al.</i> , 2010; Magalhães <i>et al.</i> , 2011
<i>Lactobacillus kefiranofaciens</i>	Chen <i>et al.</i> , 2008; Jianzhong <i>et al.</i> , 2009; Kesmen & Kacmaz, 2011
<i>Lactobacillus kefiranogrum</i>	Takisawa <i>et al.</i> , 1994; Garrote <i>et al.</i> , 2001
<i>Lactobacillus lactis ssp. lactis</i>	Kwak <i>et al.</i> , 1996; Özer & Özer, 2000
<i>Lactobacillus parabuchneri</i>	Magalhães <i>et al.</i> , 2011a
<i>Lactobacillus paracasei</i>	Magalhães <i>et al.</i> , 2011a
<i>ssp. paracasei</i>	Litopoulou-Tzanetaki & Tzanetakis, 2000; Pedrozo Miguel <i>et al.</i> , 2010
<i>ssp. tolerans</i>	Özer & Özer, 2000
<i>Lactobacillus parakefir</i>	Özer & Özer, 2000; Angulo <i>et al.</i> , 1993
<i>Lactobacillus plantarum</i>	Garrote <i>et al.</i> , 2001; Pedrozo Miguel <i>et al.</i> , 2010
<i>Lactobacillus rhamnosus</i>	Litopoulou-Tzanetaki & Tzanetakis, 2000
<i>Lactobacillus satsumensis</i>	Pedrozo Miguel <i>et al.</i> , 2010
<i>Lactobacillus uvarum</i>	Pedrozo Miguel <i>et al.</i> , 2010
<i>Lactobacillus viridescens</i>	Angulo <i>et al.</i> , 1993

^(*)also known as *Lactobacillus kefiri*

Table 3. Yeasts and mycelial fungi present in Kefir beverage and Kefir grains.

Microorganisms	Reference
Yeasts	
<i>Candida famata</i>	Bergmann <i>et al.</i> , 2010
<i>Candida holmii</i>	Witthuhn <i>et al.</i> , 2004; Latorre-García <i>et al.</i> , 2007
<i>Candida inconspicua</i>	Simova <i>et al.</i> , 2002; Bergmann <i>et al.</i> , 2010
<i>Candida kefyr</i>	Angulo <i>et al.</i> , 1993; Witthuhn <i>et al.</i> , 2004
<i>Candida krusei</i>	Latorre-García <i>et al.</i> , 2007
<i>Candida maris</i>	Simova <i>et al.</i> , 2002
<i>Candida sake</i>	Latorre-García <i>et al.</i> , 2007
<i>Cryptococcus humicolus</i>	Witthuhn <i>et al.</i> , 2005
<i>Debaromyces hansenii</i>	Loretan <i>et al.</i> , 2003
<i>Kazachstania aerobia</i>	Magalhães <i>et al.</i> , 2011a
<i>Kazachstania exigua</i>	Jianzhong <i>et al.</i> , 2009
<i>Kazachstania unispora</i>	Jianzhong <i>et al.</i> , 2009
<i>Kluyveromyces fragilis</i>	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
<i>Kluyveromyces lactis</i>	Angulo <i>et al.</i> , 1993; Kwak <i>et al.</i> , 1996; Loretan <i>et al.</i> , 2003; Magalhães <i>et al.</i> , 2011a
<i>Kluyveromyces marxianus</i>	Lin <i>et al.</i> , 1999; Loretan <i>et al.</i> , 2003; Jianzhong <i>et al.</i> , 2009
<i>Kluyveromyces marxianus var lactis</i>	Simova <i>et al.</i> , 2002
<i>Lachancea meyersii</i>	Magalhães <i>et al.</i> , 2011a
<i>Pichia</i> sp.	Tamime <i>et al.</i> , 1999
<i>Pichia fermentans</i>	Lin <i>et al.</i> , 1999; Chen <i>et al.</i> , 2009
<i>Saccharomyces cerevisiae</i>	Garrote <i>et al.</i> , 1997; Loretan <i>et al.</i> , 2003; Latorre-García <i>et al.</i> , 2007; Jianzhong <i>et al.</i> , 2009; Bergmann <i>et al.</i> , 2010; Magalhães <i>et al.</i> , 2011a
<i>Saccharomyces humaticus</i>	Latorre-García <i>et al.</i> , 2007
<i>Saccharomyces lipolytic</i>	Garrote <i>et al.</i> , 1997; Lin <i>et al.</i> , 1999
<i>Saccharomyces turicensis</i>	Chen <i>et al.</i> , 2009
<i>Saccharomyces unisporus</i>	Angulo <i>et al.</i> , 1993; Latorre-García <i>et al.</i> , 2007
<i>Torulaspora delbrueckii</i>	Libudzisz & Piatkiewicz, 1990; Angulo <i>et al.</i> , 1993
<i>Zygosaccharomyces</i> sp.	Witthuhn <i>et al.</i> , 2005
<i>Zygosaccharomyces florentinus</i>	Özer & Özer, 2000
<i>Zygosaccharomyces rouxii</i>	Loretan <i>et al.</i> , 2003
<i>Trichosporon coremiiforme</i>	Latorre-García <i>et al.</i> , 2007
Mycelial fungi	
<i>Geotrichum</i> sp.	Garrote <i>et al.</i> , 1997; Tamime <i>et al.</i> , 1999
<i>Geotrichum candidum</i>	Roginski, 1988; Witthuhn <i>et al.</i> , 2005

Table 4. Lactococci, leuconostocs, streptococci and acetic acid bacteria present in Kefir beverage and Kefir grains.

Microorganisms	Reference
Lactococci	
<i>Lactococcus filant</i>	Özer & Özer, 2000
<i>Lactococcus lactis</i>	Koroleva, 1988a; Frengova <i>et al.</i> , 2002
<i>Lactococcus lactis</i>	
<i>ssp. cremoris</i>	Kurmann <i>et al.</i> , 1992; Özer & Özer, 2000
<i>ssp. lactis</i>	Angulo <i>et al.</i> , 1993; Simova <i>et al.</i> , 2003; Witthuhn <i>et al.</i> , 2005
<i>ssp. lactis</i> var. <i>diacetylactis</i>	Özer & Özer, 2000; Garrote <i>et al.</i> , 2001
Leuconostocs	
<i>Leuconostoc dextranicum</i>	Özer & Özer, 2000
<i>Leuconostoc kefir</i>	Özer & Özer, 2000
<i>Leuconostoc mesenteroides</i>	Chen <i>et al.</i> , 2008; Jianzhong <i>et al.</i> , 2009; Kesmen & Kacmaz, 2011
<i>ssp. cremoris</i>	Libudzisz & Piatkiewicz, 1990; Witthuhn <i>et al.</i> , 2005
<i>ssp. dextranicum</i>	Kwak <i>et al.</i> , 1996; Litopoulou-Tzanetaki & Tzanetakis, 2000
<i>ssp. mesenteroides</i>	Kwak <i>et al.</i> , 1996; Litopoulou-Tzanetaki & Tzanetakis, 2000
Streptococci	
<i>Streptococcus durans</i>	Libudzisz & Piatkiewicz, 1990
<i>Streptococcus salivarium</i>	
<i>ssp. thermophilus</i>	Angulo <i>et al.</i> , 1993; Frengova <i>et al.</i> , 2002; Kesmen & Kacmaz, 2011
Acetic acid bacteria	
<i>Acetobacter</i> sp.	Angulo <i>et al.</i> , 1993; Garrote <i>et al.</i> , 2001
<i>Acetobacter aceti</i>	Kurmann <i>et al.</i> , 1992; Marshall, 1993; Tamime <i>et al.</i> , 1999
<i>Acetobacter lovaniensis</i>	Magalhães <i>et al.</i> , 2011a
<i>Acetobacter rasens</i>	Marshall, 1993; Tamime <i>et al.</i> , 1999
<i>Acetobacter syzgjii</i>	Pedrozo Miguel <i>et al.</i> , 2010

Yeasts

The yeasts present in Kefir grains have been reported to be either lactose fermenting and/or non-lactose fermenting (Simova *et al.*, 2002; Loretan *et al.*, 2003; Latorre-Garcia *et al.*, 2007). Their number is usually lower than that of the LAB, and specifically around $10^4 - 10^5$ cfu.g⁻¹ (Abraham & De Antoni, 1999; Garrote *et al.*, 2001; Latorre-Garcia *et al.*, 2007) but in some grains higher yeast counts than LAB counts have been reported (50% vs. 31.2%) (Angulo *et al.*, 1993; Zajšek & Goršek, 2010).

The overall microbial composition of Kefir grains is complex and is known to vary from region to region. The environment (cultivation, preservation and storage conditions) is the principal factor leading to the microbial diversity of Kefir grains (Zourari & Anifantakis, 1988; Stepaniak & Fetlínski, 2002; Latorre-García *et al.*, 2007).

Microbial composition of the Kefir beverage

Kefir beverage owes its microbial composition to the presence of Kefir grains. Once in milk, Kefir grain microorganisms are released and continue to multiply (Kroger, 1993) by using the available nutrients in the milk, and especially lactose that serves as the carbon and energy source. It is therefore expected that both the Kefir grain and the Kefir beverage should have a very similar composition. Even though the microbial profile of Kefir grains and Kefir beverage is very similar, it is not advised to use Kefir beverage as inoculum to make a new batch of Kefir since the grains are essential to obtain the traditional Kefir (Marshall, 1984; Simova *et al.*, 2002). In the same way, it is preferable to use Kefir grains as a starter rather than a mixture of pure cultures (Assadi *et al.*, 2000). According to Marshall (1984), the integrity of the grains is necessary to have the effervescent character and the typical yeasty flavour of Kefir associated with a creamy texture (Simova *et al.*, 2002).

Lactic acid bacteria

The LAB population of the beverage has been reported to be higher than the yeast population (Wzsolek *et al.*, 2001; Witthuhn *et al.*, 2005; Ertekin & Güzel-Seydim, 2010), especially the lactobacilli population ($10^8 - 10^9$ cfu.mL⁻¹). In contrast, other researchers (Rea *et al.*, 1996; Beshkova *et al.*, 2002) found a higher population of lactococci (10^9 cfu.mL⁻¹) and a lower population of lactobacilli (10^6 cfu.mL⁻¹). Leuconostocs were the second major group of microorganisms isolated from an Irish Kefir beverage with a count of 10^8 cfu.mL⁻¹ (Rea *et al.*, 1996). According to Robinson (1995), leuconostocs naturally grow poorly in milk and are usually found in association with lactococci.

Acetic acid bacteria

They are also found in the Kefir beverage at levels between $10^4 - 10^6$ cfu.mL⁻¹ (Rea *et al.*, 1996; Loretan *et al.*, 2003; Irigoyen *et al.*, 2005; Magalhães *et al.*, 2011a). Acetic acid bacteria are not always present in Kefir beverage and sometimes considered as contaminants (Angulo *et al.*, 1993;

Tamime *et al.*, 1999).

Yeasts

They have been reported to either be lactose or non-lactose fermenting yeasts at levels of 10^4 – 10^5 cfu.mL⁻¹ (Farnworth & Mainville, 2003; Loretan *et al.*, 2003; Irigoyen *et al.*, 2005; Jianzhong *et al.*, 2009; Pedrozo Miguel *et al.*, 2010). Yeasts are generally present in Kefir beverage (Kwak *et al.*, 1996; Simova *et al.*, 2002; Zajšek & Goršek, 2010).

E. KEFIR FLAVOUR

Kefir is described as slightly acidic, mildly alcoholic with an effervescent sensation (Duitschaever, 1989) associated with a buttery aroma (Marshall, 1993). A good Kefir foams and fizzes like a beer (Kosikowski & Mistry, 1997). Duitschaever (1989) described the flavour of a typical Kefir as yeasty, with a sharp acid taste and a prickling sensation due to CO₂. According to Koroleva (1988b), a good quality Kefir beverage contains an average 10^9 streptococci, 10^7 – 10^8 thermophilic lactobacilli and 10^4 – 10^5 cfu.mL⁻¹ AAB. The pH ranges between 4.0 and 4.4 with a lactic acid content ranging from 0.5 to 1.5% (m.v⁻¹) (Duitschaever *et al.*, 1987; Steinkraus, 1996). The ethanol content varies between 0.01 and 2.5% (m.v⁻¹) (Koroleva, 1988b; Kuo & Lin, 1999; Beshkova *et al.*, 2002) and normally depends on the age of Kefir and on the region of production (Kosikowski & Mistry, 1997) while the CO₂ content is between 0.08 and 0.2% (m.v⁻¹) (Kurmman *et al.*, 1992; Muir *et al.*, 1999). The data summarised in Tables 5, 6 and 7 show the microbiological and chemical characteristics of some LAB involved in dairy fermentations. The ratio and type of flavour compounds produced by these microorganisms differ according to species and/or strains present. This variation in the composition of the LAB can greatly affect the final product quality (Maurellio *et al.*, 2001). In cultured dairy products, it is recommended that a balanced flavour must prevail and a desirable full-flavour is achieved at diacetyl: acetaldehyde ratios of 3:1 to 5:1, whereas a green flavour or apple like defect is noticeable when the ratio drops below 3:1, and a harsh diacetyl flavour is present when the ratio exceeds 5:1 (Sandine *et al.*, 1972).

The flavour compounds responsible for the typical Kefir aroma flavour can be divided in two groups: major and minor end-products (secondary metabolites). The former group is only composed of lactic acid and the latter of flavour compounds produced especially during the stationary growth phase (Belin *et al.*, 1992). Carbonyl compounds (acetaldehyde, ethanol, diacetyl, acetoin, 2-butanone and ethyl acetate), volatile organic acids (formic, acetic, propionic, butyric) and non-volatile acids (lactic, pyruvic, oxalic and succinic) are secondary metabolites and classified as flavour-forming compounds (Fernandez-Garcia & Mc Gregor, 1994; Tamime & Robinson, 1999).

It is interesting to note that only carbonyl compounds have a decisive impact on the final flavour and aroma of Kefir (Imhof *et al.*, 1995) whereas the impact of the others is negligible

Table 5. Characteristics of LAB involved in dairy fermentations. Trace compounds are given in italics (Marshall, 1982; Saloff-Coste, 1994; Litopoulou-Tzanetaki & Tzanetakis, 2000; McSweeney & Sousa, 2000).

Genus	Morphology	Optimum temperature	Species	Major end-products	Secondary end-products
<i>Streptococcus</i>	coccus	40° – 44°C	<i>S. thermophilus</i>	L(+) lactic acid	Acetaldehyde, Acetone, Acetoin, Diacetyl, Ethanol
<i>Lactobacillus</i>	rod	40° – 44°C	<i>Lb. delbrueckii</i> <i>ssp. bulgaricus</i>	D(-) lactic acid	Acetaldehyde, Acetone, Acetoin, Diacetyl, Ethanol
		40° – 44°C	<i>Lb. helveticus</i>	DL lactic acid	Acetaldehyde, Acetic acid, Diacetyl, Ethanol
		40° – 44°C	<i>Lb. delbrueckii</i> <i>ssp. lactis</i>	D(-) lactic acid	Acetaldehyde, Acetone, Diacetyl, Ethanol
		40° – 44°C	<i>Lb. acidophilus</i>	DL-lactic acid	Acetaldehyde, <i>Ethanol</i>
		25° – 30°C	<i>Lb. casei</i> <i>ssp. casei</i>	L(+) lactic acid	Acetic acid, Ethanol
		25° – 30°C	<i>Lb. kefir</i>	DL-lactic acid	Acetic acid, Acetaldehyde, Ethanol, CO ₂
<i>Lactococcus</i>	coccus	25° – 30°C	<i>Lc. lactis</i> <i>ssp. lactis</i>	L(+) lactic acid	Acetaldehyde, Acetone, Diacetyl, Ethanol
		25° – 30°C	<i>Lc. lactis</i> <i>ssp. cremoris</i>	L(+) lactic acid	Acetaldehyde, Acetone, Diacetyl, Ethanol
		25° – 30°C	<i>Lc. lactis</i> <i>ssp. diacetylactis</i>	L(+) lactic acid, Acetaldehyde, Diacetyl, Acetoin, CO ₂	Acetone, Ethanol
<i>Leuconostoc</i>	oval	25° – 30°C	<i>Ln. mesenteroides</i> <i>ssp. cremoris</i>	D(-) lactic acid, Acetoin, Acetic acid, Diacetyl, CO ₂	Ethanol
		25° – 30°C	<i>Ln. mesenteroides</i> <i>ssp. dextranicum</i>	D(-) lactic acid, Acetoin, Acetic acid, Diacetyl, CO ₂	Ethanol
		25° – 30°C	<i>Ln. lactis</i>	D(-) lactic acid, Acetoin, Acetic acid, Diacetyl, CO ₂	Ethanol
<i>Bifidobacterium</i>	rod or bifid	35° – 38°C	<i>B. breve</i>	L(+) lactic acid	Formic acid, Succinic acid, Acetaldehyde, Acetone, Acetoin, Diacetyl, Ethanol
			<i>B. bifidum</i>	L(+) lactic acid	Formic acid, Succinic acid, Acetaldehyde, Acetone, Acetoin, Diacetyl, Ethanol
			<i>B. longum</i>	L(+) lactic acid	Formic acid, Succinic acid, Acetaldehyde, Acetone, Acetoin, Diacetyl, Ethanol
			<i>B. infantis</i>	L(+) lactic acid	Formic acid, Succinic acid, Acetaldehyde, Acetone, Acetoin, Diacetyl, Ethanol
<i>Pediococcus</i>	coccus	25° – 30°C	<i>P. acidilactici</i>	DL lactic acid	<i>Acetoin, Diacetyl</i>

Table 6. General characteristics and major metabolites of mesophilic LAB used in dairy fermentations (Hosono & Surono, 2003).

Characteristic	<i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i>	<i>Leuconostoc mesenteroides</i> ssp. <i>dextranicum</i>
Morphology	Cocci, pairs, short/long chains	Cocci, pairs, chains
Catalase	–	–
Growth temperature (°C)		
Optimum	20 – 25	20 – 25
Minimum	4 – 10	4 – 10
Maximum	37	37
Incubation temperature (°C)	22	22
Heat tolerance (60°C for 30 min)	–	–
Type of fermentation	heterofermentative	heterofermentative
Lactic acid isomers (%)	D(-) 0.1 – 0.2	D(-) 0.1 – 0.2
Acetic acid (%)	0.2 – 0.4	0.2 – 0.4
CO ₂ production	±	±
Citrate fermentation	+	+
Flavour/aroma formation	+++	+++
Alcohol production	±	±
Proteolytic activity	±	±
Lipolytic activity	±	±
Salt tolerance (% max)	6.5	6.5

Table 7. General characteristics and major metabolites of mesophilic LAB used in dairy fermentations (Hosono & Surono, 2003).

Characteristic	<i>Lactococcus lactis</i> ssp. <i>lactis</i>	<i>Lactococcus</i> <i>lactis</i> spp. <i>cremoris</i>	<i>Lactococcus lactis</i> ssp. <i>lactis</i> biovar <i>diacetylactis</i>
Morphology	Cocci, pairs, short chains	Cocci, pairs, short/long chains	Cocci, pairs, short chains
Catalase	–	–	–
Growth temperature (°C)			
Optimum	28 – 31	22	28
Minimum	8 – 10	8 – 10	8 – 10
Maximum	40	37 – 39	40
Incubation temperature (°C)	21 – 30	22 – 30	22 – 28
Heat tolerance (30min at 60 °C)	±	±	–
Type of fermentation	homofermentative	homofermentative	homofermentative
Lactic acid isomers (%)	L(+) 0.5 – 0.7	L(+) 0.5 – 0.7	L(+) 0.5 – 0.7
Acetic acid (%)	–	–	–
CO ₂ production	–	–	+
Citrate fermentation	–	–	+
Flavour/aroma formation	+	+	+++
Alcohol production	±	±	±
Proteolytic activity	+	+	+
Lipolytic activity	±	±	±
Salt tolerance (% max)	4.0 – 6.5	4.0	4.0 – 6.5

because they are present in only very low concentrations.

Lactic acid

Lactic acid is a non-volatile, odourless compound, responsible for the characteristic acidity of fermented dairy products. The total lactic acid content of Kefir varies from 0.80 to 1.15% (m.v⁻¹) (Kosikowski & Mistry, 1997) and originates from the degradation of lactose by the homofermentative and heterofermentative LAB present in Kefir grains. In Fig. 2A the metabolism of lactose by homofermentative bacteria is shown diagrammatically. In this case, lactose is translocated into the cytoplasm by a specific carrier known as the phosphoenolpyruvate-dependant phosphotransferase system (PEP-PTS). This translocation is coupled to the phosphorylation of lactose which leads to the formation of lactose-6-phosphate (Lactose-6-P). This compound is then hydrolysed by phospho- β -galactosidase (P- β -galactosidase) into D-glucose and D-galactose-phosphate (D-galactose-P). D-glucose is converted to fructose 1,6 di-phosphate (fructose 1,6-DP) by a series of phosphorylations and isomerisations. Then, fructose 1,6-DP is split to dihydroxyacetone phosphate (DHAP) and 3-glyceraldehyde-phosphate (3GP) by the enzyme aldolase. Both compounds are further converted to pyruvate which is reduced to lactic acid by lactate dehydrogenase (LDH) (Axelsson, 1998; Tamime & Robinson, 1999). The pathway by which D-glucose is converted to lactic acid is known as the Embden-Meyerhoff-Parnas pathway (EMP) or glycolysis or homofermentative pathway. D-galactose-P through the Tagatose pathway is transformed to tagatose 1,6 di-phosphate which is also split to DHAP and 3GP which enter the glycolytic pathway (Tamime & Robinson, 1999).

The heterofermentative pathway is shown in Fig. 2B. In this pathway, lactose is transported into the cytoplasm by lactose permease. Once in the cell, lactose is cleaved into D-glucose and D-galactose by β -galactosidase. They are respectively catabolised through 6-phosphogluconate-phosphoketolase pathway (6PG/PK) and the Leloir pathway (Litopoulou-Tzanetaki & Tzanetakis, 2000). D-glucose is phosphorylated to glucose-6P which undergoes dehydrogenations and decarboxylation steps leading to ribulose-5P.

Epimerisation of ribulose-5P yields to xylulose 5-phosphate which is then cleaved into 3GP and acetylphosphate (acetyl-P) by the enzyme phosphoketolase. Glyceraldehyde-3-phosphate enters the glycolytic pathway resulting in lactic acid formation whereas acetyl-P is reduced to ethanol via acetyl CoA and acetaldehyde (Johnson & Steele, 1997; Axelsson, 1998).

The galactose moiety is phosphorylated to glucose-1-P and then isomerised into glucose-6-P which enters the 6PG/PK pathway (Gottschalk, 1986; Axelsson, 1998; Adam & Moss, 2006).

The homofermentation produces only two moles of lactic acid and two ATPs per mole glucose consumed whereas the heterofermentation produces one mole each of lactic acid, ethanol, CO₂ and 1 ATP per glucose. In presence of oxygen, NAD⁺ can be regenerated by NADH oxidases and peroxidases, leaving acetyl-P available for conversion to acetic acid (Axelsson, 1998; Adam & Moss, 2006). The LAB responsible for lactic acid synthesis are either homofermentative or heterofermentative (Table 5). The former group are better acid producers than the latter (Rea *et*

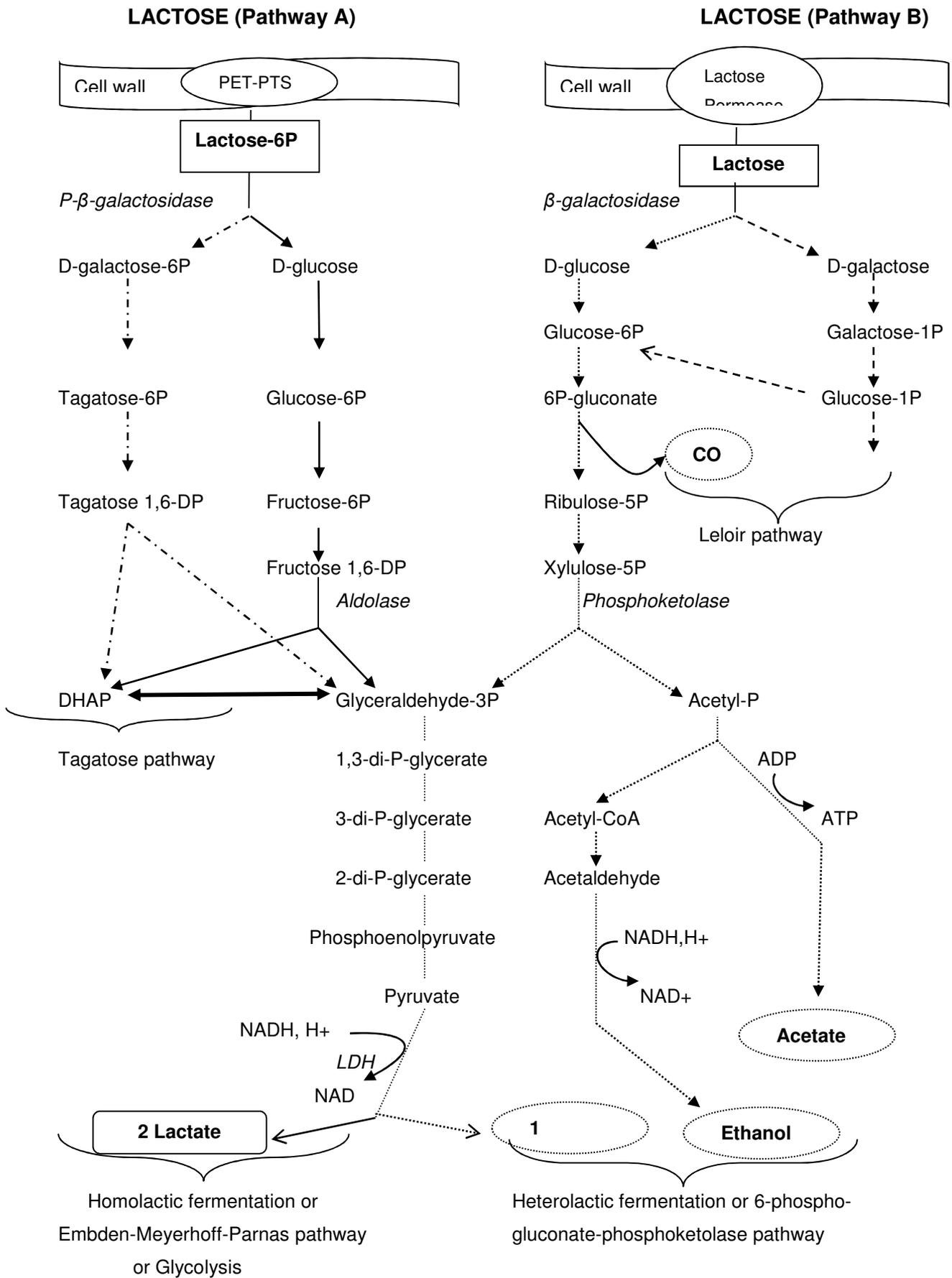


Figure 2. Lactose metabolism (LDH: Lactate dehydrogenase) (Cogan & Jordan, 1994; Axelsson, 1998; Voet & Voet, 2004; Adam & Moss, 2006).

al., 1996). The LAB belonging to the genus *Lactococcus* are homofermentative. Species of this genus are generally used in dairy fermentations for their acidification capacity, lowering the pH to about 4.5 (Hassan & Frank, 2001). *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* belong to this genus and are the principal species used as dairy starter cultures (Ray, 2001). Lactobacilli (group I or obligate homofermentative) are another group with a homofermentative pathway. They exclusively ferment hexose sugars and disaccharides (lactose and sucrose) to lactic acid by the Embden-Meyerhoff-Parnas pathway (EMP). *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lb. delbrueckii* ssp. *lactis*, *Lb. helveticus* and *Lb. acidophilus* belong to this group but *Lb. acidophilus* is mostly added into dairy foods for its probiotic benefits (Robinson, 1995).

Acetaldehyde

Acetaldehyde is considered to be the compound responsible for the characteristic aroma of yoghurt (Gilliland, 1986; Ott *et al.*, 1997) as it is responsible for the “fresh-fruity” note (Ott *et al.*, 2002). In some cultured products such as butter, buttermilk and cheese, acetaldehyde is considered as an off-flavour because it imparts “a green taste” (Sandine *et al.*, 1972). This flavour defect is present when overgrowth of lactococci takes place (Lindsay, 1967) and can be prevented by adding 25 – 50% of leuconostocs to the starter (Keenan *et al.*, 1966), which convert acetaldehyde into ethanol (Johnson & Steele, 1997).

Acetaldehyde is one of the principal aroma compounds found in Kefir with concentrations ranging from 0.5 to 10 mg.L⁻¹ in Kefir made from Kefir grains (Güzel-Seydim *et al.*, 2000; Wszolek *et al.*, 2001; Beshkova *et al.*, 2003). It was reported to be higher (13.8 – 18.3 mg.L⁻¹) in Kefir made from pure cultures resulting in a product with a strong “yoghurt” flavour (Beshkova *et al.*, 2003; Aglilara *et al.*, 2009). These variations in acetaldehyde concentrations may be due to the strains involved and their activity levels (Tamime & Robinson, 1999; Güzel-Seydim *et al.*, 2000). Indeed, it has been demonstrated that the ability to produce acetaldehyde as well as other flavours is strongly strain dependant (Ott *et al.*, 2000; Østlie *et al.*, 2003; Yüksekdağ *et al.*, 2004). For example, *Lb. bulgaricus* HP is a better acetaldehyde producer than *S. thermophilus* T15, *Lc. lactis* C15 and *Lb. helveticus* MP12 (Beshkova *et al.*, 2003). It is interesting to note that acetaldehyde was not among the 32 aromas found in pure cultures of *Lb. helveticus* (Imhof *et al.*, 1995).

Biosynthesis of acetaldehyde occurs through glucose and amino acids catabolism and to a lesser extent through DNA catabolism. The metabolic routes to acetaldehyde are illustrated in Fig. 3.

Glucose and amino acids catabolism

Ott *et al.* (2000) using ¹³C-labelled glucose, L-threonine and pyruvate demonstrated that glucose was the main precursor of acetaldehyde. Similarly, Wilkins *et al.* (1986) while using ¹³C-labelled threonine reported that only 2% acetaldehyde was found to be labelled. However, when the growth

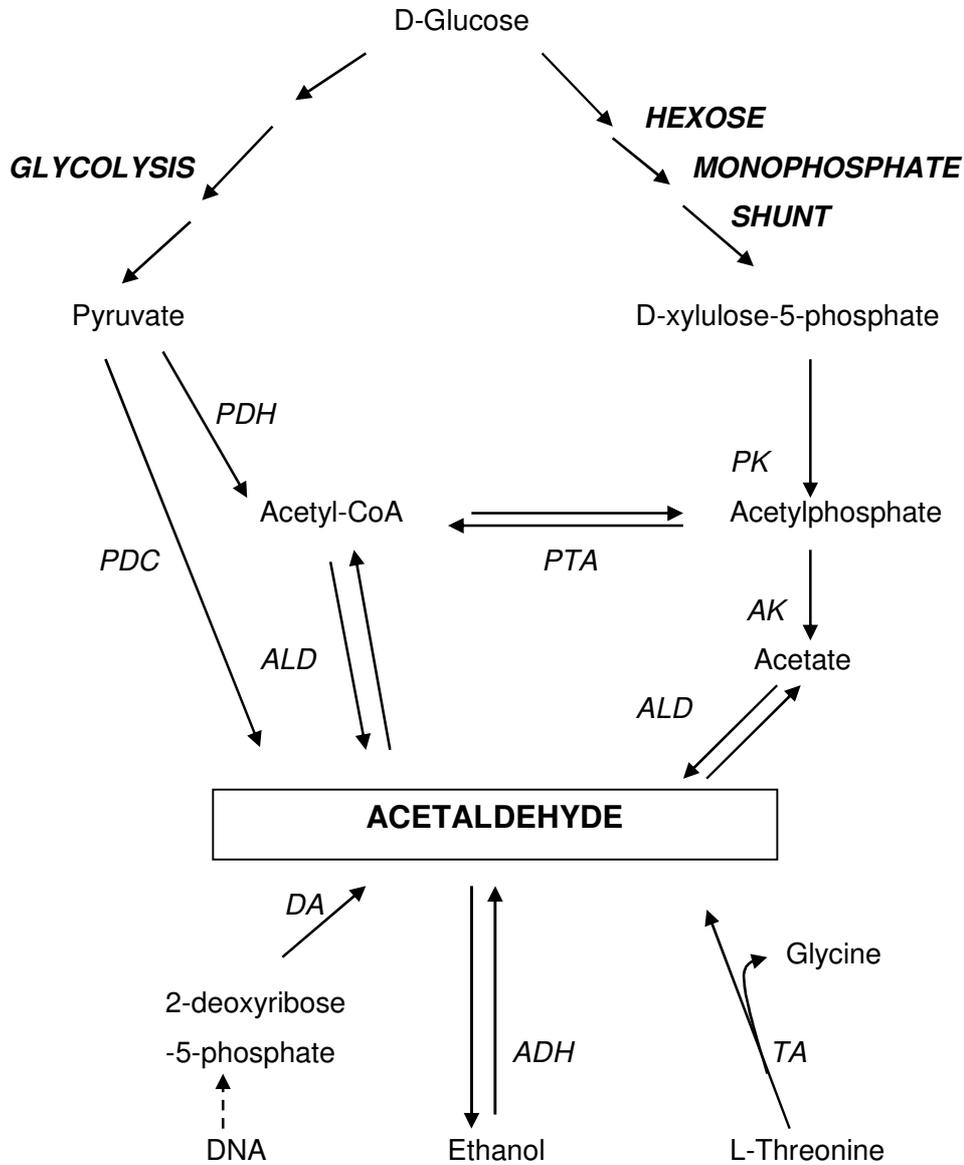


Figure 3. Routes to acetaldehyde (PDH = Pyruvate dehydrogenase, PDC = Pyruvate decarboxylase, PTA = Phosphotransacetylase, AK = Acetate kinase, TA = Threonine aldolase, ADH = Alcohol dehydrogenase, PK = Phosphoketolase, DA = 2-deoxyribose aldolase, ALD = Aldehyde dehydrogenase) (Ott *et al.*, 2002; Chaves *et al.*, 2002).

medium (milk) was supplemented with L-threonine, an increase of acetaldehyde production was found. They concluded that L-threonine also contributed to the formation of acetaldehyde but to a lesser extent (Lees & Jago, 1976; Wilkins *et al.*, 1986).

The main producers of acetaldehyde are lactococci (former N-Streptococci) but lactobacilli such as *Lb. bulgaricus* are also capable of producing acetaldehyde by the degradation of threonine via L-threonine aldolase (Lees & Jago 1976; Raya *et al.*, 1986).

In yeasts, acetaldehyde is produced through the action of pyruvate decarboxylase and threonine aldolase (Pronck *et al.*, 1996; Monschau *et al.*, 1997).

DNA catabolism

During the exponential growth phase of microbes, the frequency of DNA catabolism is low. Since acetaldehyde synthesis is directly linked to the microorganism's activity and growth, production of acetaldehyde by this mechanism is low and unlikely to occur (Ott *et al.*, 2000). In this section of acetaldehyde formation pathway, 2-deoxyribose-5-P is the precursor of acetaldehyde through the action of deoxyribose aldolase. This enzyme has been identified in some strains of *S. thermophilus* and *Lb. bulgaricus* (Lees & Jago, 1976; Raya *et al.*, 1986).

Facultative heterofermentative (Group II) and obligate heterofermentative lactobacilli (Group III) produce acetaldehyde as secondary end-products. Species belonging to these groups are *Lb. casei* ssp. *casei* and *Lb. kefir* (Hassan & Frank, 2001). *Lactococcus* species are also involved in acetaldehyde synthesis; especially *Lc. lactis* ssp. *diacetylactis* that produces it as a major compound along with lactic acid, acetoin and diacetyl.

Bifidobacteria are also able to produce acetaldehyde but they are essentially incorporated in dairy products for health-promoting attributes. Additionally, they are always used alongside other cultures since they grow poorly in milk (Robinson, 1995; Hassan & Frank, 2001).

Diacetyl

Diacetyl is also a desirable constituent of many dairy products (Cogan, 1985; Belin *et al.*, 1992) which at very low concentrations up to 5 mg.L⁻¹ is responsible for the buttery aroma of milk products (Hugenholtz, 1993; Oberman & Libudzisz, 1998). In contrast, diacetyl is seen as undesirable in the brewery and wine industries as it causes off-flavours (Collins, 1972; Belin *et al.*, 1992).

Diacetyl is considered to be an important aroma compound of Kefir (Wszolek *et al.*, 2001; Beshkova *et al.*, 2003). It has been found at different concentrations (0.30 mg.L⁻¹ to 1.85 mg.L⁻¹) in both Kefir manufactured from grains and products manufactured with pure cultures (Beshkova *et al.*, 2003; Cais-Sokolińska *et al.*, 2008; Aghlara *et al.*, 2009). It has also been reported that no diacetyl could be identified in Kefir made from grains in a study by Güzel-Seydim *et al.* (2000). Another

example to illustrate the variations in diacetyl content was the exceptionally high concentration of diacetyl (253 mg.L^{-1}) found by Liu *et al.* (2002).

Diacetyl is normally produced via the citrate metabolic pathway as illustrated in Fig. 4. However, the monitoring of citrate utilisation during Kefir fermentation has revealed that citrate decreased “insignificantly” from $1\,450 \text{ mg.L}^{-1}$ to $1\,230 \text{ mg.L}^{-1}$ in Kefir made from pure cultures and only to $1\,330 \text{ mg.L}^{-1}$ in Kefir made from grains Beshkova *et al.* (2003). Other authors have also noticed this “insignificant” decrease (Rea *et al.*, 1996; Güzel-Seydim *et al.*, 2000). For these reasons, it must be remembered that diacetyl cannot only be a product of citrate metabolism (Ramos *et al.*, 1994) but also of the co- metabolism of both glucose and milk citrate (Fig. 4) (McSweeney & Sousa, 2000; Østlie *et al.*, 2003).

Citrate, as shown in Fig. 4, enters the cell by a specific carrier known as citrate permease which is active below pH 5.5 (Collins, 1972; García-Quintáns *et al.*, 1998). Once inside the cell, the citrate is cleaved by citrate lyase to yield oxaloacetate (OA) and acetic acid. Then OA is decarboxylated by oxaloacetate decarboxylase into CO_2 and pyruvate. This reaction is very important during the production of semi-hard cheese because the production of CO_2 results in eye formation (Hugenholtz, 1993). Thereafter, two molecules of pyruvate are condensed to form α -acetolactate (α -AL) by the action of α -acetolactate synthase (Hugenholtz, 1993). Finally, the chemical oxidative decarboxylation of α -AL leads to diacetyl (Bassit *et al.*, 1995; Boumerdassi *et al.*, 1997).

The products of citrate metabolism are different in citrate positive (Cit+) *Lactococci* ssp. and *Leuconostoc* ssp. (Tables 5, 6 and 7): the former produce diacetyl along with other C4 compounds (Robinson, 1995; Hugenholtz *et al.*, 2000) but the latter produce D-lactate and acetate under all cultivation conditions (Hugenholtz & Starrenburg, 1992; Hugenholtz, 1993; McSweeney & Sousa, 2000). Only under extreme conditions, like a low pH and in the absence of sugar, can some citrate be converted to diacetyl, acetoin and 2,3 butanediol (Hugenholtz, 1993). Obligate homofermentative lactobacilli and bifidobacteria may also produce diacetyl as secondary end-products.

One aspect that must be remembered is that Cit+ LAB may lose the ability to ferment citrate because citrate permease gene (Cit P) is located on a plasmid (Kempfer & McKay, 1979; García-Quintáns *et al.*, 1998). The loss of this plasmid would result in the absence of diacetyl in the fermented milks.

Acetoin and 2,3 Butanediol

Acetoin has been reported to be present in good quality Kefir made from grains at a concentration of 9 mg.L^{-1} (Güzel-Seydim *et al.*, 2000). In contrast, it has also been reported absent in a study done by Beshkova *et al.* (2003). In Kefir made with pure cultures, up to 70 mg.L^{-1} of acetoin were found by Aghlara *et al.* (2009).

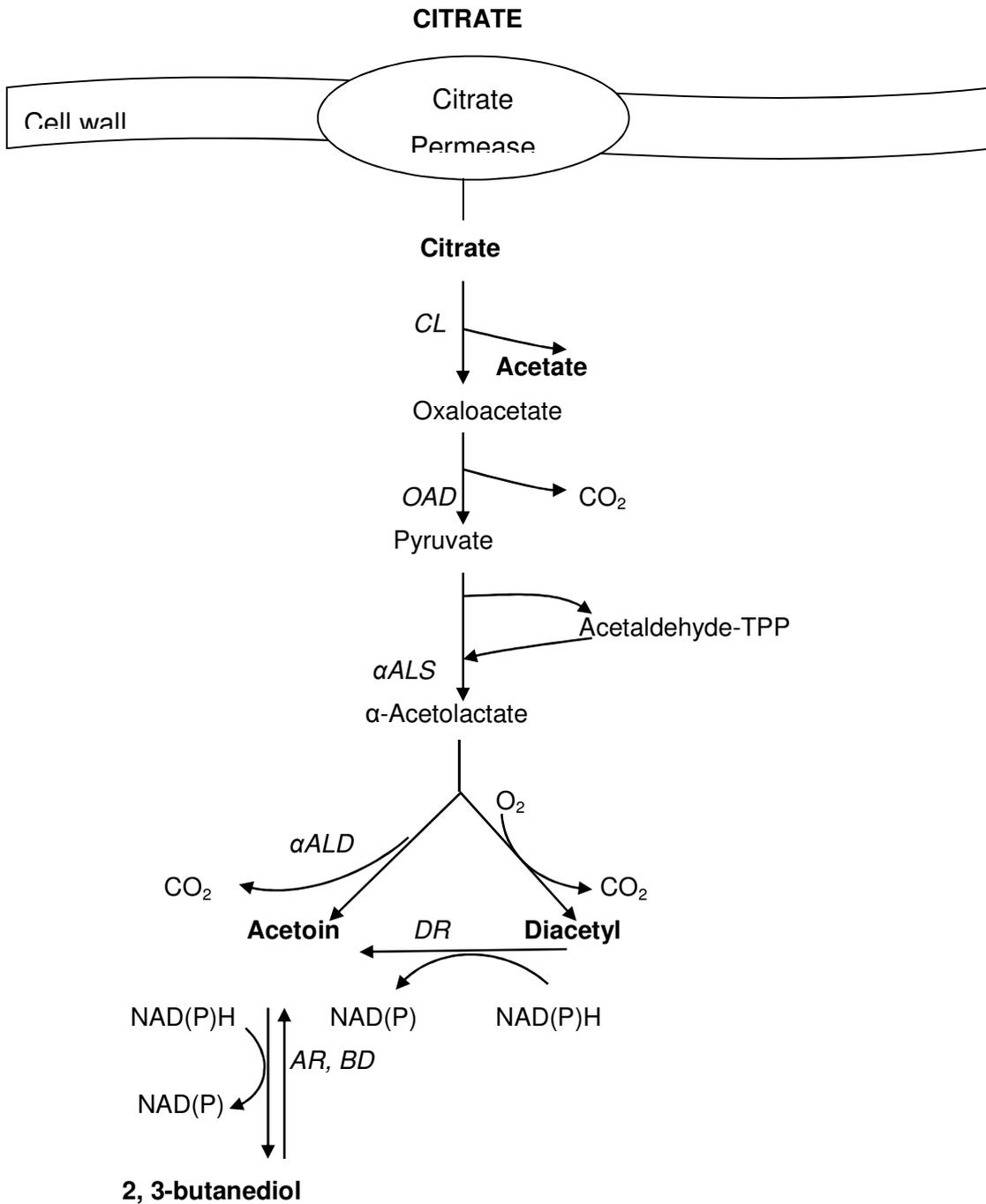


Figure 4. Citrate metabolism in citrate-utilising lactic acid bacteria (AR = Acetoin reductase, BD = Butanediol dehydrogenase, DR = Diacetyl reductase, αALD = α-Acetolactate dehydrogenase, αALS = α-Acetolactate syntase, CL = Citrate lyase, OAD = Oxaloacetate decarboxylase) (Cogan, 1981; Belin *et al.*, 1992; Hugenholtz, 1993; Boumerdassi *et al.*, 1997; McSweeney & Sousa, 2000).

Acetoin is formed through citrate metabolism (Fig. 4) by the decarboxylation of α -AL via acetolactate decarboxylase (Goupry *et al.*, 2000) or by the reduction of diacetyl by diacetyl reductase (Østlie *et al.*, 2003). Acetoin can be excreted or reduced to 2,3 butanediol through the action of acetoin reductase or butanediol dehydrogenase (Hugenholtz, 1993). Both acetoin and 2,3 butanediol are synthesised by citrate positive lactococci and leuconostocs (Rea *et al.*, 1996). At concentrations encountered in cultured products acetoin and 2,3 butanediol are usually flavourless and odourless (Lindsay, 1967; Cogan, 1985) so they may under certain circumstances be of little flavour value.

Acetone

Acetone is a normal constituent of milk and cheese (Vedemuthu *et al.*, 1966; Urbach, 1993) and it was found in Kefir prepared from Kefir and pure cultures at different concentrations (0.6 – 4.91 mg.L⁻¹) (Liu *et al.*, 2002; Beshkova *et al.*, 2003; Aghlara *et al.*, 2009). Acetone plays only a minor role in Kefir's organoleptic characteristics (Blanc, 1984) and it is believed that concentrations of acetone below 1 mg.L⁻¹ are unlikely to have a great effect on flavour (Harvey, 1960).

Acetone originates from citrate and lactose metabolisms and its production appears to be strain related (Harvey, 1960). Some lactobacilli strains such as *Lb. bulgaricus*, *Lb. helveticus* MP12 (Blanc, 1984; Beshkova *et al.*, 2003) as well as Streptococci cultures such as "*S. lactis*", "*S. cremoris*" and "*S. diacetylactis*" are able to synthesise it in small amounts (Harvey, 1960; Bills & Day, 1966; Keenan *et al.*, 1966). However, because heat treated milk contains 0.3 to 0.8 mg.L⁻¹ of acetone (Harvey, 1960; Bills & Day, 1966), Bassette & Claydon (1965) stripped the volatile constituents from heat treated milk media prior to inoculation with cultures of "*S. lactis*" and "*S. diacetylactis*" and found no acetone in the milk inoculated with these species. In contrast, Harvey (1960) showed that the difference in acetone content of milk (control) and cultures ("*S. lactis*" and "*S. cremoris*") was so large that there was no doubt that acetone was produced as a result of the metabolic activities of these microorganisms.

2-Butanone

2-butanone was detected in Kefir made with pure cultures (0.04 – 0.30 mg.L⁻¹) and in Kefir made with Kefir grains (0.06 mg.L⁻¹) (Beshkova *et al.*, 2003; Aghlara *et al.*, 2009). 2-Butanone is thought to play only a minor role in Kefir flavour (Blanc, 1984) whereas in cheddar cheese it imparts a very desirable flavour (Keen *et al.*, 1974). 2-Butanone is synthesized by specific lactobacilli strains such as *Lb. helveticus* MP12 (Beshkova *et al.*, 2003) and *Lb. plantarum* (Keen *et al.*, 1974) and formed after the dehydration of 2,3 butanediol followed by a rearrangement (Fig. 5) (Keen *et al.*, 1974).

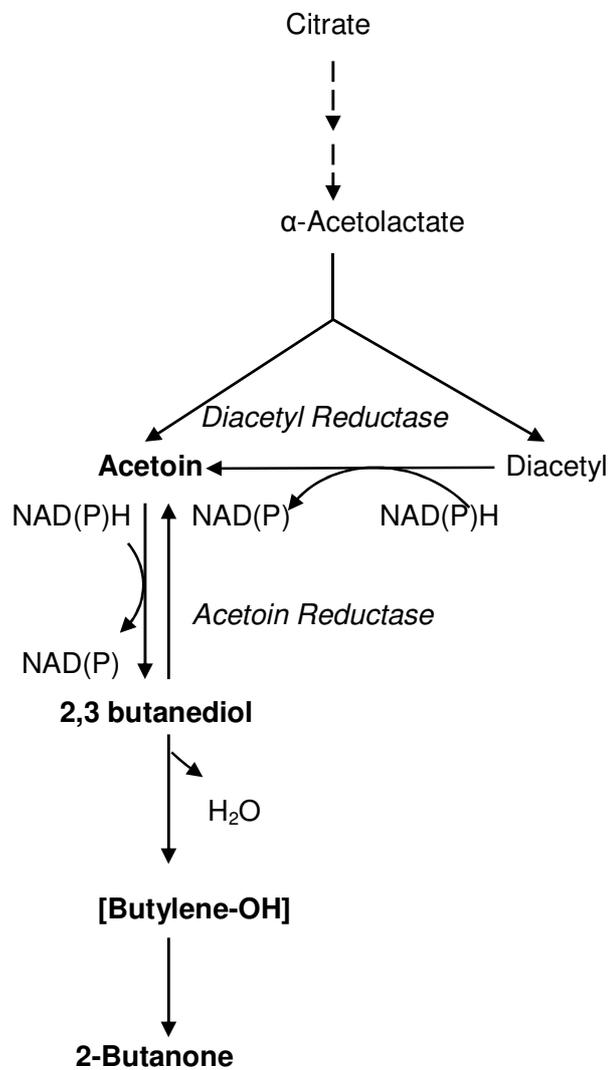


Figure 5. Proposed pathway for the formation of 2-Butanone (Scarpellino & Kosikowski, 1962; Keen *et al.*, 1974).

Ethanol

The ethanol concentration of Kefir has been reported to vary from 0.01 to 2.5% (m.v⁻¹) (Koroleva, 1988b; Kuo & Lin, 1999; Beshkova *et al.*, 2002; Chen *et al.*, 2009; Magalhães *et al.*, 2011a) depending on the starter and the method used to prepare the Kefir.

Adequate concentrations of ethanol and CO₂ are believed to give Kefir its typical yeasty flavour (Beshkova *et al.*, 2003; Magalhães *et al.*, 2011a). In contrast, some consumers feel that the excess ethanol leads to a strong and sometimes unpleasant yeasty flavour (Marshall, 1984). According to some authors, a good Kefir should only have a slight yeasty flavour (Vedemuthu, 1977; Güzel-Seydim *et al.*, 2000).

The formation of ethanol is essentially obtained by the conversion of acetaldehyde to ethanol by alcohol dehydrogenase, an enzyme present in both yeasts and LAB (Fig. 6) (Güzel-Seydim *et al.*, 2000; Bonzcar *et al.*, 2002). The activity of this enzyme (Table 8) in lactobacilli and lactococci species is mild (Imhof *et al.*, 1994; Ott *et al.*, 1999) but may vary according to the strains and the environmental conditions (Bills & Day, 1966; Lees & Jago, 1976). However, production of alcohol by LAB must not be overlooked since acetaldehyde is toxic to the organism and may therefore be catabolised to ethanol rather than being excreted (Marshall & Cole, 1983).

Yeasts and leuconostocs are considered the principal producers of ethanol. But since no ethanol is produced during co-metabolism of lactose and citrate by leuconostocs (Rea *et al.*, 1996), yeasts can be considered to be the main ethanol producers (Güzel-Seydim *et al.*, 2000). In contrast, mesophilic facultative and heterofermentative Lactococci (Tables 5 and 6) as well as Leuconostocs (Table 7) produce ethanol as secondary compounds. Facultative heterofermentative lactobacilli and obligate heterofermentative lactobacilli (Table 5) may also convert hexose sugar and disaccharides exclusively into lactic acid, acetic acid or ethanol and CO₂ via the phosphoketolase pathway (Hassan & Frank, 2001).

Two types of yeasts may be present in Kefir: non-lactose and lactose fermenting yeasts. It was demonstrated that the lactose fermenting yeasts do not have sufficient alcohol dehydrogenase activity and the final beverage obtained only had a weak yeasty flavour compared to beverages prepared with non-lactose fermenting yeasts (Beshkova *et al.*, 2002; Simova *et al.*, 2002).

Carbon dioxide originating from the alcoholic fermentation and from the heterofermentation, gives Kefir its subtle effervescence (Liu *et al.*, 2002). Industrial Kefir does not contain, or only contains very low CO₂ concentrations (Marshall & Cole, 1985; Koroleva, 1988a).

Acetic acid

Acetic acid is a short chain volatile fatty acid which has been identified in Kefir at concentrations between 200 and 850 mg.L⁻¹ (Rea *et al.*, 1996; Garrote *et al.*, 2001). However, Güzel-Seydim *et al.*

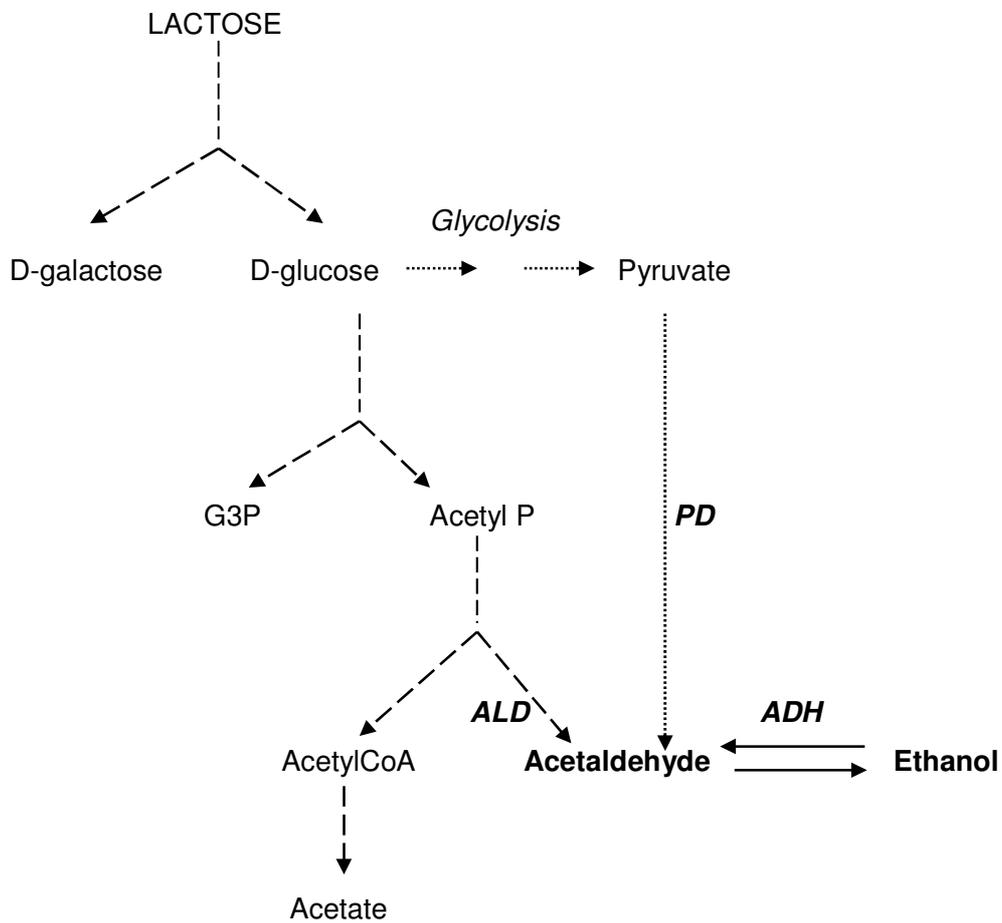


Figure 6. Ethanol metabolism in LAB (—▶) and yeasts (.....▶) (ADH = Alcohol dehydrogenase; ALD = Aldehyde dehydrogenase; PD = Pyruvate decarboxylase (Pronck *et al.*, 1996; Axelsson, 1998; Ott *et al.*, 2002).

Table 8. NAD-dependant alcohol dehydrogenase activities in some lactic acid bacteria (Lees & Jago, 1976; Marshall & Cole, 1985).

Species	Strain	Alcohol Dehydrogenase activity
<i>Lactobacillus acidophilus</i>	1748	3.3*
<i>Lactobacillus acidophilus</i>	2	5*
" <i>Lactobacillus bulgaricus</i> "	Ns	0*
" <i>Lactobacillus bulgaricus</i> "	LB1	0
" <i>S. lactis</i> ssp. <i>diacetylactis</i> "	DRC1	48.8
" <i>S. lactis</i> ssp. <i>diacetylactis</i> "	DRC2	5.8
" <i>Streptococcus cremoris</i> "	HP	8.8
" <i>Streptococcus cremoris</i> "	ML1	16.9
" <i>Streptococcus lactis</i> "	C6	5.8
" <i>Streptococcus lactis</i> "	C10	43.8
" <i>Leuconostoc cremoris</i> "	91404	59.3
" <i>Streptococcus thermophilus</i> "	CSIRO	0
" <i>Streptococcus thermophilus</i> "	NIZO	19

* $\mu\text{moles NADH}_2 \times 10^{-2}$ oxidised. min^{-1} . mg protein^{-1} ; $\mu\text{moles acetaldehyde} \times 10^{-2}$ reduced. min^{-1} . mg protein^{-1}

Ns: Not specified

(2000) did not find any acetic acid in Kefir produced in their study. Acetic acid gives a vinegar-like flavour (Bodyfelt *et al.*, 1988) but in Kefir this flavour is not predominant. It is unlikely for acetic acid to be a product of lipolysis since natural lipase in milk is destroyed during pasteurisation (McSweeney & Sousa, 2000; Kondyli *et al.*, 2002). Consequently, acetic acid must be formed from pyruvate (Fig. 7), citric acid metabolism (Fig. 4) (Hugenholtz, 1993; Rea *et al.*, 1996; Kondyli *et al.*, 2002) or amino acid catabolism (Fig. 8) (Nakae & Elliott, 1965; Lui *et al.*, 2003).

Lactic acid bacteria possess cell-envelope proteinases and peptidases that degrade caseins into small peptides and free amino acids which may act as precursors of flavour compounds (Marilley & Casey, 2004; Smit *et al.*, 2005). Amino acids undergo enzymatic reactions (Fig. 8), firstly decarboxylation to form amine and CO₂, then deamination to produce ammonia and α -keto-acids and finally transamination to form other amino acids. Amines, α -keto-acids and amino acids can be further metabolised to formaldehydes, alcohols, organic acids like acetic acid, and sulphur compounds (Liu *et al.*, 2003).

Biosynthesis of acetic acid may be from various amino acids. Nakae & Elliott (1965) demonstrated that *S. diacetylactis* DRC1 was able to form acetic acid from glycine, alanine and leucine. In the same study, *Lactobacillus* No 138 produced large quantities of acetic acid from alanine and serine (Nakae & Elliott, 1965). Liu *et al.* (2003) also showed that serine was a precursor of acetate. According to their findings, serine-degrading homo fermentative lactobacilli produced on average, higher amounts of acetate than lactococci, thermophilic streptococci and leuconostocs.

During Kefir fermentation, leuconostocs are the most likely acetate producers rather than heterofermentative lactobacilli (Rea *et al.*, 1996). Liu *et al.* (2003) demonstrated that the production of acetate was dependant on the specific species and strain within the species.

Acetate may also be formed from pyruvate in the absence or presence of oxygen (Fig. 7). In the former case and under substrate limitation, pyruvate is cleaved into formate and acetyl-CoA by pyruvate-formate lyase. Acetyl-CoA is phosphorylated to yield acetyl-P which is then converted to acetic acid by acetate kinase (Axelsson, 1998). In the latter case, NAD⁺ can be regenerated by NADH oxidases and peroxidases, leaving acetyl-P available for conversion to acetic acid (Axelsson, 1998; Adam & Moss, 2006).

Ethyl acetate

Ethyl acetate is an ester, which is scarcely mentioned in the literature of Kefir related to flavour (Beshkova *et al.*, 2003; Aghlara *et al.*, 2009). Amounts of ethyl acetate reported in the literature varied between, 0.02 and 10 mg.L⁻¹ in Kefir prepared using Kefir grains and from 0.03 to 2.77 mg.L⁻¹ in Kefir prepared using a mixed starter culture (*Lactobacillus bulgaricus* HP1, *Streptococcus thermophilus* T15, *Lactobacillus helveticus* MP12, *Lactococcus lactis* C15 and *Saccharomyces cerevisiae* A13)

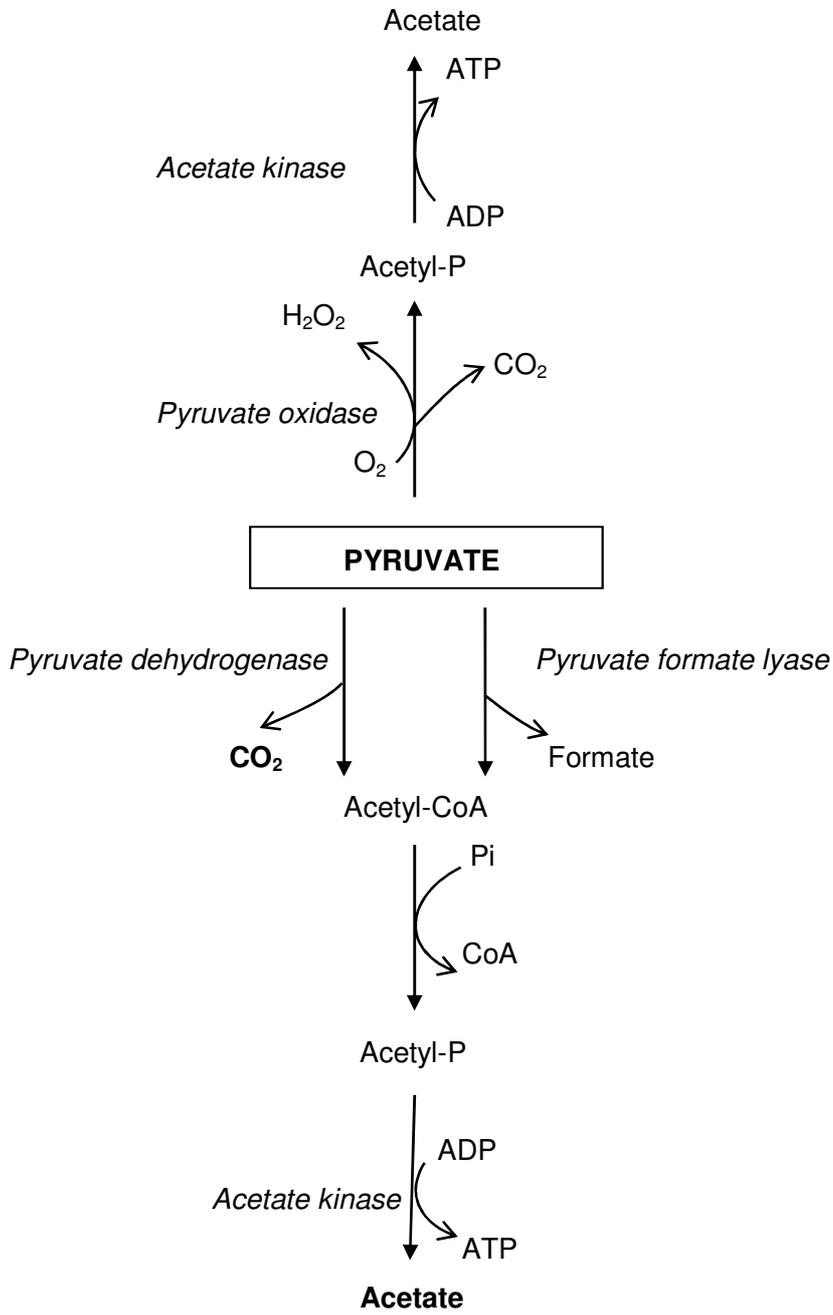


Figure 7. From pyruvate to acetate (Gottschalk, 1986; Axelsson, 1998).

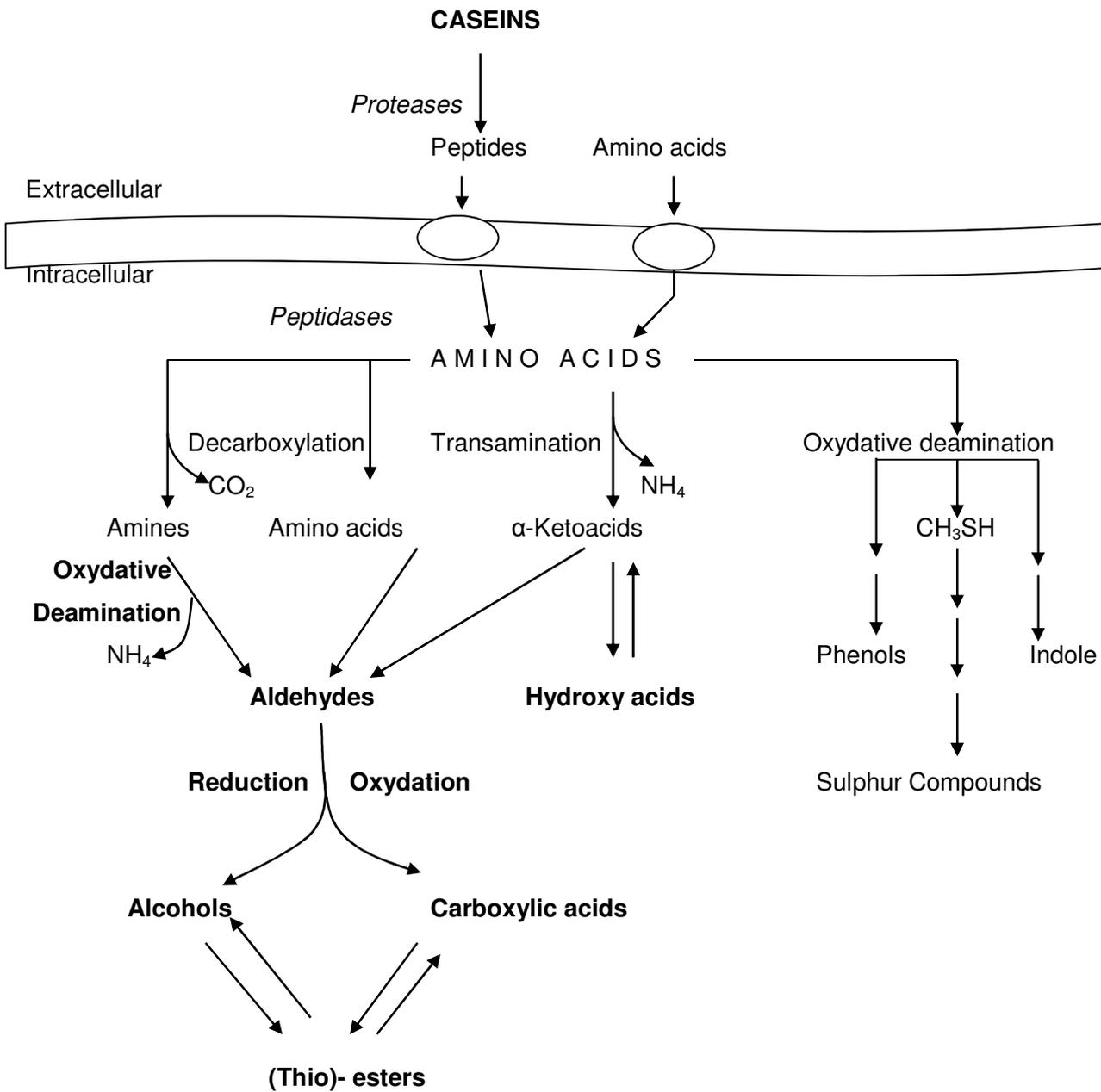


Figure 8. General amino acid catabolism pathways relevant to flavour formation in dairy fermentations (McSweeney & Sousa, 2000; Van Kranenburg *et al.*, 2002; Smit *et al.*, 2005).

(Beshkova *et al.*, 2002; Aghlara *et al.*, 2009; Magalhães *et al.*, 2011b).

Esters contribute to the development of a fruity flavour, which is seen as a positive contributor or a defect in the overall flavour of different types of fermented products such as cheeses (Nogueira *et al.*, 2005; Thierry *et al.*, 2006), beers and wines (Christiani & Monnet, 2001; Verstrepen *et al.*, 2003). Ethyl acetate, particularly, is known to give a banana or pineapple like aroma but it has also been characterised as giving a “solvent like” aroma (Liu *et al.*, 2004).

Lactic acid bacteria are capable of synthesising esters; although it seems that water activity is a critical factor in ester formation by esterases of LAB (Liu *et al.*, 1998; Fenster *et al.*, 2003). Nardi *et al.* (2002) confirmed it by reporting that the amount of esters measured at 30°C was 100-fold lower range compared to the amount measured after 88 h at -20°C. However, it is well known that yeasts are the main ester producers (Martin *et al.*, 2001).

Ethyl esters of short chain fatty acids (e.g. ethyl acetate, ethyl butanoate, ethyl hexanoate...) originate from the condensation of alcohols and short chain fatty acids, (McSweeney *et al.*, 1997), reactions catalysed by esterases, alcohol transferases and lipases (Kallel-Mhiri & Miclo, 1993; Liu *et al.*, 2004). Substrates, enzymes and environment may all determine the rate of ester formation; but according to some authors (Liu *et al.*, 2004; Thierry *et al.*, 2006) ethanol is the limiting factor of ester synthesis.

F. IMPACT OF ENVIRONMENTAL FACTORS ON FLAVOUR

Variations in the storage conditions, the growth medium and the environment, will lead to the development of a community of microorganisms that will be characteristic of the grains (Takisawa *et al.*, 1998). Therefore, the organoleptic features of Kefir will be directly linked to the microorganisms present in the grains and can be influenced by changes in the intrinsic factors of the grains (grain activity, grain to milk ratio, starter) or by one or several environmental factors which include: incubation temperature, growth medium enrichment, effect of pH and storage conditions.

Intrinsic factors

Grain activity

Kefir grains kept as dried, freeze-dried (Oberman & Libudzisz, 1998) or frozen (Garrote *et al.*, 1997) must be considered as inactive grain forms due to the fact that the microorganisms are in lag phase. Therefore, they need to be activated to their exponential growth phase before use. Physically, this can be seen when the grains float to the surface of milk (Burke, 1938) or when the milk has clotted. No standard method of activation exists; however a recommended activation process is activating in pasteurised full cream or even skimmed milk (Schoevers & Britz, 2003; Santos *et al.*, 2003),

incubation at room temperature ranging from 20° to 25°C for 18 to 24 h. The activation process can last up to one week when frozen grains are used (Micheli *et al.*, 1999; Santos *et al.*, 2003; Wittuhn *et al.*, 2005) and up to one month for lyophilised grains (Simova *et al.*, 2002). The grains are transferred daily (Schoevers & Britz, 2003; Magalhães *et al.*, 2011a) into a new batch of milk or twice to thrice a week (Angulo *et al.*, 1993; Güzel-Seydim *et al.*, 2000; Liu & Lin, 2000).

Grain to milk ratio

The impact of the inoculum size on the characteristics of Kefir beverage, especially pH, lactococci concentration, apparent viscosity and CO₂ content, were studied by Garrote *et al.* (1998). They demonstrated that there were significant differences in the characteristics of the Kefir obtained with an inoculum size of 1% and 10%. The former inoculum size gave a highly viscous and low acid beverage whereas the latter inoculum size gave a low viscosity, highly acidic and effervescent product. Some authors (Korovkina *et al.*, 1978; Kuo & Lin, 1999) agree that an inoculum size of 5% (m.v⁻¹) is suitable to make the traditional high-quality refreshing Kefir beverage with a prickling and slight yeasty taste associated with a clear acid taste without bitterness, a smooth texture and a pleasant flavour (Assadi *et al.*, 2000).

Starter

The strains present in the starter, whether as pure cultures or as Kefir grains, can affect the quality of Kefir. Indeed, it has been shown that the amount of aroma compounds vary according to the strains present (Cogan, 1975; Liu *et al.*, 2003). Burrow *et al.* (1970) showed that the amount of diacetyl produced by “*S. diacetylactis*” strains varies from 0.07 to 3.72 mg.L⁻¹ whereas none of the other lactococci strains isolated from Kefir produced diacetyl (Yüksekdağ *et al.*, 2004). The irreversible conversion of diacetyl to acetoin which is further reduced to 2.3 butanediol and volatilisation, are responsible for the low level of diacetyl and acetoin in cultured products especially during long incubation periods (Collins, 1972; Østlie *et al.*, 2003; Maurello *et al.*, 2001). In the case of acetaldehyde, which is toxic to the organism, it may be reduced to ethanol by alcohol dehydrogenase rather than excreted (Marshall & Cole, 1983). Thus, accumulation of acetaldehyde in the growth medium will depend on the level of alcohol dehydrogenase activity (Gonzales *et al.*, 1994; Østlie *et al.*, 2003).

Environmental factors

Incubation temperature

The incubation temperature is an important parameter in the manufacture of the final Kefir since it may

enhance or inhibit the activity of a specific group of microorganisms (Zajšek & Goršek, 2010). The result is that specific desirable or even undesirable flavours may develop. A good Kefir is obtained with an inoculum size of 5% (m.v⁻¹) and incubation at 25°C (Korovkena *et al.*, 1978). But according to Koroleva (1988a), fermentation at 25° – 27°C leads to an atypical product whereas fermentation at lower temperature (20° – 22°C) permits the development of all the characteristic microorganisms. Consequently, the cycle of manufacture should last 24 h and consist of two steps: the first is fermentation at 20° – 22°C for 10 – 12 h and the second step is maturation at 8° – 10°C for the remaining 12 h. Professor Britz (Food Science Department, Stellenbosch, South Africa, Personal Communication, 2005) found that incubation at 22°C for 24 h followed by a maturation period of 5 – 6 h at 4°C gives a tasty Kefir product.

Growth medium enrichment

Glycolysis may only proceed if NADH₂ is re-oxidised to NAD⁺. In LAB, this re-oxidation is possible by using pyruvate as a hydrogen acceptor to form lactic acid. As a result, unless other hydrogen acceptors are present in the media, little diacetyl will be synthesised (Collins, 1972). The addition of citrate to milk creates excess pyruvate which is converted into acetaldehyde, diacetyl and acetoin (Collins, 1972; Güzel-Seydim *et al.*, 2000; Beshkova *et al.*, 2003), whereas the addition of threonine rather enhances acetaldehyde production (Marshall, 1984). Metal ions and vitamins may also contribute to flavour synthesis. A study revealed that addition of metal ions such as Mn²⁺ may also stimulate diacetyl and acetoin production in *Leuconostoc* species whereas it has no effect on *Lactococcus lactis* species (Cogan, 1975). It was demonstrated that addition of 4 µg.mL⁻¹ of Mn²⁺ plus citrate to pre-incubated cultures of *Leuconostoc cremoris* stimulated growth and citrate utilisation. In contrast, in *Lc. lactis* ssp. *diacetylactis* DRC1 and DRC3 addition of Mn²⁺ or Mn²⁺ plus citrate had no stimulatory effect on both citrate utilization and flavour compounds production (Cogan, 1975; Drinan *et al.*, 1976).

Richter *et al.* (1979) showed that ascorbic acid added to milk inoculated with mixed cultures of *Ln. cremoris* with either “*S. cremoris*”, “*S. lactis*” or both had a stimulatory effect on diacetyl concentration without affecting the acid production. Ascorbic acid concentrations of 0.1% (w.v⁻¹) were sufficient to stimulate diacetyl synthesis. A greater concentration in ascorbic acid did not induce greater diacetyl production.

Effect of pH

Citrate permease (Cit-P) is the key enzyme of the citrate metabolic pathway because it is the means by which citrate is transported into the cell (Samaržija *et al.*, 2001). Thus, possibly due to pH

constraints, citrate uptake may limit the rate of citrate utilisation and may therefore directly affect the yield of aroma compounds. Studies have demonstrated that Cit-P optimum activity lies between pH 4.5 and 5.5 in *Lc. lactis* ssp. *diacetylactis* (Magni *et al.*, 1996; García-Quintáns *et al.*, 1998) and between pH 5.0 to 6.0 in some other species (Hugenholtz, 1993). Under these conditions, citrate is metabolised and converted to flavour compounds. Thus, lack of flavour in Kefir may be attributed to inadequate pH due to a short fermentation time or the absence of a diacetyl producer such as *Lc. lactis* ssp. *diacetylactis* among Kefir grains microflora.

Storage conditions

The absence of diacetyl in dairy products such as cultured buttermilk and sour cream is mainly due to the irreversible conversion of diacetyl into acetoin by diacetyl reductase which is widely spread among LAB, but its activity varies among species and among strains within species (Seitz *et al.*, 1963; Keenan & Lindsay, 1967; Levata-Javanovic & Sandine, 1996). This enzyme has been found in several species including strains of "*S. diacetylactis*", "*L. cremoris*", "*L. dextranicum*" (Seitz *et al.*, 1963; Levata-Javanovic & Sandine, 1996). Reduction of diacetyl proceeds rapidly at high temperatures and decreases with decreasing temperatures (Bassit *et al.*, 1995). Therefore, to stabilise (Sandine *et al.*, 1972) and even increase (Pack *et al.*, 1968) diacetyl content of cultured products including Kefir it is recommended that they be kept at refrigerated temperatures (4° – 5°C). However, it is interesting to note that of "*S. diacetylactis* 18 - 16" possessing 100 units of diacetyl reductase per milligram of enzyme protein was able to reduce 9 mg.L⁻¹ of diacetyl in 10 min. This highlights the importance of choosing the right combination of species in a mixed culture.

Diacetyl reductase was also found in coliforms (*E. coli*) and psychrophilic bacteria (*Pseudomonas putrefaciens*, *Pseudomonas fragi*) (Elliker, 1945; Seitz *et al.*, 1963). While diacetyl reductase activity is generally low in *Leuconostoc* and *Lactococcus* species, the opposite is true for coliforms and psychrophilic species that exhibit activities ranging from 3 to 345 units.mg⁻¹ of enzyme protein (Seitz *et al.*, 1963). Thus, defects in refrigerated cultured products where diacetyl is the main flavour compound may then be attributed to contamination by spoilage psychrophilic bacteria.

G. CONCLUSIONS

Kefir is not commercially manufactured in Southern Africa and is thus unknown to the South African consumer. However, it is well established that in Southern Africa, Kefir is made by a few households (Loretan *et al.*, 2003). It has been reported that value-added foods of all kinds, are growth areas for the South African food industry (IDF, 2006). Kefir can be considered a typical value-added food because of its high nutritive value and health properties which makes it ideal for people of all ages

and health status (Ötles & Cagindi, 2003).

However, the limitation is the availability of good mass-cultured grains. The use of starter cultures like Kefir grains may significantly contribute to an improvement of quality, safety and nutrition of consumers (Holzapfel, 1997) and will be of low cost since the grains are indefinitely reusable (Britz, 2003). Not only is Kefir a nutritious dairy product, but it has also therapeutic attributes due to the presence of probiotic species (Sarkar, 2007). Besides having probiotic properties, Kefir also has the potential to help prevent and/or reduce the damage caused by free radical activities and thus reduce the risk of degenerative diseases like cancer and diabetes. Indeed, it was demonstrated that microbes present in fermented milks and Kefir showed antioxidant activity (Wang *et al.*, 2006; Virtanen *et al.*, 2007). Furthermore, a sensory study revealed that the degree of liking of Kefir compared to Maas, a South African traditional fermented milk was equal; and anti-microbial study showed that Kefir inhibits the growth of pathogens (Van Wyk *et al.*, 2002).

Regarding all these attributes, Kefir is the appropriate healthy fermented dairy product that would benefit the South African consumers. Thus, there is an urgency to develop good mass cultured Kefir grains that could be distributed and/or sold in South Africa.

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CHAPTER 3

METABOLIC PROFILES OF KEFIR UNDER CONTROLLED AND UNCONTROLLED CONDITIONS USING DIFFERENT KEFIR GRAINS

Summary

Kefir grains (LG ‘laboratory Kefir grains’, MG ‘mass cultured Kefir grains’ and TG ‘Traditional Kefir grains’) were used to prepare Kefir under controlled (24 h at 22°C – LG22 Kefir and MG22 Kefir) and under uncontrolled (Traditional Kefir and MG-home Kefir) temperature conditions. Traditional Kefir obtained by inoculating 20 g of active TG Kefir grains in 300 mL pasteurised milk, was the best quality Kefir. Under these conditions, the Traditional Kefir had a pH varying between 4.0 and 4.3 with TA values ranging from 0.8 to 1.0 % (m.v⁻¹). The flavour compounds identified were acetaldehyde (2 – 4 mg.L⁻¹), ethanol (100 – 200 mg.L⁻¹), acetone (1 – 3 mg.L⁻¹), diacetyl (3 – 6 mg.L⁻¹) and acetic acid (300 – 700 mg.L⁻¹). These flavour compounds were also found in MG22 Kefir, whereas LG22 Kefir and MG-home Kefir only contained acetaldehyde, ethanol and acetic acid. Ethyl acetate and 2-butanone were not found in any Kefir. The concentrations of the flavour compounds found in LG22 Kefir, MG22 Kefir and MG-home Kefir were generally higher than those found in the Traditional Kefir. The pH and TA were in the same range as found for the Traditional Kefir.

The Traditional Kefir had a buttery flavour associated with an acidic “background”. Although containing diacetyl, but at concentrations lower than in the Traditional Kefir, MG22 Kefir did not have a noticeable buttery flavour but was characterised by a strong acidic taste. Similarly, LG22 Kefir and the MG-home Kefir had an atypical taste compared to the Traditional Kefir, probably caused by the high level of ethanol and the “lack” of diacetyl. This study highlighted that environmental conditions affect the quality of Kefir but also the importance of stabilised microbial consortium within the Kefir grains as it produces, over time, both stable and acceptable Kefir.

Introduction

Fermented milk products have gained in popularity in recent years due to the public awareness of their health attributes (Van de Water, 2004; Ebringer *et al.*, 2008). Around the world, different types of fermented products are consumed, some being very similar but have different names. In Southern Africa, variants of Maas, a traditional fermented milk, are produced on industrial scale and sold under the name of Inkomasi or Amasi. Unfortunately, the retail price of Maas and fermented dairy products derivatives has steadily increased over the years, making these products unaffordable.

Kefir is a suitable fermented dairy product that can be home-made and therefore becomes

very affordable as Kefir grains are a live starter that can, if carefully handled, last a lifetime. Kefir has numerous advantages among which it can be kept at room temperature without being spoiled and is a highly nutritious beverage with therapeutic properties. Additionally, a sensory evaluation study conducted in 2002 revealed no significant sensory difference between Kefir and Maas (Van Wyk *et al.*, 2002).

Kefir is made from Kefir grains which are microbially rich structures containing yeasts and lactic acid bacteria. The beverage contains 0.8 to 1.15% lactic acid, carbon dioxide and ethanol. The main flavour compounds reported are acetaldehyde, ethanol, acetone, diacetyl and 2-butanone. Acetoin and 2,3-butanediol can also be found but they do not have an impact on the flavour as they are flavourless. Other compounds such as acetic acid and ethyl acetate have been also detected (Fernandez-Garcia & Mc Gregor, 1994; Tamime & Robinson, 1999; Beshkova *et al.*, 2003; Magalhães *et al.*, 2011a).

The Traditional Kefir prepared with traditional Kefir grains has an acidic, slightly alcoholic and pleasant buttery flavour and a subtle fruity flavour. However, the growth rate of the grain is slow, from 5 to 7% per day (Libudzisz & Piatkiewicz, 1990) and consequently, the commercialisation of these grains cannot be effective if the supply is not constant. Thus, mass cultured Kefir grains (MG) were developed by adding yeast extract and urea to milk containing Kefir grains (Schoevers & Britz, 2003), but the Kefir beverage obtained using these grains is always very acidic and tasteless. It is hypothesised that the lack of flavour of Kefir prepared with MG is due to the absence or the low concentration of the most important aroma compounds. Consequently, it is important to develop microbiologically stable mass cultured Kefir grains that can be commercially viable as it impacts on the characteristics of the final product. Indeed, it is the consumer expectation to always consume a product with similar sensory qualities.

In this regard, the first aim of this study was to standardise the headspace gas chromatographic method that will be used to quantify the volatile organic compounds produced by different Kefir grains. Headspace gas chromatography (HSGC) was chosen because it is less time consuming and it allows the qualitative and quantitative analysis of several compounds at the same time. The second aim was to determine the impact of controlled and uncontrolled incubation conditions that results in the production of a good quality Kefir, on the metabolic profiles of Kefir using different sources of Kefir grains.

Material and methods

Grain activation

Frozen mass cultured Kefir grains (MG) were obtained from the University of Stellenbosch, Department of Food Science and defrosted at ambient temperature for 24 h. The grains (20 g) were

activated by inoculation in 300 mL full cream pasteurised milk and incubated at 25°C for 24 h. At the end of the incubation period the grains were recovered by sieving through a stainless steel sieve and added to a new batch of milk. The grains were considered to be active after the fifth batch of milk.

Activated traditional grains (TG) were obtained from Professor T.J. Britz (Department of Food Science – University of Stellenbosch) and divided into two batches, labelled 'laboratory' Kefir grains (LG) and TG. The former Kefir grains were used to make Kefir under laboratory conditions (controlled) and the latter Kefir grains were used to make Kefir under home conditions (uncontrolled).

Headspace gas chromatography analysis

Separation and identification of volatile organic compounds (VOCs) was determined using a Fisons 8000 Series gas chromatograph (Fisons Instruments S.p.A., Milan, Italy) equipped with a flame ionisation detector (FID) and a 60 m DB 5 capillary column bonded with a methyl-5% phenyl silicone layer as stationary phase (film thickness 0.25 μm ; diameter 0.25 mm Quadrex Corporation, Newhaven).

Operating parameters were: injector and detector temperatures set at 150° and 200°C, respectively; helium was used as carrier gas at a flow rate of 1.1 mL.min⁻¹. The oven heating cycle was programmed at 30°C for 2 min followed by an increase of 5°C.min⁻¹ to 220°C for 10 min. A 1.5 mL aliquot of the headspace gas was withdrawn using a warmed (70°C) Hamilton gas-tight syringe and split-injected into the gas chromatograph at a split ratio of 1:100 (Human, 1998).

Quantitative determination of the metabolite compounds was done by integration of the peak areas using an external standard calibration and Borwin Version 1.2 integration software (JMBS Developpements, Le Fontanil, France).

Standards solutions

All chemicals used were of analytical reagent grade. Acetaldehyde, ethyl acetate, acetone and 2-butanone were purchased from Merck. Ethanol, diacetyl, DL-lactic acid (DL-LA), sodium chloride (NaCl) and tetrahydrofuran (THF) were purchased from Sigma-Aldrich. Preliminary tests showed that the THF peak did not overlap with the peaks of acetaldehyde, ethanol, acetone, diacetyl, 2-butanone and ethyl acetate, thus, the THF was chosen as external standard (ES). Ultra High Temperature (UHT) milk was purchased at local supermarkets and the UHT milk was, where necessary, degassed using a vacuum pump.

Three standard stock solutions (SSS) (Table 1) were prepared using distilled water (SSS1), UHT milk (SSS2) and degassed UHT milk (SSS3) as solvents. Each SSS contained THF (200 mg.L⁻¹), acetaldehyde (200 mg.L⁻¹), ethanol (600 mg.L⁻¹), acetone (200 mg.L⁻¹), diacetyl (200 mg.L⁻¹), 2-butanone (100 mg.L⁻¹) and ethyl acetate (100 mg.L⁻¹). The SSS1 was kept at 4°C and stock solutions

Table 1. Description of the standard stock solutions (SSS) and working standard solutions (WSS) used to determine the reproducibility, repeatability and linearity of the headspace gas chromatography (HSGC) method.

Standard stock solutions (SSS)	Working standard solutions (WSS)	Vial's content
SSS1	WSS 1-1	Distilled water
	WSS 1-2	Distilled water + NaCl
SSS2	WSS 2-3	UHT milk
	WSS 2-4	UHT milk + NaCl
SSS3	WSS 3-5	Degassed UHT milk
	WSS 3-6	Degassed UHT milk + NaCl

(SSS2 and SSS3) with UHT milk as solvent were prepared daily. The working standard solutions (WSS) were obtained by dilution of the specific SSS.

The specific working standard solution WSS (10 mL) (Table 1) was placed in a 20 mL glass vial with or without 2.5 g of NaCl (WSS1 and WSS2) (Ulberth, 1991). The vials were crimp-sealed with silicone-PTFE seals and aluminum caps, and incubated in a waterbath at 95°C for 50 min and mixed several times while in the waterbath. Each WSS (Table 1) was used to determine the reproducibility, repeatability and linearity of the headspace gas chromatography (HSGC) method.

Validation of the headspace gas chromatography method

Recovery

Accuracy of the HSGC method was determined by calculating the percentage metabolite recovery (Ulberth, 1991; Romeu-Nadal *et al.*, 2004; Yang & Choong, 2001) from Kefir samples that had been spiked with acetaldehyde (20 mg.L⁻¹), ethanol (60 mg.L⁻¹), acetone (20 mg.L⁻¹), diacetyl (20 mg.L⁻¹), 2-butanone (10 mg.L⁻¹) and ethyl acetate (10 mg.L⁻¹). The recovery rate of each volatile was calculated as the mean of three replicates.

Precision

To determine the precision of the HSGC method, samples were injected five to seven times (repeatability) and on two to three days (reproducibility). The reliability of the method was determined in terms of percent coefficient of variation (Green & Payne, 1989; Xanthopoulos *et al.*, 1994).

Linearity

The sensitivity of the HSGC method was determined by adding a constant amount of the external standard (THF) (20 mg.L⁻¹) to increasing concentrations of aqueous acetaldehyde (10, 20, 30, 40, 50 and 60 mg.L⁻¹), ethanol (30, 60, 90, 120, 150 and 180 mg.L⁻¹), acetone (2, 4, 6, 8, 10 and 12 mg.L⁻¹), diacetyl (4, 8, 12, 16, 20 and 24 mg.L⁻¹), 2-butanone (2, 4, 6, 8, 10 and 12 mg.L⁻¹) and ethyl acetate (2.5, 5.0, 7.5, 10.0, 12.5 and 15 mg.L⁻¹). Sample peaks were integrated and the average area of each compound recorded. The ratio (y) of the peak area of each compound to the peak area of THF (y = area peak compound/area THF) versus each compound concentration (x), was plotted. The linearity of the method was assessed by determining the multiple correlation coefficients (R²) (Xanthopoulos *et al.*, 1994; Romeu-Nadal *et al.*, 2004) using the SigmaPlot software (2001).

Kefir flavour compounds

A Kefir sample (9.75 mL) was placed in a 20 mL glass vial containing 2.5 g of NaCl and 0.25 mL of THF. The vial was crimp-sealed with a silicone-PTFE seal and aluminum cap and incubated in a waterbath for 50 min at 95°C. This temperature was chosen to optimise the volatilisation of the VOCs

in the headspace. The vial's content was mixed several times while in the waterbath. Kefir compounds were identified by comparing the retention time of the unknown compounds to those of the analytical grade standards. Quantitative determination of the compounds was done by integration of the peak areas using external standard calibration and Borwin Version 1.2 integration software (JMBS Developpements, Le Fontanil, France). The waterbath was set at 95°C to optimise the volatilisation of the volatile compound in the headspace (Dr. Sigge, G.O, Lecturer, Food Science Department, Stellenbosch, South Africa, Personal Communication, 2004).

Short chain volatile fatty acids determination

A standard solution of short chain volatile fatty acids (VFAs) (acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids) was prepared by mixing 1 mL of each fatty acid and 0.5 mL of n-hexanol in a 1 000 mL volumetric flask with 250 mL of formic acid and 750 mL of distilled water.

Samples of Kefir were prepared as follows: 10 mL of Kefir was centrifuged (10 min at 10 000 g) and the supernatant filtered through Whatman No. 1 filter to remove solid particles and obtain a clear supernatant. One millilitre of formic acid (35% v.v⁻¹) and 2 µL of n-hexanol (as internal standard) were added to 3 mL of the filtered supernatant. The GC injection volume was 1 µL and the run time 20 min.

The VFAs were determined using a Varian gas chromatograph equipped with a flame ionisation detector and a 30 m bonded phase Nukol (Supelco, Inc., Belafonte, PA) fused silica capillary column (0.53 mm diameter and 0.5 µm film thickness). The oven heating cycle program was held at 105°C for 2 min followed by an increase of 10°C per min to 190°C for 10 min. Injector and detector temperatures were 150°C and 300°C, respectively. The flow rate of nitrogen, the carrier gas was 6.1 mL.min⁻¹. The VFAs were quantified using the Borwin Version 1.2 integration software (JMBS Developpements, Le Fontanil, France) using the internal standard method (Sigge *et al.*, 2005).

Environmental conditions

Preliminary study

To determine the incubation temperature at which Kefir should be prepared under controlled conditions, a preliminary study was done, where 20 g Kefir grains (LG and MG) were added to 500 mL double pasteurised milk and incubated for 24 h at 25°C. This temperature was chosen because Korovkina *et al.* (1978), Güzel-Seydim *et al.* (2000) and Schoevers (1999) reported that incubation of Kefir at 25°C gives a beverage with the best organoleptic attributes. After the incubation period, the grains were removed by sieving and the pH, the titratable acidity (TA) (James, 1999) and the metabolic profile (VOCs and VFAs) of the Kefir beverage were determined. Kefir was prepared on a daily basis for 9 days but only analysed every three days. Different length of incubation periods were

not studied because it was assumed that in an household environment, an incubation period of 24 h would be the best option.

Controlled incubation temperature: 22°C for 24 h

Kefir was prepared in the following way: 20 g of Kefir grains (LG22 and MG22) were inoculated into 300 mL of full cream pasteurised milk and incubated for 24 h at 22°C to favour the activity of heterofermentative lactococci and leuconostoc (Robinson, 1995). At the end of the fermentation, the grains were separated by using a sieve. The VOC and VFA contents as well as the pH and TA were monitored every day for a period of nine days.

Uncontrolled incubation temperature: Traditional Kefir (Home-made)

To obtain the profile of traditional home-made Kefir, which served as the control, 'TG' Kefir grains were used. These grains have been used for several years in the "home" production of Kefir, which involves no special sterilisation steps or temperature control. The fermenting room temperature varied from 15° to 28°C. It was therefore assumed that the microbial population had stabilised (Prof. Britz, T.J., Food Science Department, Stellenbosch, South Africa, Personal Communication, 2008). Kefir was prepared in the following way: 20 g of Kefir grains (TG) were inoculated into 300 mL of full cream pasteurised milk and incubated at room temperature. After 24 h of incubation, the grains were separated by using a sieve and re-inoculated into a new batch of milk. This was repeated for a period of 9 days. The VOC and VFA contents and the pH and TA were monitored every day.

pH and Titratable acidity

The pH of the Kefir was measured with an Orion pH meter and a glass electrode (Hanna Instruments). The TA was measured in triplicate by the titration of 10 mL sample with 0.11 N sodium hydroxide (NaOH) until the pink phenolphthalein end-point (James, 1999).

The pH and the TA are measurements of acidity. They differ in that pH describes the degree of acidity of an aqueous medium by measuring the concentration of the hydronium ions whereas the TA measures the amount of acids present in solution. In Kefir, the major acid is lactic acid but acetic acid, which is also present, might influence the TA. Thus, TA will be expressed as % total acids instead of % lactic acid, using the following formula:

$$\text{TA (as \% total acids)} = (\text{X mL 0.11 N NaOH used}) / 10$$

Gas chromatography of DL-lactic acid

Separation and identification of DL-lactic acid (DL-LA) was determined using a Fisons 8000 Series gas chromatograph (Fisons Instruments S.p.A., Milan, Italy). The gas chromatograph was equipped

with a flame ionisation detector (FID) and a 30 m Innowax capillary column bonded with a polyethylene glycol layer as stationary phase (film thickness 0.25 μm ; diameter 0.25 μm , J&W Scientific, USA). A pre-column was attached to the column to prevent impurities from reaching the column. Helium was used as carrier gas at a flow rate of 2 $\text{mL}\cdot\text{min}^{-1}$ and the injector and detector temperatures were set at 150° and 200°C, respectively. The oven heating cycle was programmed at 50°C for 1 min followed by an increase of 6°C $\cdot\text{min}^{-1}$ to 180°C for 10 min then a final increase of 10°C $\cdot\text{min}^{-1}$ to 230°C for 5 min. The injection mode was splitless and 0.1 μL of sample DL-LA (0.125 $\text{g}\cdot 100\text{ mL}^{-1}$) was directly injected.

Quantitative determination of DL-LA was done by integration of the peak areas using an external standard calibration and Borwin Version 1.2 integration software (JMBS Developpements, Le Fontanil, France).

Sensory evaluation

The sensory evaluation of Kefir beverages produced was done by two trained panellists.

Results and discussion

Validation of the headspace gas chromatography method

The aim of this section of the study was to optimise the HSGC method for the detection of volatile organic compounds (acetaldehyde, ethanol, acetone, diacetyl, 2-butanone and ethyl acetate) present in Kefir. For the validation of the HSGC method, samples of distilled water, full cream UHT milk (degassed and non-degassed) and Kefir prepared with LG, were used.

Recovery

The data in Table 2 show the recovery rates of each compound. The recovery rates ranged from 95.9 to 106.0%. The recovery rates, lower or higher than 100%, may have resulted from the formation or conversion of volatile compounds to other compounds by microbial enzymes or from losses due to volatilisation. All recovery rates were satisfactory for the five compounds (Table 2). The results demonstrated good accuracy of the HSGC method.

Precision

The reproducibility and repeatability of the HSGC method was assessed by calculating the coefficients of variation (Yang & Choong, 2001). The data in Table 3 show the results of the precision of the HSGC method with WSS1-1 (distilled water) and WSS1-2 (distilled water + NaCl 2.5 g). It can be

Table 2. Recovery rates for the HSGC determination of VOCs in Kefir. Known amounts of each compound were added to the spiked Kefir.

Compound	*VOC in	VOC added	*Total VOC	**Recovery (%)
	Kefir (mg.L ⁻¹)	(mg.L ⁻¹)	Detected (mg.L ⁻¹)	X ± SD
Acetaldehyde	10.4	20.0	31.0	103.0 ± 1.20
	3.53	20.2	22.9	95.9 ± 1.77
Ethanol	69.6	59.1	129.9	102.0 ± 2.15
	40.0	60.5	101.0	101.0 ± 2.54
Acetone	1.19	20.0	21.2	100.0 ± 2.26
	0.00	20.0	22.0	102.0 ± 3.50
Diacetyl	1.90	19.8	21.9	101.0 ± 1.97
	2.79	20.0	24.0	106.0 ± 2.80
2-butanone	0.00	10.9	11.5	106.0 ± 0.50
	0.00	9.98	9.96	99.8 ± 1.10
Ethyl acetate	0.00	10.0	10.1	101.0 ± 2.19
	0.00	10.0	9.8	98.0 ± 0.77

*Each data point represents the mean of three replicates of duplicate studies

**Recovery = [(Total amount detected - VOC in Kefir) X 100] / VOC added

Table 3. Precision of the HSGC method, expressed as CV (%), using standards prepared with distilled water (WSS1-1) and distilled water + 2.5 g NaCl (WSS1-2).

Compound	Coefficient of variations (CV%)	
	WSS1-1 [□]	WSS1-2 [□]
Acetaldehyde	1.9 – 9.4	2.6 – 4.7
Ethanol	2.1 – 4.7	2.3 – 3.9
Acetone	1.6 – 12.2	3.5 – 6.2
Diacetyl	2.5 – 19.0	2.3 – 4.8
2-butanone	3.3 – 11.1	2.2 – 3.1
Ethyl acetate	1.0 – 5.5	3.7 – 4.6

[□]Mean range of triplicate studies

CV = describes the magnitude of the sample values and the variation within them; CV = [(mean / sd) x 100]

seen that with WSS1-2, the precision was better than with the WSS1-1 as demonstrated by the values of the coefficient of variation that were all below 10%. Indeed, in gas chromatography, it is generally accepted that a high reproducibility and repeatability is characterised by a coefficient of variation (CV) below 10% (Green & Payne, 1989; Xanthopoulos *et al.*, 1994). The data showed that the addition of salt contributed to a decrease in the solubility of the polar organic compounds and an increase in their transfer into the headspace. As a result, the precision of aqueous standards containing NaCl was better than the precision obtained in aqueous standards without added salt, except for diacetyl.

The data in Table 4 show the CV of WSS2-3, WSS2-4, WSS3-5 and WSS3-6. The results show that the reproducibility and repeatability of the VOC in WSS2-3 (UHT milk), WSS2-4 (UHT milk + NaCl), WSS3-5 (degassed UHT milk) and WSS3-6 (degassed UHT milk+ NaCl) were generally very poor, with 75% of CV found being higher than 10%.

However, the precisions found for diacetyl were neither reproducible nor repeatable when milk was used as solvent (degassed, not degassed, with or without 2.5 g NaCl) as shown by the coefficient of variations (CV) in Table 4, which varied between 9.0 and 24.1%. The inaccuracies in the detection of the diacetyl were also reported by other researchers (Veringa & Schrijver-Davelaar, 1970). Monnet *et al.* (1994), while using skimmed milk as solvent, reported a very low coefficient of variation (< 3.4%) for diacetyl compared to the data in this study where full cream milk was used as solvent. It is possible that diacetyl was entrapped to the milk fat, which could affect the release of diacetyl.

The data obtained in this study clearly show that it is preferable to use distilled water as solvent rather than milk. Additionally, the inclusion of salt (NaCl) generally proved to improve the results compared to the samples without added salt. The use of distilled water instead of milk has also the advantage of minimising the interactions that might occur between volatile compounds and milk constituents and affect the release of those compounds into the headspace. However, if milk has to be used, low fat could be a preferable option, to minimise the interactions between compounds and the fat portion of milk.

Linearity of the method

The linear regression plots of acetaldehyde, ethanol, acetone, diacetyl, 2-butanone and ethyl acetate are illustrated in Fig. 1. The plots were linear over the range of concentrations studied for acetaldehyde, ethanol, acetone, diacetyl, 2-butanone and ethyl acetate with coefficients of correlation (R^2) of 0.996, 0.964, 0.991, 0.985, 0.998 and 0.997, respectively. According to the literature (Beebe & Gilpin, 1983; Ulberth, 1991), a coefficient of correlation approaching a value of 1 indicates a good response from the gas chromatograph detector. Consequently, from the results obtained, it is clear that the response of the flame ionisation detector (FID) of the gas chromatograph used in this study

Table 4. Precision of the HSGC method, expressed as CV (%), using standards prepared with UHT milk (WSS2-3), UHT milk + 2.5 g NaCl (WSS2-4), degassed UHT milk (WSS3-5) and degassed UHT milk + 2.5 NaCl (WSS3-6).

Compound	Coefficient of variations (CV%)			
	WSS2-3 [□]	WSS2-4 [□]	WSS3-5 [□]	WSS3-6 [□]
Acetaldehyde	7.9 – 16.9	2.6 – 5.6	6.9 – 9.6	3.2 – 4.2
Ethanol	9.3 – 13.5	1.1 – 13.1	13.4 – 22.2	2.9 – 12.5
Acetone	8.2 – 14.9	2.7 – 5.0	8.1 – 11.7	4.9 – 7.0
Diacetyl	9.0 – 24.1	17.9 – 19.5	14.3 – 17.9	14.7 – 16.8
2-butanone	7.7 – 14.6	3.8 – 4.8	8.0 – 8.2	3.4 – 5.3
Ethyl acetate	6.0 – 8.0	4.2 – 7.0	6.1 – 9.0	4.5 – 6.0

*Mean of duplicate studies

CV = describes the magnitude of the sample values and the variation within them; $CV = [(mean / sd) \times 100]$

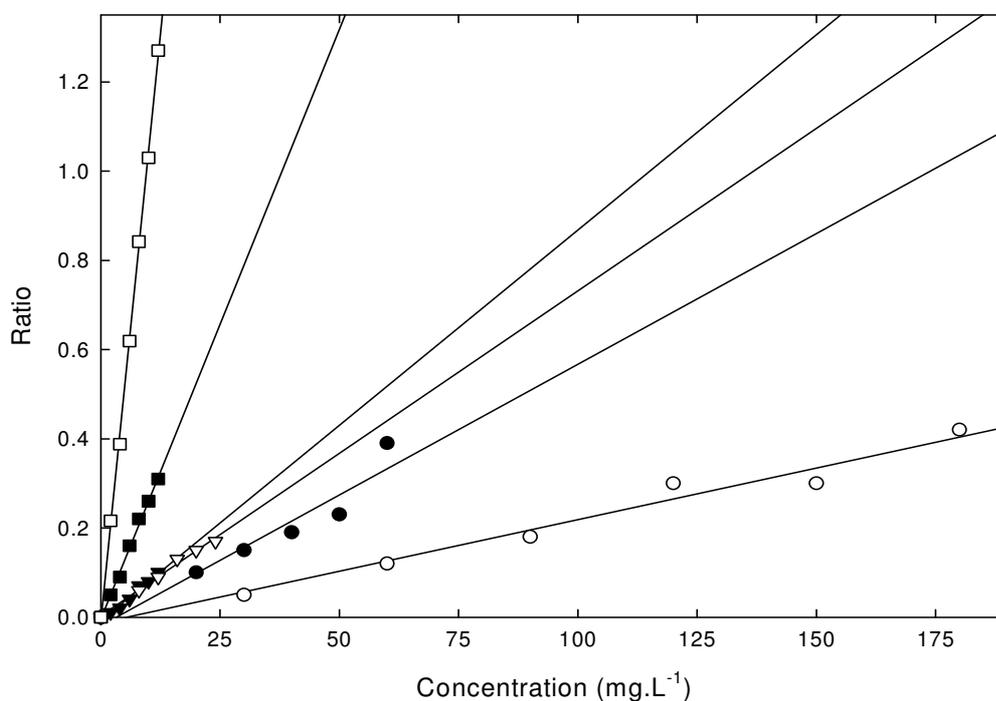


Figure 1. Linearity of the flame ionisation detector of the headspace gas chromatograph to increasing concentrations of acetaldehyde (●), ethanol (○), acetone (▼), diacetyl (▽), 2-butanone (■) and ethyl acetate (□).

was good and linear. In addition, the results show that HSGC is sensitive and can be used as an analytical tool for the detection of flavour compounds in Kefir.

Conclusion to HSGC method

In conclusion, it can be stated that the HSGC method as used in this study was found to be reliable, accurate and sensitive for the analysis of the flavour compounds of Kefir. It was thus decided that for analysis of the metabolites of Kefir samples, NaCl would be added to the solvent as it increases the volatilisation of the VOC in the headspace and enhances the precision of the HSGC.

Preliminary study

The data in Table 5 show the values of pH and TA found in Kefir prepared with either LG or MG Kefir grains and incubated at 25°C for 24 h.

The data in Table 5 show that the pH values of LG Kefir, as found in this preliminary study, varied between 4.07 and 5.08 and the TA was found to vary between 0.66% and 1.13 %. The TA values found were within the range (0.8 – 1.0%) reported in the literature, whereas the range of the pH is generally smaller (4.0 – 4.5) (Garrote *et al.*, 1998; Schoevers, 1999).

In the case of MG Kefir, the pH was higher (4.81 – 5.23) and the TA lower (0.52 – 1.05%) when compared to the data found in the literature (Beshkova *et al.*, 2002; Simova *et al.*, 2002). This suggests that the grains were not in their active state. The activation is a process whereby the microorganisms of Kefir grains, initially in the lag phase (inactive Kefir grains), reach their exponential growth phase (active Kefir grains) after successive transfers in milk.

From these results, it was observed that incubation at 25°C led to a rapid acidification where separation of the whey and the curd was visible. Additionally, the products had also a noticeable acidic taste. No ethyl acetate or 2-butanone was found. The results obtained for the flavour volatile compounds (acetic, acetaldehyde, ethanol, acetone, and diacetyl) showed that the concentrations of these compounds were in the same range for both LG Kefir and MG Kefir (Table 5). In addition, although diacetyl was present at concentrations between 1 and 2 mg.L⁻¹, the buttery taste imparted by diacetyl could not be detected in both types of Kefir. This could probably be ascribed to the presence of various acids such as lactic acid, which is known to mask the buttery flavour of a product (Vedemuthu, 2006).

It was thus decided that since the Kefir incubated at 25°C had a noticeable acidic taste and showed whey and curd separation, the Kefir, for the further studies, would be incubated at 22°C, which is known to be more favourable for the activity of diacetyl producing bacteria (Robinson, 1995). In addition, the inoculum size would be kept constant 20 g in 300 mL volumes instead of the 500 mL volumes. Under these environmental conditions, acidification would be slower and more uniform,

Table 5. Concentration ranges of volatile organics acids, volatile short chain fatty acids, pH and TA of Kefir prepared with LG and MG Kefir grains.

Kefir grains	Incubation temperature	Time (d)	pH	TA (% total acids)	mg.L ⁻¹					
					Acetic acid*	Diacetyl*	Acetaldehyde*	Ethanol*	Acetone*	Ethyl acetate*
LG	24 h at 25°C	9	5.08 ± 0.01 –	0.66 ± 0.01 –	458 ± 15.5 –	1.20 ± 0.15 –	2.88 ± 0.26 –	237.5 ± 12.1 –	1.24 ± 0.19 –	nd
			4.07 ± 0.005	1.13 ± 0.01	1394 ± 47.3	1.93 ± 0.19	17.6 ± 0.49	481.5 ± 20.5	2.28 ± 0.30	
MG	24 h at 25°C	9	5.23 ± 0.01 –	0.52 ± 0.005 –	468 ± 29.6 –	1.07 ± 0.11 –	2.84 ± 0.18 –	211.6 ± 22.3 –	1.22 ± 2.10 –	nd
			4.81 ± 0.01	1.05 ± 0.01	1018 ± 37.9	2.02 ± 0.54	11.0 ± 0.53	466.9 ± 6.96	2.10 ± 0.01	

(*)The data represent the mean ± the standard deviation.

The range represents the lowest and highest values obtained over 9 days.

nd = not detected

without leading to whey and curd separation and resulting in a beverage with the more acceptable buttery taste.

Metabolic profiles of Kefir prepared daily over a period of 9 days

The aim of this study was to determine the impact of controlled and uncontrolled incubation conditions that lead to the production of a good quality Kefir. To achieve this, the quantitative (metabolic profiles of VOCs, VFAs, pH and TA) and qualitative attributes of Kefir prepared with Kefir grains under controlled (22°C for 24 h) (LG22 Kefir and MG22 Kefir), and uncontrolled (home-made) (MG-home Kefir and Traditional Kefir) temperature conditions were studied.

pH and TA profiles

The profiles of pH and TA found for the Kefir prepared under controlled and uncontrolled temperature conditions and obtained over 9 days are shown in Fig. 2.

pH and TA profiles of Traditional Kefir and MG-home Kefir

It can be seen (Fig. 2) that the pH profile of the Traditional Kefir was found to drop to 4.25 by day 2. From day 3 to day 9, the pH stabilised, varying between 4.20 and 4.10. The pH profile of MG-home Kefir was similar to that of the Traditional Kefir: the pH was found to drop to 4.16 by day 2, then stabilised from day 3 to 9, ranging from 4.05 to 4.13.

The TA of Traditional Kefir increased to 0.80% by day 2 and then fluctuated for the rest of the study, with TA values ranging between 0.80 and 1.00%. For the MG-home Kefir, the TA increased up to 0.90 by day 3 and then steadily decreased for the remaining days, with TA values ranging between 0.82 and 0.92%. Both Kefirs had similar profiles.

pH and TA profiles of LG22 Kefir and MG22 Kefir

LG22 Kefir and MG22 Kefir had the same profiles (Fig. 2). The pH dropped to 4.23 and 4.1 by day 3, respectively for LG22 Kefir and MG22 Kefir, then stabilised until day 9. (3.9 – 4.1). The patterns of pH observed for LG22 Kefir and MG22 Kefir were similar to the pattern of Traditional Kefir. However, it can be noticed that Kefir made under controlled temperature condition had a slightly lower pH than both Traditional Kefir and MG-home Kefir.

Under these controlled temperature conditions, the TA of LG22 Kefir, increased to 0.83%, then plateaued with values ranging between 0.90 and 1.10%. For MG 22 Kefir, the TA steadily increased till day 6 before slightly decreasing, with values ranging between 0.60 and 1.00%. These profiles are similar to the profile of the Traditional Kefir and those found in the literature (Garotte *et al.*, 1998; Schoevers, 1999; Chen *et al.*, 2009).

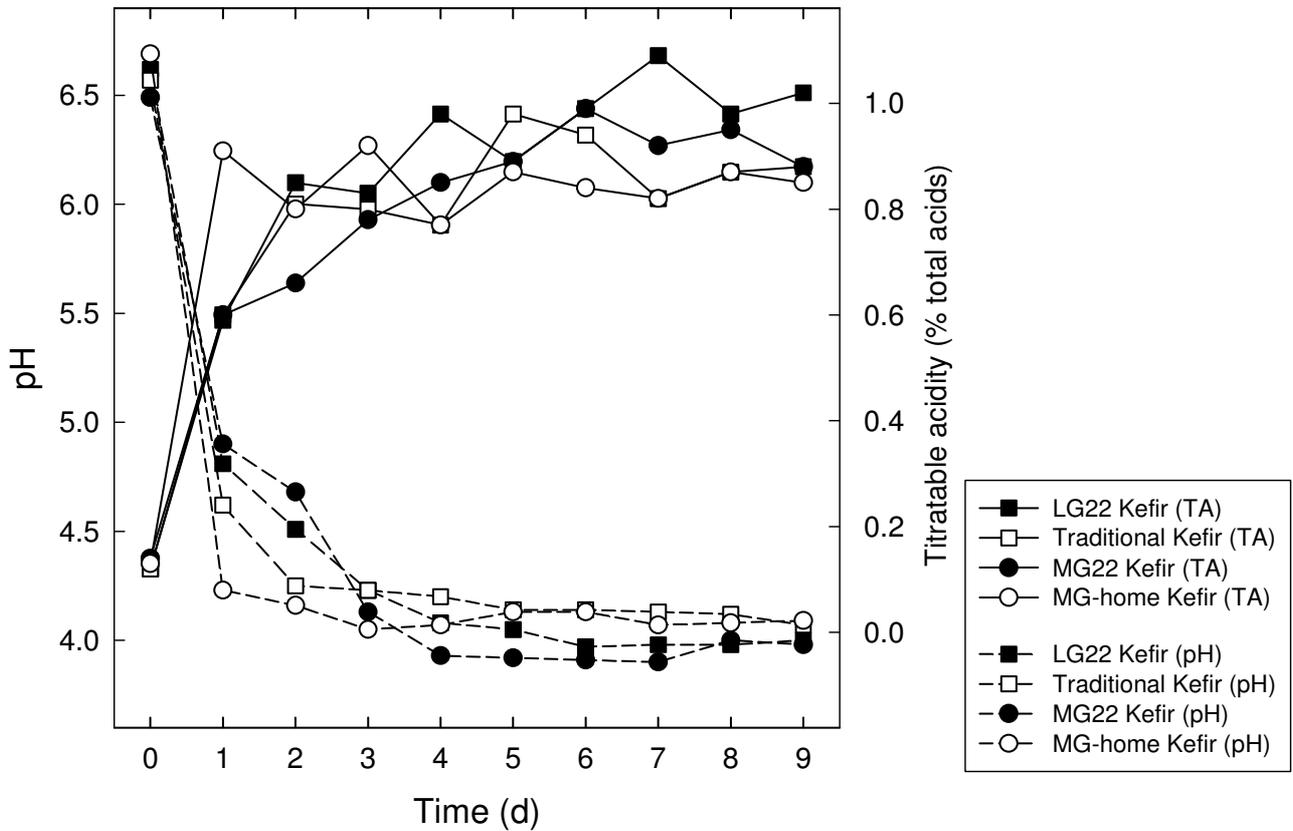


Figure 2. Impacts of the different incubation temperature conditions (controlled and uncontrolled) on pH and titratable acidity found in Kefir prepared with LG, TG and MG over 9 days. The values are the mean of three replicates and the variations (standard deviation) are not shown because they are below 10%.

In conclusion, the incubation temperature conditions did not seem to drastically affect the pH and the TA of Kefir prepared with LG and MG since the pH and the TA found were generally within the range found for the Traditional Kefir. Additionally, the pH and TA profiles of LG and MG showed similarities with the pH and TA profiles of the Traditional Kefir suggesting similarities in the microbial population involved in acid production.

The importance of the acid in Kefir is due to lactic acid, which is essentially responsible for the acidic taste of Kefir, but also acts as a preservative inhibiting many spoilage and pathogenic bacteria (Vedemuthu, 2006).

Metabolic profiles of VOCs and VFAs

Acetaldehyde profile

The profiles of acetaldehyde concentrations found in Kefir prepared under controlled and uncontrolled temperature conditions and obtained over 9 days are illustrated in Fig. 3.

Acetaldehyde profile of the Traditional Kefir and MG-home Kefir

The Traditional Kefir was prepared with Kefir grains (TG) that had been used for a long period of time. Therefore, it was assumed that the microbial population had stabilised (Prof. Britz, T.J, Food Science Department, Stellenbosch, South Africa, Personal Communication, 2005).

In Fig. 3, it can be seen that the concentrations of acetaldehyde, produced in the Traditional Kefir, varied between 2.0 and 3.5 mg.L⁻¹ and the profile obtained was generally stable throughout the 9 days study period. These levels of acetaldehyde fall within the range (0.4 to 10 mg.L⁻¹) reported in the literature (Gawel & Gromadka, 1978; Güzel-Seydim *et al.*, 2000; Beshkova *et al.*, 2003; Magalhães *et al.*, 2011a).

The data in Fig. 3 also show that the concentrations of acetaldehyde found in the MG-home Kefir were found to vary drastically between 7.63 and 20.5 mg.L⁻¹. A general decrease from day 1 to day 4 was observed, followed by a stabilisation phase (8.1 – 10.1 mg.L⁻¹). On the contrary to the Traditional Kefir, levels of acetaldehyde found in the first few days are outside the range (0.4 to 10 mg.L⁻¹) found in the literature (Güzel-Seydim *et al.*, 2000; Wszolek *et al.*, 2001; Beshkova *et al.*, 2003; Magalhães *et al.*, 2011a). This could be attributed to the fact that the MG microbial population was probably not stabilised yet. It must also be taken into account that with the MG-home Kefir, temperature was not controlled and this could also have influenced the formation of acetaldehyde.

Acetaldehyde profiles of LG22 Kefir and MG22 Kefir

For the LG22 Kefir, it was found that a fairly slow increase in acetaldehyde concentrations occurred from day 1 to 5, followed by a faster increase from day 5 to 7. This increase was followed by a

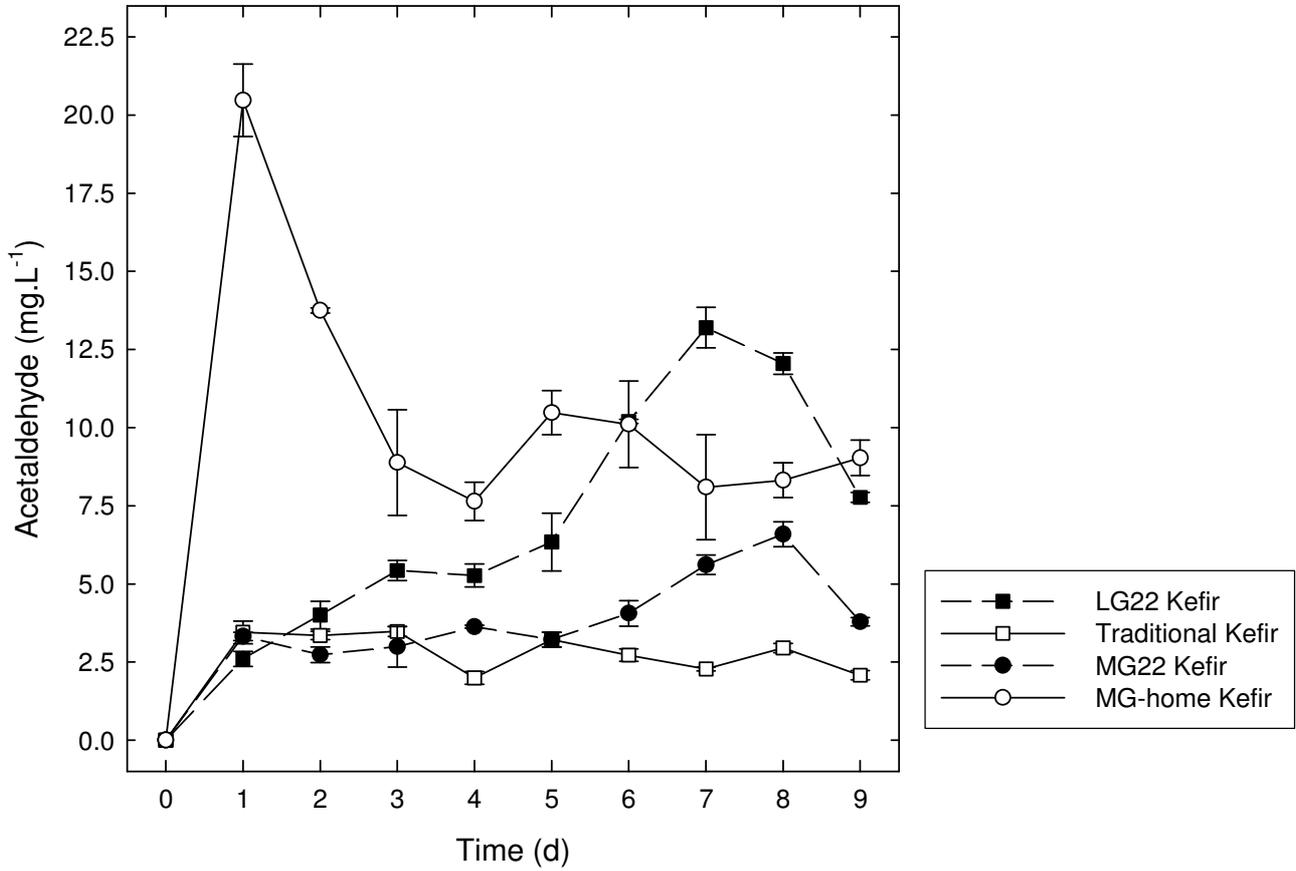


Figure 3. Impacts of the incubation temperature conditions (controlled and uncontrolled) on acetaldehyde concentrations found in Kefir prepared with LG, TG and MG over 9 days. Error bars represent the standard deviation.

decrease to 7.77 mg.L⁻¹ (Fig. 3).

In the MG22 Kefir, acetaldehyde concentrations were found to vary between 2.7 and 3.63 mg.L⁻¹, the first four days. From day 5, the concentration of acetaldehyde increased to 6.6 mg.L⁻¹ (day 8) and finally decreased to 3.8 mg.L⁻¹ by day 9. These patterns are fairly similar but higher than the pattern for the Traditional Kefir.

The decrease in acetaldehyde observed can be explained by its conversion into ethanol, acetic acid or acetyl CoA (Chaves *et al.*, 2002; Ott *et al.*, 2002).

The highest concentrations of acetaldehyde were found on day 7 (13.2 mg.L⁻¹) for LG22 Kefir and on day 8 (6.6 mg.L⁻¹) for MG22 Kefir. The corresponding pH observed for those days were 3.98 and 4.0 (Fig. 2). According to Tamime & Robinson (1999), the production of acetaldehyde in yoghurt is maximal at a pH of 4.2 and stabilises at pH 4.0; consequently it was assumed that the presence of higher concentrations in the different profiles (Fig. 3) were linked to the pH of the growth environment. One difference found was that the Traditional Kefir had a pH around 4.0 and 4.2 but the profile of the Traditional Kefir did not show similar peaks as observed in the profiles of Kefir prepared with LG22 Kefir and MG22 Kefir. Therefore, it was assumed that the pH was not the only factor that contributed to the presence of these higher concentrations. This was confirmed in the Kefir prepared with MG and incubated at 25°C for 24 h (preliminary study) where the higher concentration of acetaldehyde was found on day 9 (11 mg.L⁻¹) for a pH of 4.8. Additionally, these maximum concentrations of acetaldehyde cannot be attributed to the increase in the microbial population of the Kefir grains because the doubling time of lactic acid bacteria does not permit the microbial population to increase so fast. It can also be, as mentioned by Koroleva (1991), that Kefir grains could self-regulate their microbial population after being mishandled. Consequently, the presence of these peaks and more generally, the pattern of acetaldehyde observed, might suggest that a self regulation mechanism is active in the grains. This self-regulation would consist of a self adjustment of the microbial population (Koroleva, 1991). However, it is not clear what mechanisms are put in place within Kefir grains for self-regulatory mechanism to take place.

The presence of acetaldehyde is responsible for the “yoghurt flavour” in fermented dairy products (Bodyfelt *et al.*, 1988). Yoghurt is well known for having a strong yoghurt flavour due to high concentrations of acetaldehyde (2 – 41 mg.L⁻¹) produced by the synergistic effect of *Lb. delbrueckii* and *St. thermophilus* (Tamime & Robinson, 1999). In Kefir, which has a balanced flavour, high concentrations of acetaldehyde can impart a strong yoghurt flavour. This was observed in a study of Kefir prepared with a starter culture of *Streptococcus thermophilus* T15, *Lactococcus lactis* C15, *Lactobacillus helveticus* MP12 and *Saccharomyces cerevisiae* A. The acetaldehyde concentration was 18 mg.L⁻¹ compared to Kefir prepared with Kefir grains where the acetaldehyde concentration was 9 mg.L⁻¹ (Beshkova *et al.*, 2002; 2003). However, in Kefir, the presence of acetaldehyde is not directly

linked to the presence of typical yoghurt's microorganisms since some Kefir grains do not contain them (Lin *et al.*, 1999; Garrote *et al.*, 2001; Witthuhn *et al.*, 2005; Magalhães *et al.*, 2011b).

Ethanol profile

The profiles of ethanol concentrations found in Kefir prepared under controlled and uncontrolled temperature conditions over 9 days are illustrated in Fig. 4.

Ethanol profiles of the Traditional Kefir and MG-home Kefir

In the Traditional Kefir, the concentrations of ethanol varied between 99 and 207 mg.L⁻¹ and the profile of ethanol concentrations shows a constant ethanol production over the 9 days (Fig. 4).

In the MG-home Kefir, from day 1 to 5, a sharp decrease in ethanol concentrations was observed (3 351 to 1 131 mg.L⁻¹), followed by a gradual increase to 2 028 mg.L⁻¹ by day 9. This profile is very different from the Traditional Kefir ethanol profile.

Ethanol profiles of LG22 Kefir and MG22 Kefir

In the LG22 Kefir, a continuous increase in ethanol was found up to day 8 (2 433 mg.L⁻¹), after which there was a decrease (Fig. 4). It is not unusual for ethanol concentrations to be so high in Kefir as Marshall (1984) reported that Kefir may contain ethanol concentrations of up to 2 000 mg.L⁻¹ and similar or even higher ethanol concentrations (1 700 to 9 000 mg.L⁻¹) were reported (Kuo & Lin, 1999; Beshkova *et al.*, 2003; Magalhães *et al.*, 2011a). However, it is surprising that the same set of grains produced a Kefir beverage with such distinctively different concentrations of ethanol. Indeed, Kefir grains (LG) used for this profile (22°C for 24 h) were the grains previously used for the preparation of Kefir incubated at 25°C for 24 h (preliminary study), where the concentration of ethanol varied between 287 and 481 mg.L⁻¹. One explanation for the high concentrations could be as the result of increased yeast growth and metabolic activities at the lower temperatures. The same explanation is applicable to the huge differences in ethanol concentrations observed between the Traditional Kefir (99 – 207 mg.L⁻¹) and the LG Kefir incubated at 22°C for 24 h (784 – 2433 mg.L⁻¹). Additionally, it is important to note that lactobacilli species such as *Lactobacillus kefir*, which has been isolated from different Kefir grains (Garrote *et al.*, 2001; Chen *et al.*, 2008, Pedrozo Miguel *et al.*, 2010; Magalhães *et al.*, 2011b) is able to produce up to 2 500 mg.L⁻¹ ethanol in Kefir (Marshall, 1984). Consequently, even if Kefir grains do not contain yeasts, ethanol could still be produced by the LAB population.

In the MG22 Kefir, the concentrations of ethanol remained fairly stable, then gradually increased to reach a peak (640 mg.L⁻¹) on day 8, followed by a decrease (Fig. 4). The patterns observed for ethanol were very similar to the pattern of Traditional Kefir.

A study by Witthuhn *et al.* (2005) showed that in Kefir made on an uninterrupted basis from

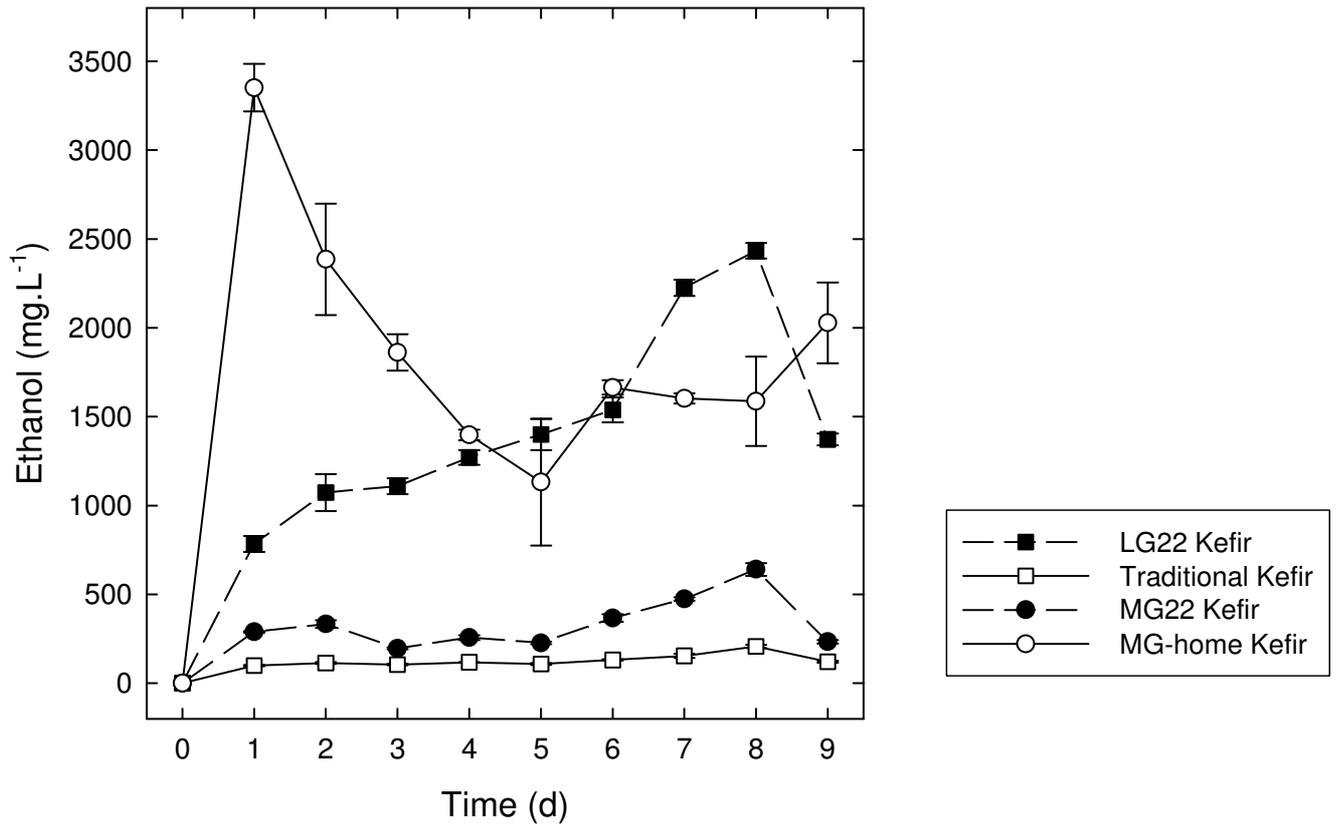


Figure 4. Impacts of the incubation temperature conditions (controlled and uncontrolled) on ethanol concentrations found in Kefir prepared with LG, TG and MG over 9 days. Error bars represent the standard deviation.

MG and TG grains, the proportion of yeasts was higher in MG Kefir grains compared to TG Kefir grains. This could explain the higher concentrations of ethanol and acetaldehyde observed throughout the study period in MG-home Kefir and MG22 Kefir compared to the Traditional Kefir.

The profiles of the ethanol and acetaldehyde concentrations gave similar patterns, which suggests, as previously mentioned for acetaldehyde, that self regulation takes place, and possibly affects all groups of microorganisms, including the alcohol producers.

The general decrease observed in ethanol and acetaldehyde profiles in MG-home Kefir (Fig. 3 and Fig. 4) could originate from a microbial self stabilisation process inside the grains.

The presence of ethanol is essential as it imparts the typical yeasty flavour found in Traditional Kefir. Some authors believe that Traditional Kefir must have a slight yeasty flavour (Vedemuthu, 1977; Kroger, 1993), whereas others (Beshkova *et al.*, 2003) do not support this view. In other cases, a too strong yeasty flavour is seen as a defect and it is believed to be caused by the presence of yeast such as *Saccharomyces cerevisiae* (Wszolek *et al.*, 2006).

Diacetyl profile

The profiles of diacetyl concentrations found in Kefir prepared under controlled and uncontrolled temperature conditions over 9 days are illustrated in Fig. 5.

Diacetyl profile of the Traditional Kefir and MG-home Kefir

The profile of the diacetyl formation (3.4 to 6.0 mg.L⁻¹) found in the Traditional Kefir is illustrated in Fig. 5. No diacetyl was found in MG-home Kefir, it was possible that the concentrations of diacetyl present were below the detection limit of 0.30 mg.L⁻¹.

The balance between different flavour compounds, particularly acetaldehyde and diacetyl must be present to obtain a good flavour. Indeed, in most good quality cultured dairy products, the ratio of diacetyl to acetaldehyde varies between 3 and 5 (Sandine *et al.*, 1972). However, in this study, the ratios found for the Traditional Kefir varied between 0.75 and 1.75 suggesting that a lower ratio might be more applicable to this Kefir. In the literature of Kefir, the ratio of diacetyl to acetaldehyde can vary between 0.10 and 1.24 (Gawel & Gromadka, 1978; Wszolek *et al.*, 2001; Beshkova *et al.*, 2003). Such differences in the ratio originate from the differences in the microbial composition of Kefir grains as well as the inoculum size used, which affect the qualitative and quantitative composition of Kefir.

Diacetyl profiles of LG22 Kefir and MG22 Kefir

For unknown reasons, diacetyl was not detected in LG22 Kefir (Fig. 5). It was possible that the concentrations of diacetyl were below the detection limit of 0.30 mg.L⁻¹. In the MG22 Kefir, the diacetyl concentration was found to vary between 1.70 and 3.87 mg.L⁻¹ to finally decrease to 1.85 mg.L⁻¹ by day 9 (Fig. 5).

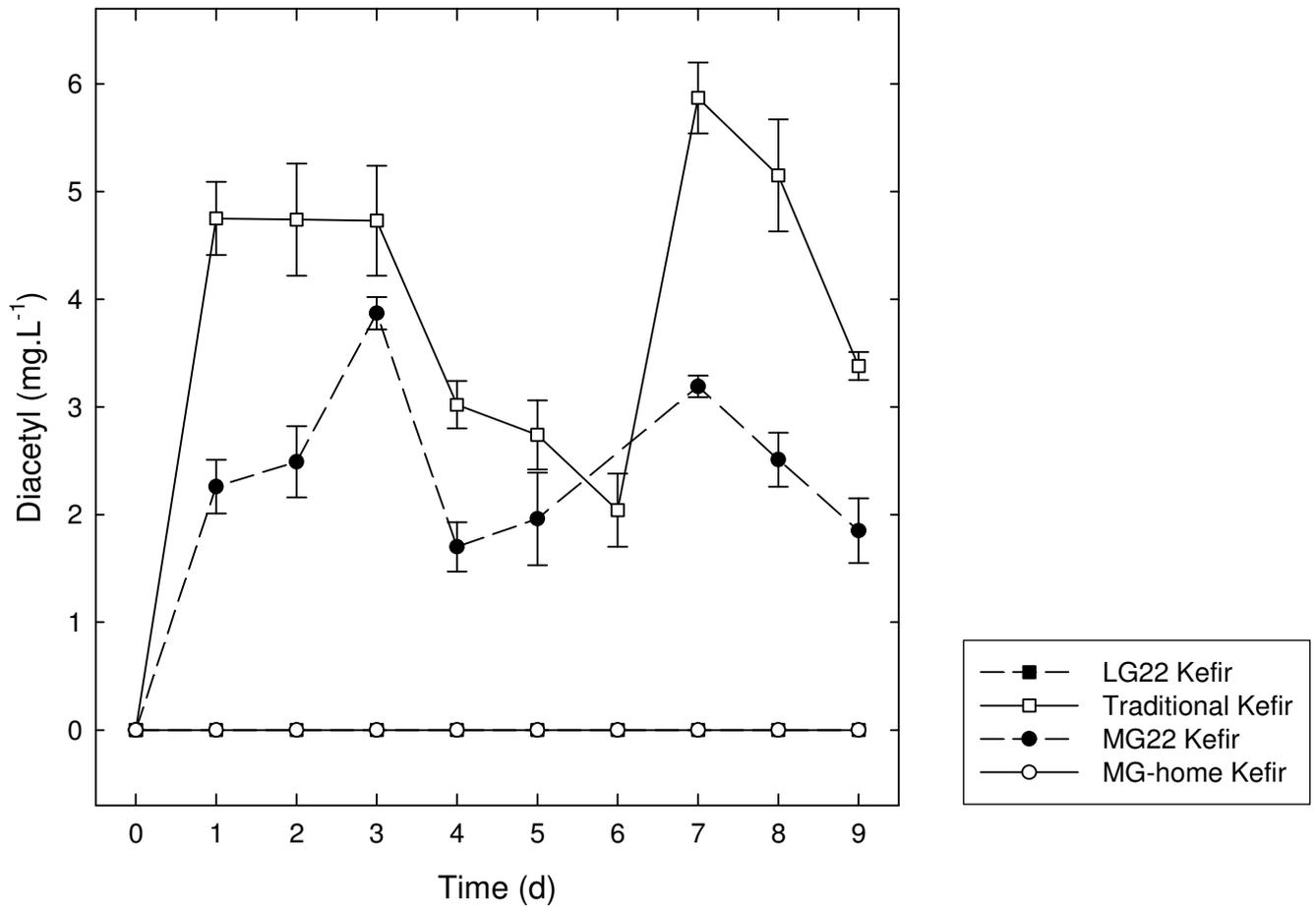


Figure 5. Impacts of the incubation temperature conditions (controlled and uncontrolled) on diacetyl concentrations found in Kefir prepared with LG, TG and MG over 9 days. Error bars represent the standard deviation.

The concentrations in diacetyl found in the Traditional Kefir were higher than those found in MG22 Kefir. This might be ascribed to higher numbers of diacetyl producers in the Kefir grains used to make the Traditional Kefir compared to MG22 Kefir. Additionally, the presence of diacetyl was not surprising because the pH was favourable ($4.90 < \text{pH} < 3.90$) for diacetyl production. Research has shown that the initiation of diacetyl production is pH dependant and starts with the activation of the citrate carrier citrate permease Cit P, which is activated at a pH range of 6.0 to 5.5 (Collins, 1972; García-Quintáns *et al.*, 1998). Additionally, the presence of diacetyl indirectly suggests the presence of aroma forming bacteria such as *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* ssp. *cremoris* among the microbial population of Kefir grains.

Furthermore, although the presence of diacetyl is reported in the literature to be pH dependant (Cogan, 1975; García-Quintáns *et al.*, 1998), the concentrations of diacetyl found in this study did not appear to be pH dependant. In other words, for similar pH values, the concentrations of diacetyl varied widely. This finding also applies to the Traditional Kefir.

In the study of the reproducibility and repeatability of the headspace gas chromatography method, the data obtained in Table 4 shows that the CVs of diacetyl varied between 14.3 and 24.1%. This means that the concentrations detected by the GC (the solvent was milk) were not very precise. Indeed, in gas chromatography, it is generally accepted that a high reproducibility and repeatability is characterised by a coefficient of variation (CV) below 10% (Green & Payne, 1989; Xanthopoulos *et al.*, 1994). Consequently, the variations observed in the Traditional Kefir and the MG 22 Kefir may probably have been caused by the poor precision of the GC method when the medium was milk.

According to Vedemuthu (2006), the diacetyl concentrations in cultured buttermilk and sour cream are usually at their highest when the titratable acidity (TA) reaches 0.75 – 0.80%. This assumption appears to be applicable to Kefir, since the highest diacetyl concentrations, in the Kefir incubated at 22°C for 24 h, 3.87 mg.L⁻¹ (MG 22 Kefir) and the Traditional Kefir, 5.87 mg.L⁻¹ were obtained, when the TA values were 0.78% and 0.82% (Fig. 2), respectively. However, the highest production of diacetyl was observed on day 7 (TA = 0.82%). This suggests, as previously mentioned for acetaldehyde and ethanol that a self regulation might possibly be taking place within the grains.

Acetic acid profile

The profiles of acetic acid concentrations found in Kefir prepared under controlled and uncontrolled temperature conditions over 9 days are illustrated in Fig. 6.

Acetic acid profiles of the Traditional Kefir and MG-home Kefir

The acetic acid concentrations in the Traditional Kefir increased for 3 days then stabilised at around 600 mg.L⁻¹ (Fig. 6). In the MG-home Kefir, acetic acid concentrations varied between 363

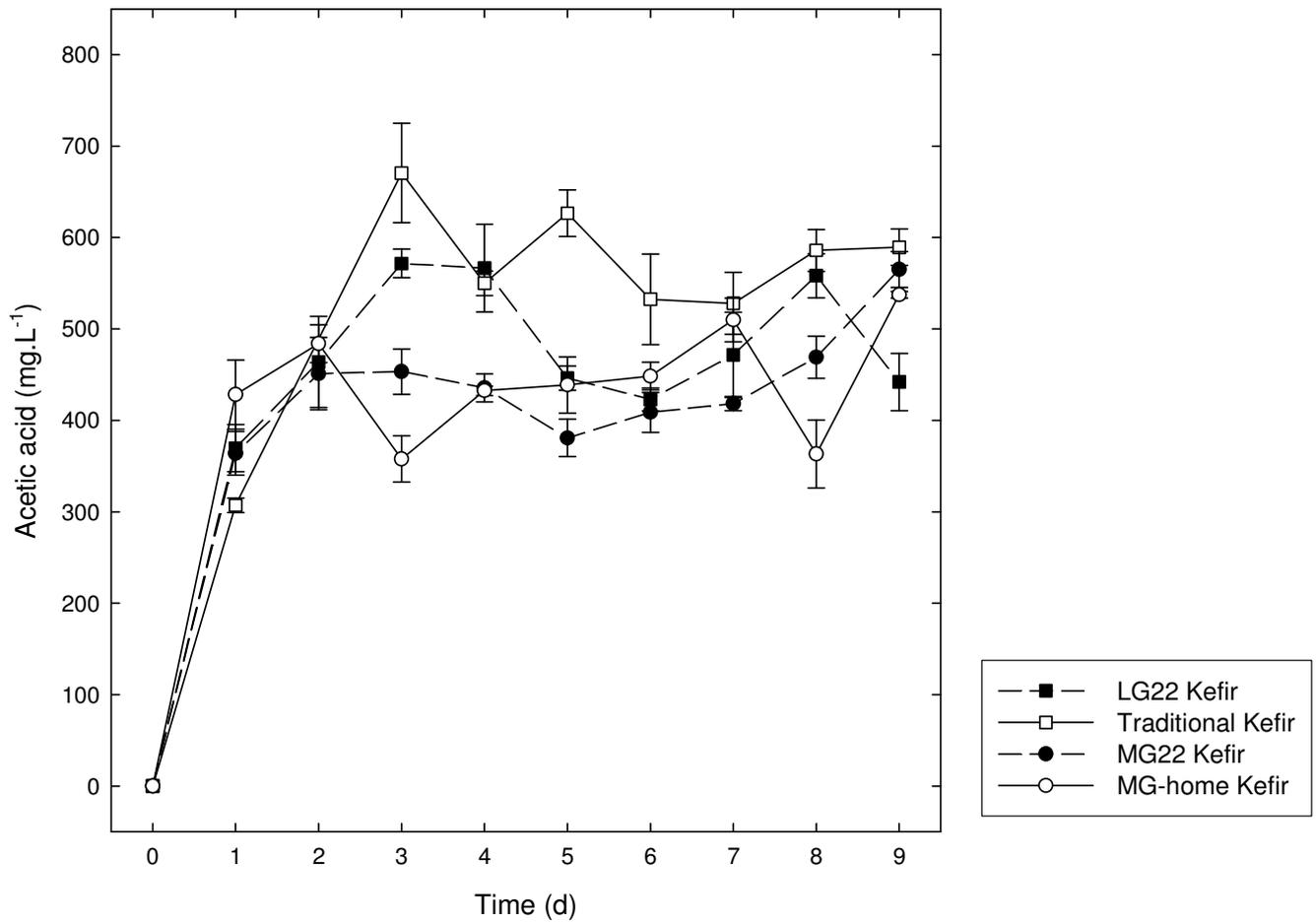


Figure 6. Impacts of the incubation temperature conditions (controlled and uncontrolled) on acetic acid concentrations found in Kefir prepared with LG, TG and MG over 9 days. Error bars represent the standard deviation.

and 537 mg.L⁻¹; and were lower than the concentrations found for the Traditional Kefir.

Acetic acid profiles of LG22 Kefir and MG22 Kefir

In the LG22 Kefir, the concentrations of acetic acid varied between 370 and 572 mg.L⁻¹; and the pattern was similar to the pattern of the Traditional Kefir (Fig. 6). In the MG22 Kefir, the acetic acid concentrations increased from day 1 to 3 (from 364 to 453 mg.L⁻¹) and remained fairly stable with a slight increase by day 9 (565 mg.L⁻¹) (Fig. 6). Both profiles had generally lower acetic acid concentrations than those found in the Traditional Kefir.

Acetic acid formation probably has multiple origins in Kefir but the activity of heterofermentative LAB through citrate catabolism and heterofermentation could be the main pathways leading to the formation of this organic acid in Kefir.

Acetic acid is generally considered an organic acid with a preservative effect but is also a contributor to the flavour of Kefir, but to a lesser extent than found with lactic acid. However, excessive production of acetic acid in Kefir is considered a serious defect, which imparts an undesirable “vinegar aroma” (Wszolek *et al.*, 2006) to the final beverage. It is difficult to say at which concentration, acetic acid would cause a defect because the typical flavour of Kefir does not depend on one compound but originates from the balance between diacetyl and several other compounds (acetic acid, acetaldehyde, ethanol and acetone).

Acetone profile

The profiles of acetone concentrations found in Kefir prepared under controlled and uncontrolled temperature conditions over 9 days are illustrated in Fig. 7.

Acetone profiles of the Traditional Kefir and MG-home Kefir

The concentration of acetone found in the Traditional Kefir was fairly low and varied between 1.06 and 2.96 mg.L⁻¹. No acetone was found in the MG-home Kefir (Fig. 7).

Acetone profiles of LG 22 Kefir and MG 22 Kefir

No acetone was found in the LG22 Kefir (Fig. 7). The concentrations might have been too low to be detected using the HSGC parameters of this study. However, in the MG22 Kefir, the acetone concentrations were found to vary between 1.41 and 3.00 mg.L⁻¹ and similar to the variations observed for diacetyl (Fig. 5). Such a similarity suggests that the presence of acetone was essentially due to the microorganisms involved in diacetyl production (e.g. *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*) and therefore that acetone probably originates from citrate metabolism. These results are in agreement with the findings of Bills & Day (1966) and Keenan *et al.* (1967), who reported that acetone was synthesised by dairy lactococci and leuconostoc. However, it cannot be excluded that lactobacilli species such as *Lb. bulgaricus* and *Lb. helveticus* could have

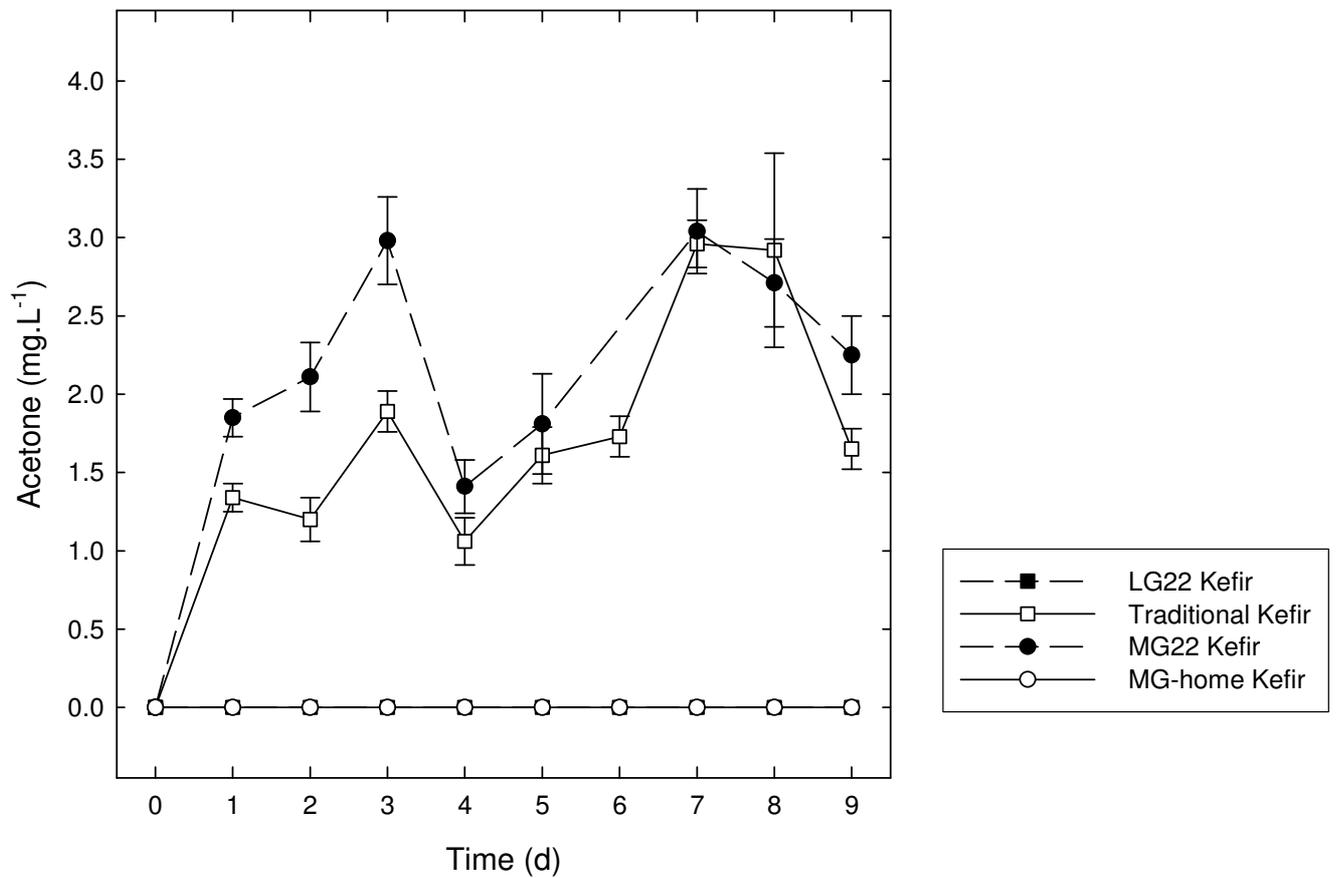


Figure 7. Impacts of the incubation temperature conditions (controlled and uncontrolled) on acetone concentrations found in Kefir prepared with LG, TG and MG over 9 days. Error bars represent the standard deviation.

also contributed to synthesis of acetone (Blanc, 1984; Beshkova *et al.*, 2003) as they have been shown to be present in the grains used in this study (Cronjé, 2003; Garbers, 2003).

Acetone is not known to be an important flavour contributor in fermented dairy products (Keenan *et al.*, 1967; Blanc, 1984). Therefore, the impact of acetone on the flavour of Kefir can be considered negligible. However, the presence of concentrations of acetone higher than those usually found ($< 5 \text{ mg.L}^{-1}$) in Kefir (Liu *et al.*, 2002; Beshkova *et al.*, 2003) can impart a flavour defect known as “cowy”, which is characterised by an unpleasant and lingering after taste (Bodyfelt *et al.*, 1988).

Ethyl acetate profile

No ethyl acetate (EA) was found in any types of the Kefir prepared under controlled and uncontrolled conditions. The presence of EA ($0.02 - 10 \text{ mg.L}^{-1}$) has previously been mentioned in both Kefir prepared with Kefir grains and with a starter culture (Beshkova *et al.*, 2003; Aghlara *et al.*, 2009; Magalhães *et al.*, 2011a).

Although EA is an ester that imparts a fruity flavour (Liu *et al.*, 2004; Magalhães *et al.*, 2011a), this often goes unnoticed due to the acidity. This was confirmed in Kefir prepared with Kefir grains and different types of milk (bovine, caprine and ovine) where the fruity attribute obtained the lowest score (3.0 – 5.0) compared to the acid/sour attribute (18 – 43) (Wszolek *et al.*, 2001; Irigoyen *et al.*, 2005).

Gas chromatographic detection of DL-lactic acid

DL-lactic acid (DL-LA) could be detected (Fig. 8) but the repeatability and reproducibility of this compound, as expressed by the coefficient of variation (CV%), were found to be high ($> 20\%$). It is generally accepted that in gas chromatography, a high reproducibility and repeatability is characterised by a coefficient of variation (CV) below 10% (Green & Payne, 1989; Xanthopoulos *et al.*, 1994). The results obtained in this study clearly showed a lack of accuracy and precision when using the method of Yang & Choong (2001), which is in contrast with the good results that they obtained with direct injection (CV $< 9.4\%$). A likely explanation for the high CV% may be ascribed to the fact that in presence of high temperature, DL-LA is highly unstable (possibility of dehydration); and it is highly polar causing non-reproducible interactions (Brotz & Schaefer, 1987; Richardson *et al.*, 1989; Dr Scheffer, A, Senior analytical chemist, Haward Technology, Abu Dhabi, Unites Arab Emirates, Personal Communication, 2004).

It has been reported that stabilisation of DL-LA can be achieved by derivatization, (Schooley *et al.*, 1985; Xiao *et al.*, 2007) and good results (CV $< 8.70\%$) have been reported in the literature (Schooley *et al.*, 1985; Buglass & Garnham, 1991). However, in this study, derivatization was not evaluated since it is time consuming (Richardson *et al.*, 1989) and the requirement of this study was to develop an accurate reference method for quantification of DL-LA in Kefir.

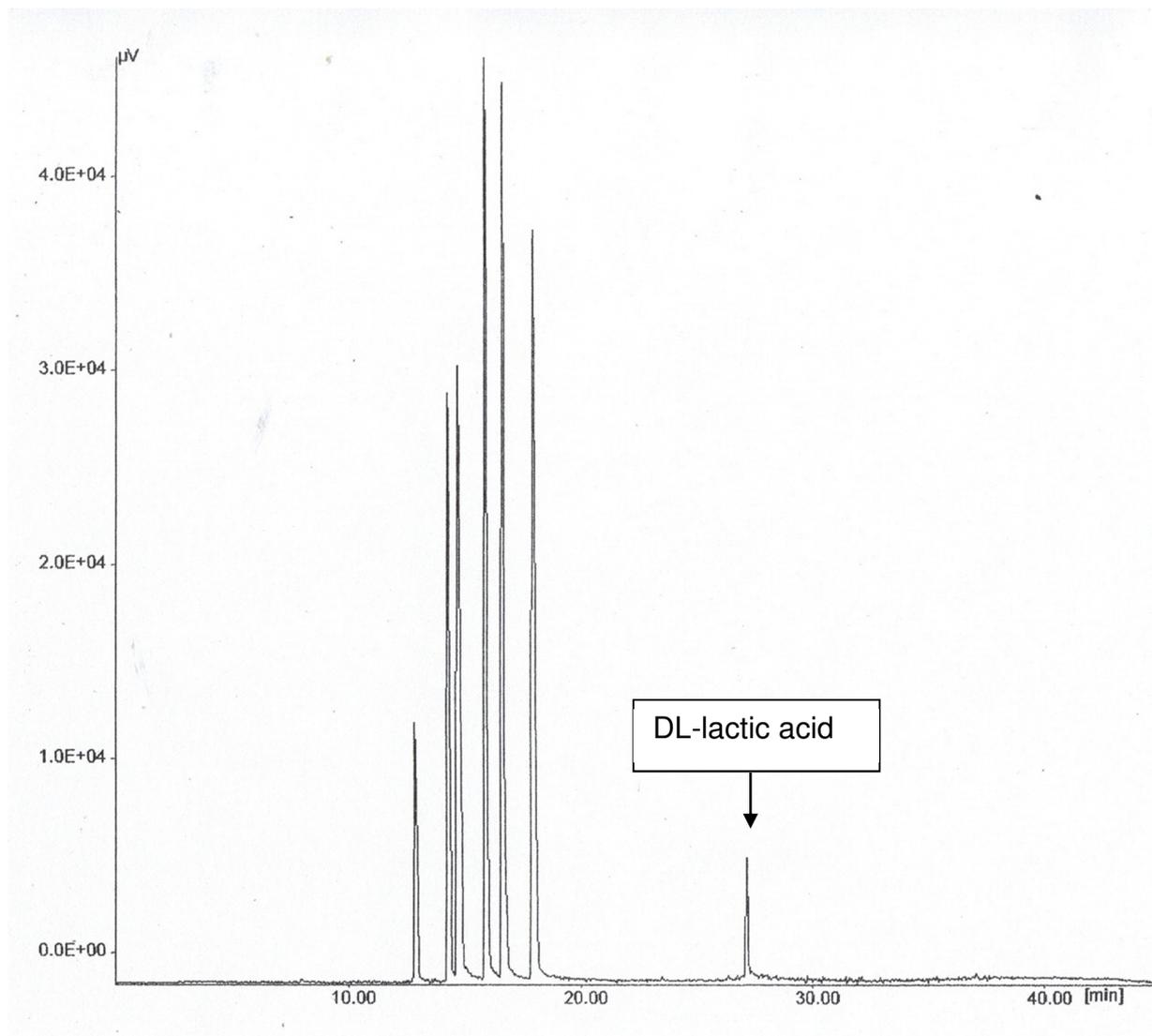


Figure 8. Qualitative detection of DL-Lactic acid in mixture with short chains fatty acids (DL- lactic, acetic, propionic, isobutyric, butyric, iso-valeric and valeric acids).

Conclusions

For the manufacture of a good quality Kefir product with sensory attributes that will be acceptable to the consumer, it is essential to optimise the environmental factors. It is also of importance to know what the flavour compound variations would be if different Kefir grains are used under different environmental conditions. In addition, knowing which flavour contributing compounds are formed and in what concentrations they can be found is also important for assessing the quality and preventing flavour defects.

In this regard, incubation at 25°C, led to over acidification. The latter is a major problem since it can mask the buttery flavour of a product (Vedemuthu, 2006) and affect the sensory acceptability of the final product. Thus, incubation at 22°C was chosen as incubation temperature for Kefir prepared under controlled conditions. However, the inoculum size was kept at 20 g of Kefir grains in 300 mL pasteurised milk for both Kefir prepared under controlled and uncontrolled conditions.

Amongst all Kefir produced, Traditional Kefir presented the best characteristics (acidic ‘background’ associated with buttery flavour). This probably originates from the fact that under home-made conditions, the temperature is not controlled and fluctuates between 15° and 28°C throughout 24 h. This fluctuation allows yeasts and bacteria to take part in the fermentation process by producing flavour compounds. On the contrary, by fermenting at the same temperature for 24 h, not all microorganisms will actively be involved in the fermentation due to the fact that the chosen incubation temperature will not be the optimum growth temperature for all microorganisms present in the grains.

The metabolic profiles of Kefir produced with the different Kefir grains under controlled and uncontrolled conditions over 9 days, presented similarities in terms of acidity but differences in terms of flavour compound production. Each Kefir type had its own sensory identity (aroma / flavour), depending on the flavour compounds present and their concentration. It was noticed that the balance between diacetyl and acetaldehyde in Kefir prepared with mass cultured Kefir grains (MG22 Kefir) was not achieved when compared to the balance found in the Traditional Kefir. This highlights the need to promote the production of diacetyl in MG22Kefir. This could be achieved by stabilising the microbial population of mass cultured Kefir grains with selected diacetyl producing starter cultures. Alternatively, this could also be done by supplementing milk with appropriate substrates or precursors. In addition, since DL-lactic acid is a very important contributor to flavour, as well as acetic acid to a minor extent, an accurate and quick detection method would be beneficial for the quantification of DL-lactic acid and acetic acid in Kefir, as well as the acidity parameters pH and titratable acidity (TA).

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CHAPTER 4

QUANTITATIVE ANALYSIS OF DL-LACTIC ACID AND ACETIC ACID IN KEFIR USING NEAR INFRARED REFLECTANCE SPECTROSCOPY

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Abstract

To be able to monitor in real time the concentrations of DL-lactic acid and acetic acid present in Kefir, a self-carbonated fermented milk product, near infrared (NIR) calibration models were constructed based on NIR spectra of 174 samples. Enzymatic tests and gas liquid chromatography were the reference methods used for DL-lactic acid and acetic acid, respectively. The fit of the models and their prediction power were evaluated using segmented cross-validation and an external validation set. The models obtained for DL-lactic acid were found to be acceptable for both cross-validation ($R^2 = 0.90$, $SECV = 0.110 \text{ g} \cdot 100 \text{ mL}^{-1}$ and $RPD = 3.16$) and external validation ($R^2 = 0.87$, $SEP = 0.156 \text{ g} \cdot 100 \text{ mL}^{-1}$ and $RPD = 2.57$). In contrast, the models for acetic acid were found to be unacceptable. The results obtained for both cross-validation ($R^2 = 0.80$, $SECV = 0.013 \text{ g} \cdot 100 \text{ mL}^{-1}$ and $RPD = 2.21$) and external validation ($R^2 = 0.44$; $SEP = 0.017 \text{ g} \cdot 100 \text{ mL}^{-1}$ and $RPD = 1.17$) suggested this model requires further development. The application of principal component analysis (PCA) to the entire sample set showed Kefir prepared with laboratory Kefir grains (LG), traditional Kefir grains (TG) and mass cultured Kefir grains (MG) resulted in similar PCA score values in spite of the Kefir grains not having the same origin. PCA was also able to differentiate between fermented and 'milky body-like' samples. The findings of this study could serve the dairy industry in monitoring more efficiently the acidity in terms of DL-lactic acid of fermented dairy products.

Introduction

Kefir is a self-carbonated nutritious fermented milk traditionally made by incubating a starter culture called Kefir grains in milk (Schoevers & Britz, 2003). The activity of the unique microbial consortium present in the grains results in a beverage with a refreshing characteristic taste imparted by lactic acid, ethanol, carbon dioxide, acetaldehyde, acetone and diacetyl as well as other minor metabolites.

The measurement of the production of metabolites is the most reliable method for monitoring the different fermentation phases. For example, acidification is an important phase of the fermentation process because it leads to the production of lactic acid, which is responsible for the acidic taste of fermented dairy products. Therefore, monitoring key parameters such as acidity through measurement of lactic acid is of utmost importance as over-acidification causes flavour

defects due to the excessive production of lactic acid (Vedemuthu, 2006). Currently, acidity is monitored by pH and titratable acidity (TA) but these do not indicate the concentration of lactic acid present in the sample.

Near infrared (NIR) spectroscopy has proven to be an important rapid, non-destructive technology for the food industry (Woodcock *et al.*, 2008), requiring little sample preparation, no chemical treatments and producing no toxic waste. The dairy industry has used NIR spectroscopy for a number of decades in a broad range of applications. It has been used for the quantitative determination of the major components of milk (lactose, fats, proteins, total solids, moisture) and milk products such as milk powder, cheese, cream cheese, yoghurts and related products (Díaz-Carrillo *et al.*, 1993; Rodríguez-Otero & Hermida, 1996) NIR spectroscopy has also been used for qualitative purposes such as discrimination of ripened cheeses according to maturity (young, matured and extra-matured cheeses (Frankhuisen, 1992), determination of the degree of freshness (fresh, intermediate and old cheeses) (Cattaneo *et al.*, 2005), and identification of geographical origin (Karoui *et al.*, 2005). Sensory attributes and instrumental texture measurements of dairy products have also been predicted using NIR spectroscopy (Downey *et al.*, 2005; Blaquez *et al.*, 2006). Although the range of applications of NIR spectroscopy in dairy products is wide (Sørensen & Jepsen, 1998; Cimander *et al.*, 2002; Navrátil *et al.*, 2004) applications of this technique for the quantification of acids responsible for the acidity in fermented dairy products in general and Kefir in particular, are scarce. Thus, the purpose of this study was to develop calibration models for the quantification of DL-lactic acid (DL-LA) and acetic acid in Kefir beverage using NIR spectroscopy.

Material and methods

Samples

Kefir samples ($N = 140$) used in this study were produced with three different sources of Kefir grains: laboratory Kefir grains (LG; $N = 92$), traditional Kefir grains (TG; $N = 34$), and mass cultured Kefir grains (MG; $N = 14$). LG and TG were natural Kefir grains whereas MG were Kefir grains obtained by mass culturing, which is a process that leads to 582% mass increase of Kefir grains in 20 days (Schoevers & Britz, 2003).

Kefir was prepared daily over a period of 3 months by adding 20 g of LG Kefir grains in 300 mL of milk and incubating the samples from either 5 to 10 h at 25°C or 15 to 24 h at 25°C in a controlled temperature incubator. At the end of each incubation period (5, 8, 15, 18 and 24 h), Kefir was separated from the grains by sieving and matured overnight at refrigeration temperatures (4°C). Kefir samples prepared using the TG and MG Kefir grains were traditionally made (home-made) at room temperature without temperature control. The Kefir was again separated from the grains by sieving and refrigerated overnight. The Kefir samples, prepared from the respective

grains, will also be referred to as LG, TG and MG.

The range of DL-LA concentrations found in Kefir depends on the environmental conditions (inoculum size, incubation period, incubation temperature). According to Kosikowski & Mistry (1997) the total lactic acid content of Kefir usually varies from 0.80 to 1.15 g.100 mL⁻¹. However, depending on the fermentation time and for the purpose of this study, it was decided DL-LA should cover a range from 0 to 2 g.100 mL⁻¹. Therefore, the range of DL-LA found in the Kefir samples was extended with samples of pasteurised milk spiked with DL-lactic acid sodium salt (60% m.m⁻¹) (LAC) as well as fresh pasteurised full-cream milk (FM) and non-fresh pasteurised milk (NFM). The latter refers to pasteurised milk that has passed the “sell by date”, i.e. acidification had already commenced.

Similarly, the concentration of acetic acid in Kefir usually ranges between 0.2 and 0.85 g.100 mL⁻¹ (Garrote *et al.*, 2001). However, preliminary studies showed acetic acid content in Kefir could reach 1.2 g.100 mL⁻¹, therefore, for the purpose of this study it was decided that the acetic acid concentration should range from 0 to 1.4 g.100 mL⁻¹. Similar to extending the range of the DL-LA content, the range of acetic acid content was extended with samples of pasteurised milk spiked with acetic acid (99.8%) (ACE) to obtain concentrations below 0.3 g.100 mL⁻¹ and above 1.2 g.100 mL⁻¹. The acetic acid content in FM and NFM was also measured.

Lactic acid measurements

The quantification of DL-LA was carried out using a DL-lactic acid enzyme kit (Boehringer Mannheim - R Biopharm, Germany). One gram of Kefir was diluted to 100 mL with distilled water (solution A). Ten millilitres of solution A were centrifuged (15 min at 10 000 g) and the supernatant filtered through Whatman paper No. 1 to remove solids and obtain a clear supernatant. Only single analyses were performed. Duplicate analyses were deemed unnecessary, as the precision of the method (difference between duplicates) is known to be between 0.015 and 0.03 g 100 mL⁻¹ (DL-Lactic acid UV-method, Cat. No. 11112 821 035, R-BIOPHARM AG, Darmstadt, Germany).

The enzymatic assays were performed as follows: glycyglycine buffer (1 mL), nicotinamide adenine dinucleotide lyophilisate (0.2 mL) (NAD), glutamate-pyruvate transaminase suspension (0.02 mL), distilled water (0.9 mL) and solution A (0.1 mL) were mixed in a cuvette and left for 5 min at room temperature for the reaction to proceed. The absorbance (Absorbance 1) was recorded at 340 nm using a spectrophotometer (Spectronic20 Genesys, Spectronic Instruments, Cape Town). Then, D-lactate dehydrogenase solution (0.02 mL) was added to the mixture and the absorbance was recorded again after 30 min (Absorbance 2). Finally, the L-lactate dehydrogenase solution (0.02 mL) was added and the absorbance recorded after 30 min (Absorbance 3). The concentrations of D-lactic acid (D-LA) and L-lactic acid (L-LA) were calculated using the following formula: D-LA (g L⁻¹) = (2.018 / 6.3) x ΔA and L-LA (g L⁻¹) = (2.036 / 6.3) x ΔA, where, 6.3 (l x mmol⁻¹ x cm⁻¹) is the extinction coefficient of NADH (reduced NAD) at 340

nm and ΔA is the difference in absorbencies. The total DL-LA was obtained by combining the results obtained for D-LA and L-LA, respectively.

For 'milky body-like' samples (samples with pH above the isoelectric point of caseins pH > 5.5 – 5.6), 5 mL of trichloroacetic acid (TCA) was added to 1 g of sample to precipitate the proteins. The volumetric flask was then filled up to 50 mL with distilled water and the same procedure, for determination of DL-LA, as described above was performed.

Acetic acid measurements

Gas chromatography (GC) was used to quantify the concentrations of acetic acid present in the samples (Sigge *et al.*, 2005). A standard solution of short chain volatile fatty acids (VFAs) (acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids) was prepared by mixing 1 mL of each fatty acid and 0.5 mL of hexanol (carrier for internal standard) in a 1000 mL volumetric flask with 250 mL of formic acid (to put milk in acid form) and made up to 1000 mL with distilled water.

Ten mL of each sample was centrifuged (15 min at 10 000 g) and the supernatant filtered through Whatman paper No. 1 to remove the solids to obtain a clear supernatant. One millilitre of formic acid (35% v.v⁻¹) and 2 μ L of n-hexanol (as internal standard) were added to 3 mL of the filtered supernatant. The GC injection volume was 1 μ L and the run time 20 min.

The VFAs were determined using a Varian 3700 GC equipped with a flame ionisation detector and a 30 m bonded phase Nukol (Supelco, Inc., Belafonte, PA) fused silica capillary column (0.53 mm diameter and 0.5 μ m film thickness). The oven heating cycle program was held at 105°C for 2 min followed by an increase of 10°C per min to 190°C for 10 min. Injector and detector temperatures were 150°C and 300°C, respectively. The flow rate of the nitrogen carrier gas was 6.1 mL.min⁻¹. The VFAs were quantified using the Borwin Version 1.2 integration software (JMBS Developpements, Le Fontanil, France) using the internal standard method (Sigge *et al.*, 2005). The centrifugation of 'milky body-like' samples was facilitated by the addition of 1 mL 1 N HCl to precipitate the proteins.

Near infrared spectroscopy measurements

A Büchi NIRFlex N-500 Fourier transform near infrared spectrophotometer (Büchi Labortechnik AG, Flawil, Switzerland) with NIRWare (version 1.2) measurement software (Büchi Labortechnik AG, Flawil, Switzerland) was used to perform the NIR measurements in diffuse reflectance mode. Samples were left at room temperature until they reached 22°C, mixed well and presented to the instrument in a rotating glass petri-dish. The NIR spectra were collected as 32 co-added scans from 10 000 to 4000 cm⁻¹ (1000 to 2500 nm) at a resolution of 8 cm⁻¹ and intervals of 4 cm⁻¹ resulting in 1501 data points. All samples (N = 174) were scanned in duplicate to ensure the reproducibility of the data. Duplicate spectra were not averaged, but given the same reference values. Spectral outliers were identified as spectra that were abnormal compared to the spectra of

the entire data set and eliminated.

Near infrared spectroscopy calibration model development

The Unscrambler® (V9.2) software (Camo Process AS, Oslo, Norway) was used for the development of the calibration models of pH, TA, DL-LA and acetic acid content. Different spectral pre-treatments were applied to the data, before model development, to select the best quality model. To improve the signal-to-noise ratio, the spectral data for the DL-LA calibration model were smoothed by using the window average method with a segment size of 5. Other spectral pre-treatments evaluated were multiplicative scatter correction (MSC), standard normal variate (SNV) and Savitzky-Golay derivative. Partial least squares (PLS) were used for all calibration model development.

In this work, the calibration models were developed using segmented cross-validation (10 segments containing 12 to 13 samples) ($N = 112$ to 126 , depending on removal of outliers). The calibration set (using cross-validation) comprised Kefir (LG, TG and MG) as well as milk (LAC, ACE, FM and NFM) samples. Subsequently the best quality model was selected, depending on pre-treatment used, and validated using an external validation set ($N = 48$). The external validation set also comprised Kefir and 'milky body-like' samples. These samples were, however, prepared at a later stage independent of the calibration sample set.

The accuracy of the calibration models and the predictions were expressed by means of the coefficient of determination (R^2), the standard error of cross-validation ($SECV$) and the standard error of prediction (SEP). The efficiency of a calibration was shown by the RPD which is the ratio of the standard error of prediction (SEP) to the standard deviation of the reference data of the validation set (Williams, 2001). When cross-validation was used the standard deviation of the reference data of the entire samples set was used to calculate the RPD. The bias, which indicates the difference between reference and NIR data (Williams, 2001), was also indicated.

Principal component analysis

A principal component analysis (PCA) was performed to identify similarities or differences between the beverages made using the different Kefir grains using the Unscrambler® (V9.2) software (Camo Process AS, Oslo, Norway). PCA was applied to the entire spectral data set ($N = 174$).

Results and discussion

Spectral characterisation

The NIR spectra of Kefir prepared with LG, TG and MG Kefir grains, as well as the LAC, ACE, FM and NFM samples are presented in Fig. 1. The spectra were typical of milk based products

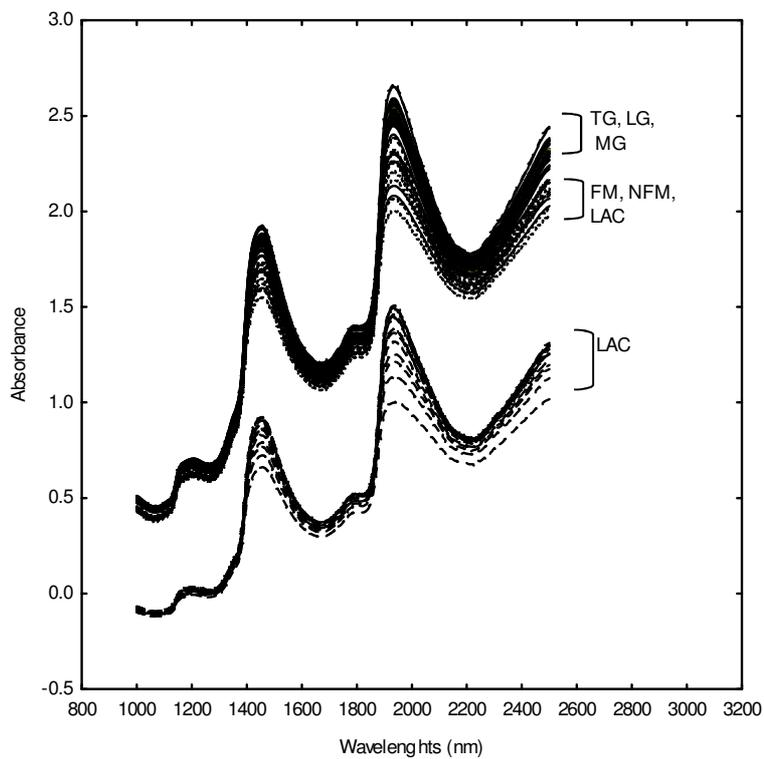


Figure 1. NIR spectra of Kefir samples (LG, TG and MG), milk spiked with LAC, milk spiked with acetic acid (ACE), fresh (FM) and non-fresh pasteurised milk (NFM).

(Laporte & Paquin, 1999). The PCA loading line plots (Fig. 2) illustrate zones where differences in absorbance coincide with the most relevant wavelengths known to be associated with molecular bonds (Williams, 2001) typically found in milk products. Absorption bands were visible between 1800 and 2000 nm, which are known to be the absorption regions of pure lactic acid (1950 nm) (Williams, 2001) and pure acetic acid (1678, 1720 and 2254 nm) (Yano *et al.*, 1997).

However, in the Kefir spectra (Fig. 1), apart from the water peaks (1450 and 1930 nm), no other peaks are clearly visible because the broader peaks of water tend to overlap other peaks. Overlapping peaks are often the case in food with high water content (Tamburini *et al.*, 2003; González-Sáis & Esteban-Díez, 2008).

Principal component analysis

Figure 3 represents the score plot of the Kefir (LG, TG and MG) as well as the FM, NFM and LAC samples whereas Fig. 4 represents the score plot of the Kefir (LG, TG and MG) as well as the FM, NFM and ACE samples. The PCA score plot (PC1 vs PC2) in Fig. 3 shows two groups (I, II). Group I is represented by LG samples obtained after 15 to 24 h at 25°C as well as TG and MG samples. Group II contains LG samples obtained after 5 to 10 h at 25°C as well as FM, NFM and LAC samples. Group I comprises all Kefir samples which were fully coagulated ($> 0.4 \text{ g} \cdot 100 \text{ mL}^{-1} \text{ DL-LA}$) whereas the samples in Group II contains the milk samples and the 'milky body-like' Kefir samples. These are the Kefir samples that were incubated only up to 10 h where no coagulation was visible ($< 0.3 \text{ g} \cdot 100 \text{ mL}^{-1} \text{ DL-LA}$).

The PCA score plot (Fig. 3) clearly shows similarities between the LG samples, incubated at 25°C for 15 to 24 h, TG and MG samples (Group I). Mass cultured Kefir grains are grains that are grown quickly (582% in 20 days), using urea and yeast extract (Schoevers & Britz, 2003) whereas LG and TG are natural Kefir grains. The similarities in the PCA scores therefore suggest that Kefir prepared with MG is similar to Kefir prepared with LG and TG.

The scores distribution in Fig. 3 shows that within group II the LAC samples formed two clusters. This was also clearly visible in Fig. 1 where a large baseline shift was apparent in the spectra of these samples. Applying MSC removed this baseline shift (Fig. 5). Consequently, it can be deduced that this separation was possibly caused by the difference in homogenisation of the two sources of milk used (e.g. difference in fat globule size) that might have caused different scattering properties.

The PCA score plot (PC1 vs PC2) in Fig. 4 also depicts two groups (I and II). Group I contains LG samples obtained after 15 to 24 h at 25°C as well as TG and MG samples. Group II contains LG samples obtained after 5 to 10 h at 25°C as well as FM, NFM and ACE samples. A differentiation between 'milky body-like' samples (Group II) and fermented samples (Group I) was again observed as previously discussed.

In this study the Kefir samples were refrigerated after fermentation. This process is

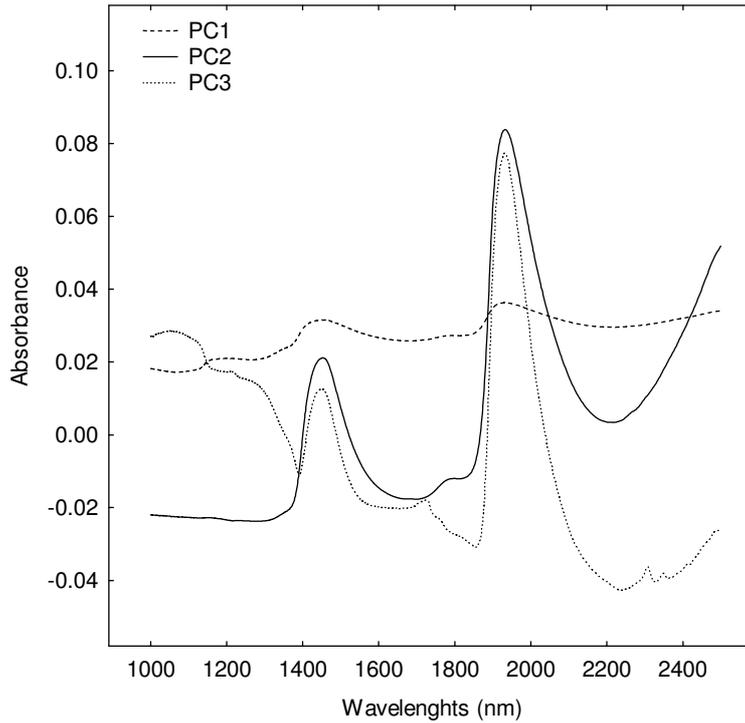


Figure 2. Principal component analysis loading line plots for PC1, PC2 and PC3.

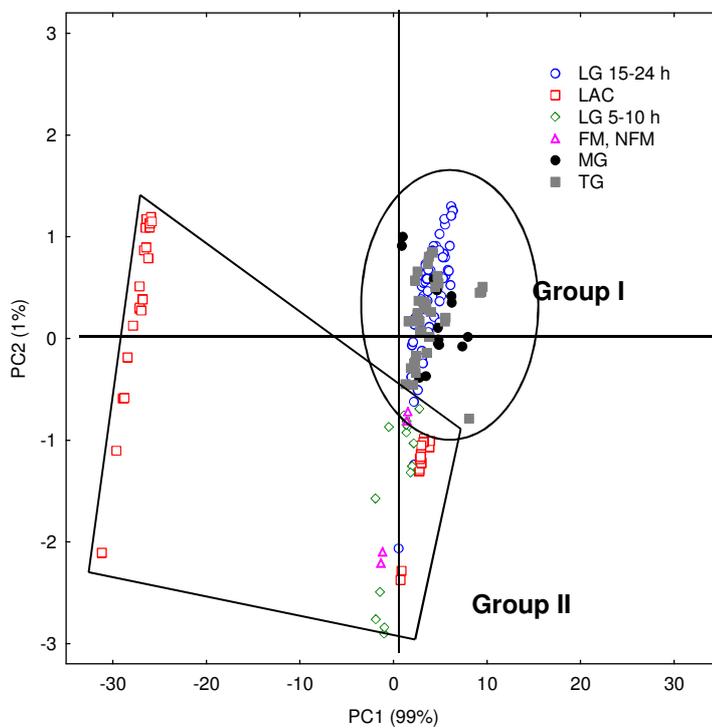


Figure 3. Principal component analysis score plot (PC1 vs PC2) of Kefir samples (LG, TG and MG), fresh milk (FM), non-fresh pasteurised milk (NFM) and milk spiked with LAC.

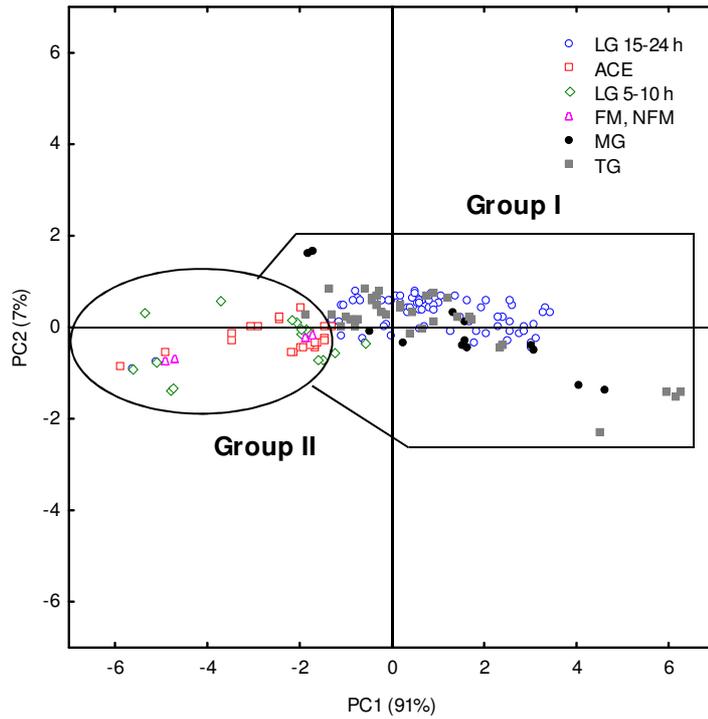


Figure 4. Principal component analysis score plot (PC1 vs PC2) of samples (LG, TG and MC), fresh milk (FM), non fresh-pasteurised milk (NFM) and milk spiked with ACE.

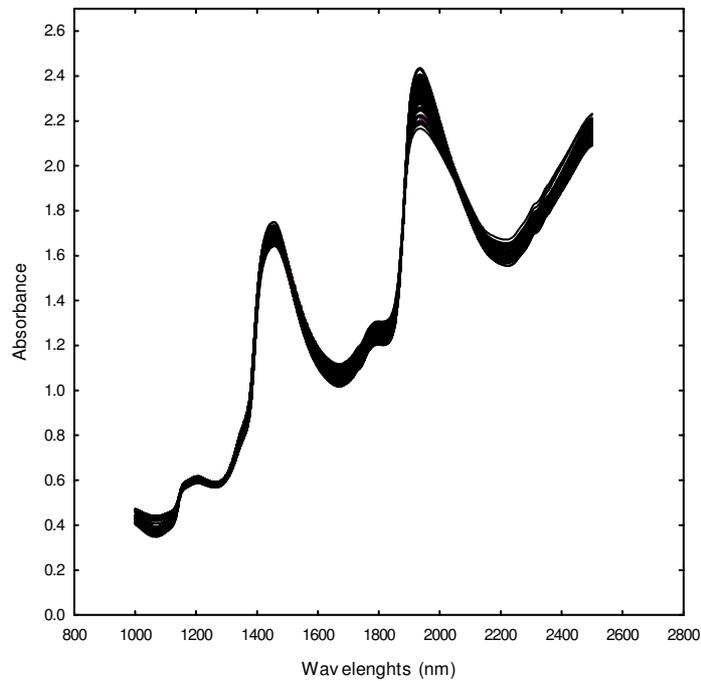


Figure 5. NIR spectra of Kefir samples (LG, TG and MG), milk spiked with LAC, milk spiked with ACE, fresh (FM) and non-fresh pasteurised milk (NFM) after MSC treatment.

known as maturation and applied in order to improve flavour and mouth-feel (Vedemuthu, 2006). Maturation is, however, not essential for Kefir production. Therefore, the extension of the data set with non-matured Kefir samples in future studies would give a more representative sample set.

Quantification of DL-lactic acid and acetic acid using NIR spectroscopy

The data in Table 1 shows the descriptive statistics for the reference data for the NIR calibration and validation sets used for the quantification of DL-LA and acetic acid.

According to Czarnik-Matusewicz *et al.* (1999) milk contains scattering particles in the form of fat globules and protein micelles. Consequently, to remove the effects of these particles, mathematical pre-treatments such as multiplicative scatter correction (MSC) (Geladi *et al.*, 1985) or standard normal variate (SNV) (Barnes *et al.*, 1989) are appropriate to apply (Cen & He, 2007). Using cross-validation (Table 2), baseline correction followed by MSC resulted in slightly better results whereas for external validation (Table 3), it was found that smoothing with a segment window of five was the most suitable method for the calibration of DL-LA. The results were, however, only marginally better. For acetic acid no pre-treatment was found necessary. The latter results are reported in more detail in Table 4.

DL-lactic acid calibration and validation

The validation plots for DL-LA using cross-validation and an external validation set are shown in Fig. 6, respectively. The R^2 for cross-validation and the $SECV$ obtained for DL-LA were 0.90 and 0.110 g.100 mL⁻¹, respectively. The RPD evaluates how well a calibration and prediction will work for analytical purposes (Cen & He, 2007), and in our study was found to be 3.16. According to Williams (2001) RPD values greater than 5, and at least 3 indicate the NIR model is efficient. RPD values between 2.4 and 3 permit a rough screening whereas a value below 2.3 indicates the model is not the recommended method to predict the compound of interest. An RPD of 1 means the model has not predicted the compound of interest at all.

The prediction of DL-LA using an external validation set gave an R^2 of 0.87 and a SEP of 0.156 g.100 mL⁻¹. The RPD was found to be 2.57. Such a RPD value indicates that the model is only good for screening purposes. However, this result could be improved by expanding the ranges in the calibration and validation sets and ensuring the calibration set has an even distribution.

The number of PLS factors used to develop the calibration model was 14. It is generally acknowledged too many PLS factors might be a sign of over-fitting, where the model integrates some noise. Over-fitting through a large number of factors might result in a good model but will not predict unknown samples very well (Williams, 2001). However, in the case of this study, the model developed for DL-LA was not over-fitted as demonstrated by the results obtained with the external validation set where the SEP was within the limit value of $1.5 \times SECV$. A $SEP = 2 \times SECV$

Table 1. Descriptive statistics of reference data used for the quantification of DL-lactic acid and acetic acid in Kefir.

Cross-validation			External validation		
Parameters	DL- lactic acid	Acetic acid	Parameters	DL-lactic acid	Acetic acid
<i>N</i>	123	108	<i>N</i>	44	44
Range*	0.040 – 1.670	0.004 – 0.112	Range*	0.050 – 1.670	0.007 – 0.083
Mean*	0.82	0.058	Mean*	0.78	0.04
Standard deviation*	0.35	0.029	Standard deviation*	0.4	0.02
SEL*	0.015 – 0.030 [#]	0.005	SEL*	0.015 – 0.030 [#]	0.005

*g.100 mL⁻¹[#]Precision as reported by manufacturer of analysis kit

Table 2. NIR spectroscopy cross-validation results for the quantification of DL-lactic acid and acetic acid in Kefir using different pre-treatment techniques.

Compounds	Cross-validation				
	Pre-treatments	R ²	SECV*	Bias	PLS factors
DL-lactic acid ^a	No pre-treatment	0.898	0.111	0.0028	14
	Smoothing, 5 points	0.900	0.110	-0.0009	14
	MSC	0.866	0.116	0.0012	11
	Baseline, MSC	0.902	0.109	-0.0020	12
	SNV	0.899	0.110	0.0009	12
	SNV + MSC	0.893	0.114	-0.0015	11
	1 st der, 5 points ^c	0.643	0.208	0.0013	7
	2 nd der, 5 points ^d	0.353	0.286	4.10 ⁻⁴	4
Acetic acid ^b	No pre-treatment	0.797	0.013	0.0031	14
	Smoothing, 5 points	0.790	0.013	0.0002	10
	MSC	0.792	0.014	0.0001	8
	Baseline, MSC	0.715	0.015	0.0004	8
	SNV	0.763	0.014	0.0004	7
	SNV + MSC	0.801	0.013	0.0004	8
	1 st der, 5 points ^c	0.539	0.022	0.0015	9
	2 nd der, 5 points ^d	0.243	0.028	0.0001	4

^a N = 123; ^b N = 108^c1st derivative Savitzky-Golay, 2nd polynomial order, 5 points smoothing^d2nd derivative Savitzky-Golay, 3rd polynomial order, 5 points smoothing* g.100 mL⁻¹

Table 3. NIR spectroscopy external validation results for the quantification of DL-lactic acid and acetic acid in Kefir using different pre-treatment techniques.

Compounds	Pre-treatments	External validation			
		R ²	SEP*	Bias	PLS factors
DL-lactic acid ^a	No pre-treatment	0.86	0.163	0.0620	14
	Smoothing, 5 points	0.867	0.156	0.1416	14
	MSC	0.756	0.201	0.0907	11
	Baseline, MSC	0.828	0.169	0.1400	12
	SNV	0.312	0.582	6.4127	12
	SNV + MSC	0.840	0.212	0.2242	11
	1 st der, 5 points ^c	0.07	648.9	5090	5
	2 nd der, 5 points ^d	0.13	71.64	-742.2	6
Acetic acid ^b	No pre-treatment	0.439	0.017	0.0020	14
	Smoothing	0.440	0.017	0.0020	10
	MSC	0.436	0.017	0.0040	8
	Baseline, MSC	0.345	0.028	-0.3785	8
	SNV	0.369	0.068	1.4965	8
	SNV + MSC	0.450	0.015	0.0333	8
	1 st der, 5 points ^c	0.049	8901	18.28	5
	2 nd der, 5 points ^d	0.309	6666	14.98	6

^a N = 44; ^b N = 44^c 1st derivative Savitzky-Golay, 2nd polynomial order, 5 points smoothing^d 2nd derivative Savitzky-Golay, 3rd polynomial order, 5 points smoothing* g.100 mL⁻¹

Table 4. NIR validation for the quantification of DL-lactic acid and acetic acid in Kefir.

Cross-validation			External validation		
Parameters	Lactic acid	Acetic acid	Parameters	Lactic acid	Acetic acid
R^2	0.9	0.8	R^2	0.87	0.44
Slope	0.92	0.83	Slope	0.99	0.71
SEC^*	0.07	0.011			
$SECV^*$	0.11	0.013	SEP^*	0.16	0.017
Bias	-0.0008	0.0031	Bias	0.142	0.0021
PLS factors	14	11	PLS factors	14	11
Outliers	3	4	Outliers	4	4
RPD	3.16	2.21	RPD	2.57	1.17

*g 100 mL⁻¹

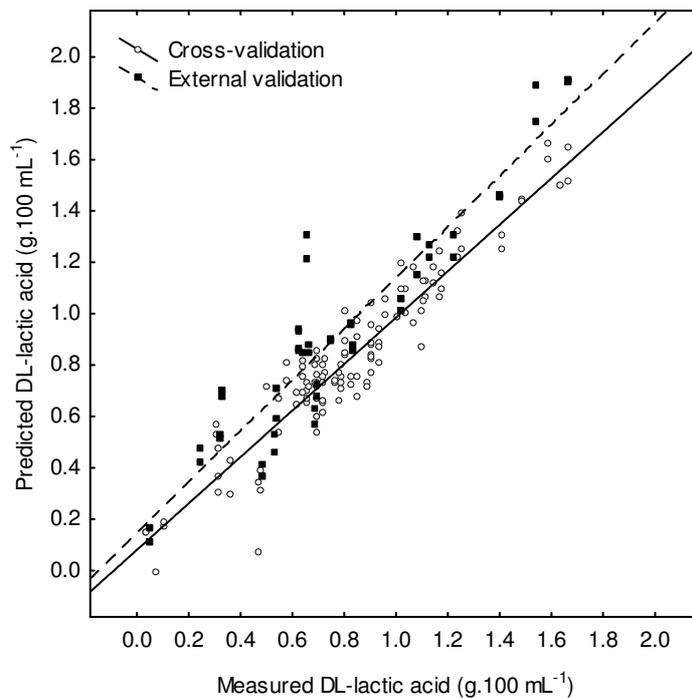


Figure 6. Validation plots of the predicted DL-lactic acid values versus the measured (enzymatic kits) DL-lactic acid for (○) the cross-validation set ($N = 126$) and (●) the external validation set ($N = 48$).

demonstrates a high degree of over-fitting (Williams, 2001).

The calibration model developed for DL-lactic acid could be used in research environments or dairy plants where acidity is currently measured in terms of pH and titratable acidity (TA). Since, these parameters (pH and TA) do not indicate the amount of DL-LA present in products, implementation of NIR spectroscopy would undoubtedly lower manufacturing costs.

Acetic acid calibration and validation

Figure 7 shows the validation plots for the quantification of acetic acid using cross-validation and external validation, respectively. The R^2 for cross-validation and the $SECV$ obtained for acetic acid were 0.80 and 0.013 g.100 mL⁻¹, respectively. According to Williams (2001), the R^2 was acceptable; however, the RPD of 2.21 indicated the model would not be reliable in this case.

It is well known that results obtained with cross-validation are generally better than those obtained using external validation. Therefore, if the cross-validation results are not promising, it is unlikely that those obtained using unknown samples would be. This is confirmed in the case of the prediction of acetic acid using a set of samples that has not been used for calibration. The R^2 and the SEP were 0.44 and 0.017 g.100 mL⁻¹, respectively.

Such an R^2 shows poor correlation between the data obtained with the reference method and the predicted data obtained by NIR spectroscopy. It can, therefore be concluded that with the set of samples available, it was not possible to build a suitable NIR model. Additionally, the inability of NIR spectroscopy to predict acetic acid concentrations, as shown by a RPD of 1.17 in Kefir, is a possible indication the reference method was not adequately accurate.

The ACE samples with concentrations above 0.01 g.100 mL⁻¹ were removed from the external prediction set because their inclusion led to a very low and negative slope (< 0.5) and R^2 (< 1). According to Tamburini *et al.* (2003) the use of samples spiked with pure compounds is not recommended because the complexity of interactions found in the raw samples cannot be reproduced or predicted. Interestingly, in the case of milk spiked with DL-LA for our study, the results indicated such a problem did not occur.

Conclusion

This work is a proof of concept that NIR spectroscopy can be used to estimate DL-LA in Kefir in particular and fermented dairy products in general. Under the conditions of this study the DL-lactic acid production of Kefir prepared with temperature control (laboratory made Kefir) or without temperature control (home-made Kefir) did not depend on the type of grains (LG, TG and MG). This suggests the presence of self-regulation within each type of Kefir grain.

Although the calibration and validation results of acetic acid content were not successful,

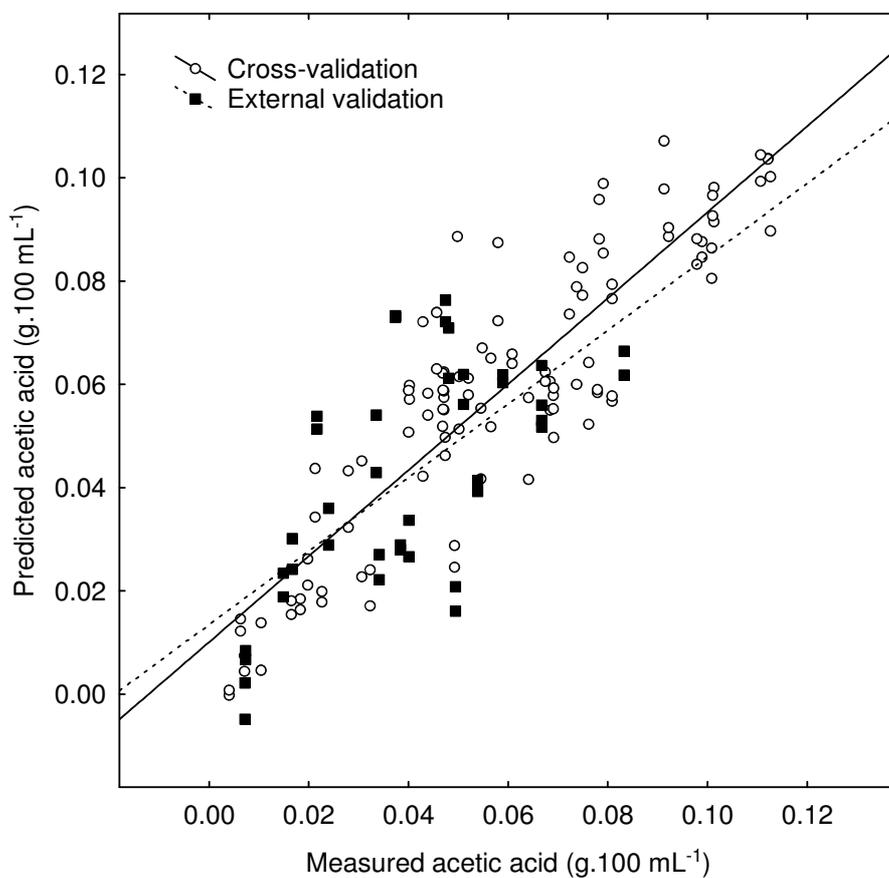


Figure 7. Validation plots of the predicted acetic acid values versus the measured (gas chromatography) acetic acid for (o) the cross-validation set ($N = 112$) and (•) the external validation set ($N = 48$).

results can still be improved with increased accuracy of the reference method. In addition, the number of samples and range for the traits of interest could be increased. Extending the sample set with the addition of non-matured Kefir samples could also lead to improved accuracy and robustness of the model for DL-LA. In further studies, the identification of the wavelengths associated with DL-LA and acetic acid could be done in order to use multilinear regression (MLR) for developing simpler calibration models.

PCA has shown that similar Kefirs could be prepared from different sources of grains and that it was possible to differentiate between fermented and 'milky body-like' samples. The potential to distinguish between milk samples not homogenised to the same extent was also observed. Confirmation of this and subsequent implementation could also be of great value to the dairy industry.

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CHAPTER 5

SIMULTANEOUS PREDICTION OF ACIDITY PARAMETERS (pH AND TITRATABLE ACIDITY) IN KEFIR USING NEAR INFRARED REFLECTANCE SPECTROSCOPY

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Abstract

Acidity in terms of pH and titratable acids (TA) influences texture and flavour of fermented dairy products, such as Kefir, however, the methods for determining pH and TA are time consuming. Near infrared (NIR) is a non-destructive method, which simultaneously predicts multiple traits from a single scan and can be used to predict pH and TA.

The best pH NIR calibration model was obtained with no spectral pre-treatment applied whereas smoothing was found to be the best pre-treatment to develop the TA calibration model. Using cross-validation, the prediction results were found acceptable for both pH and TA. With external validation, similar results were found for pH and TA; and both models were found to be acceptable for screening purposes.

Introduction

Fermented dairy products are characterised by an acidic taste originating from the presence of lactic acid, a by-product of lactic fermentation. Titratable acidity (TA) and pH are commonly used as measurements of acidity to determine the quality of milk before and during the production of fermented dairy products. In fermented dairy products, DL-lactic acid and acetic acid are also indicators of acidity but are not used routinely, as are pH and TA, either by at-line or on-line monitoring. This is due to lactic and acetic acid requiring more costly and complex validation methods including the enzymatic determination of the acids. Although there is no direct relationship between pH and TA, a general relationship exists; pH decreases as TA increases (Walstra *et al.*, 2006). In addition, the mathematical equations developed to determine pH from known values of TA, or TA from known values of pH, (Nagel & Herrick, 1989) have drawbacks as these models may not be applicable under different environmental conditions. Thus, monitoring both these indicators of acidity, i.e. pH and TA, to ensure good quality products is important.

Near infrared (NIR) spectroscopy is a very useful tool for the food industry, since it is a fast and reliable method that requires almost no sample preparation (Wüst & Rudzik, 2003). With NIR spectroscopy, it is possible to predict several compounds simultaneously, making it one of the fastest and most cost effective technologies currently available. NIR spectroscopy correlates the spectral information (spectroscopic data in the NIR region 800 nm to 2500 nm) with the property

(e.g. concentration) of the compound of interest, to develop calibration models. This enables the quantification of the compound of interest in unknown samples.

In 1957, an NIR spectrum of powdered milk was obtained for the first time (Rodríguez-Otero *et al.*, 1997). Milk analysers based on mid-infrared (MIR) spectroscopy were commercially available in the 1960s and have been used extensively. Nowadays, the use of NIR spectroscopy in dairy products for qualitative and quantitative analyses is well documented (Cimander *et al.*, 2002; Karoui *et al.*, 2005), however, studies on acidity using NIR spectroscopy are scarce for dairy products.

Recently, Ntsame Affane *et al.* (2009) developed models for the quantification of DL-lactic acid in Kefir (standard error of prediction (SEP) = 0.156 g.100 mL⁻¹, coefficient of determination (R²) = 0.87 and RPD = 2.57) and acetic acid (SEP = 0.017 g.100 mL⁻¹, R² = 0.44 and RPD = 1.17). The RPD is the ratio of the standard error of prediction to the deviation of the reference data of the validation set. Kefir is a self-carbonated alcoholic fermented milk, originating from the Caucasian Mountains (Garrote *et al.*, 1997) and can be made from any type of processed or raw milk such as cow, ewe, goat or sheep milk (Cais-Sokolińska *et al.*, 2008). The range of pH normally reported for Kefir samples is 3.5 to 4.5 and the range of TA varies between 0.50 and 1.50 g.100 mL⁻¹ (Simova *et al.*, 2002; Chen *et al.*, 2009).

The acidity of Kefir is important throughout production since insufficient or excess acidity has been shown to mask the buttery character of Kefir but also alters the structure of the product (Vedemuthu, 2006). Thus, the purpose of the current study was to develop NIR spectroscopy calibration models for the simultaneous prediction of the acidity parameters, pH and TA, in a Kefir beverage.

Material and Methods

Kefir samples

Kefir grains used to make Kefir were obtained from the Department of Food Science (Stellenbosch University) as well as from a private household (Schoevers & Britz, 2003). Kefir was prepared daily as previously described by Ntsame Affane *et al.* (2009) by adding 20 g of Kefir grains to 300 mL of milk and incubating the samples for 5, 10, 15, 18 h or 24 h at 25 °C. The Kefir beverage was then separated from the grains and matured overnight at 4°C. Traditional Kefir was prepared by incubating at room temperature without any temperature control; the grains separated by sieving followed by overnight maturation at 4°C (Ntsame Affane *et al.*, 2009).

Although the ranges of pH and TA normally reported for Kefir samples vary from 3.5 to 4.5 and from 0.50 and 1.50 g.100 mL⁻¹ respectively (Simova *et al.*, 2002; Chen *et al.*, 2009), for the purpose of this study, the pH and TA ranges were extended (3.5 to 6.7 for pH and 0.01 to 2 g.100 mL⁻¹ for TA) by spiking fresh pasteurised milk samples with DL-lactic acid using fresh pasteurised

full-cream milk (FM) for the lower ranges and non-fresh pasteurised milk (NFM) for the higher ranges. The latter refers to pasteurised milk that has passed the “sell by date”, i.e. extended acidification had already commenced (Ntsame Affane *et al.*, 2009).

Titrateable acidity and pH measurements

The pH values of the Kefir (measured after overnight maturation) and milk samples were measured with a microprocessor pH meter (Hanna Instruments model 221, Michigan, USA) equipped with a glass electrode and a temperature probe. Certified buffers (pH 7.00 and pH 4.00, LASEC, Cape Town, SA) were used to calibrate the electrode.

The TA of the Kefir (measured after overnight maturation) and milk samples were measured in duplicate by the titration of a 10 mL sample with 0.11 N NaOH until the pink phenolphthalein end-point (James, 1999).

Near infrared spectroscopy measurements

Near infrared spectra of the Kefir samples were collected in reflectance mode using a Büchi NIRFlex N-500 Fourier transform NIR spectrophotometer (Büchi Labortechnik AG, Flawil, Switzerland). Dedicated measurement software (NIRWare v.1.2, Büchi Labortechnik AG, Flawil, Switzerland) was used. Samples were left at room temperature until they reached 22°C, mixed well and presented to the instrument in a rotating glass petri-dish as previously described by Ntsame Affane *et al.* (2009). The NIR spectra were collected from 10 000 to 4000 cm^{-1} (1000 to 2500 nm) as 32 co-added scans. The resolution was set at 8 cm^{-1} with spectral intervals of approximately 4 cm^{-1} , resulting in 1501 data points. All samples (N = 174) were scanned twice. The duplicate spectra were not averaged and the same reference values allocated to each.

Near infrared spectroscopy calibration model development

The development of the pH and TA calibration models was carried out using the Unscrambler® (V9.2) software (Camo Process AS, Oslo, Norway). Different spectral pre-treatments (multiplicative scatter correction (MSC), standard normal variate transformation (SNV), baseline followed by MSC, five points smoothing, 1st derivative Savitzky-Golay and 2nd derivative Savitzky-Golay) were evaluated to determine the best model. Spectral pre-treatment is often applied to, (1) remove the effects of scattering particles, MSC and SNV (Czarnik-Matusiewicz *et al.*, 1999), (2) enhance the signal-to-noise ratio (smoothing), (3) remove baseline shifts, and (4) remove background noise and increase spectral resolution using 1st and 2nd derivatives (Cen & He, 2007). Partial least squares (PLS) regression was used for calibration model development. The calibration models were developed using segmented cross-validation (10 segments containing 14 samples each). The best model obtained was independently validated using an external validation set (N = 64), which comprised samples separately prepared to those used in the calibration set.

The criteria used for the assessment of the best calibration model and validation were the coefficient of determination (R^2), standard error of cross-validation (SECV), and standard error of prediction (SEP). The bias, which is the difference between the reference and NIR data (Williams, 2001), was also indicated. The efficiency of a calibration was shown by the RPD, which is the ratio of the standard error of prediction (SEP) or the standard error of cross-validation (SECV) to the deviation of the reference data of the validation set (Williams, 2001). RPD values of at least three indicate an acceptable efficiency of the NIR model; values between 2.4 and 3 suggest that the model can only be used for rough screening whereas values below 2.3 indicate that the NIR model is not to be recommended (Williams, 2001).

Statistical analysis

Statistica 8.0 (Statsoft, Johannesburg, SA) was used to determine the Spearman's correlation (r) between DL-lactic acid (DL-LA) and pH as well as DL-LA and TA. The p-values were calculated to highlight any significant differences. The DL-lactic acid values were determined as described by Ntsame Affane *et al.* (2009).

Results

Measurements of pH and titratable acidity using NIR spectroscopy

The descriptive statistics (N, mean and standard deviation) of the reference data used in the NIR calibration development and validation of pH and TA predictions are summarised in Table 1.

Calibration of pH and validation

From all the pre-treatments evaluated for the pH (Table 2) the best calibration model was obtained using the raw data (no pre-treatment). A possible explanation for this finding is that liquid dairy products assessed by reflectance NIR have less light scattering than powdered dairy products. The validation plots, as well as the NIR statistical results obtained for the measurement of pH using both cross and external validation, are shown in Fig. 1. The R^2 and SECV values, obtained for pH using cross-validation, were 0.95 and 0.238, respectively. With the external validation, the R^2 and SEP values were 0.89 and 0.324, respectively. The RPD of 2.90 indicated the model is suitable for screening purposes. The external validation results were better compared to those obtained in an earlier study by Růžičková and Šustová (2006), where yoghurt was used as the sample material (N = 50, R = 0.788, R^2 = 0.621, SEP = 0.038 and RPD = 1.58). This may have been due to a more narrow pH range (4.00 – 4.24) when compared to the pH range of 3.87 to 6.49 used in the present study.

Table 1. Descriptive statistics of reference data used for the measurements of pH and TA in Kefir.

	pH		TA	
	Cross-validation	External validation	Cross-validation	External validation
N	140	64	140	64
Range (g.100 mL ⁻¹)*	3.66 – 6.59	3.87 – 6.49	0.17 – 1.28	0.22 – 1.11
Mean ± SD (g.100 mL ⁻¹)*	4.74 ± 1.05	5.07 ± 0.95	0.76 ± 0.36	0.60 ± 0.3

*g.100 mL⁻¹: only applies to TA

Table 2. NIR spectroscopy cross-validation and external validation results for the measurements of pH in Kefir using different pre-treatment techniques.

Cross-validation						
Compounds	Pre-treatments	R²	SECV*	SEP*	Bias	PLS factors^g
pH ^a	No pre-treatment	0.95	0.238		0.0056	10
	Smoothing, 5 points	0.93	0.271		0.0031	10
	MSC ^c	0.93	0.276		0.0122	10
	Baseline + MSC	0.9	0.332		-0.0053	8
	SNV ^d	0.94	0.267		0.0117	12
	1 st der, 5 points ^e	0.88	0.361		-0.0038	7
	2 nd der, 5 points ^f	0.73	0.542		0.0048	3
External validation						
pH ^b	No pre-treatment	0.89		0.324	-0.0663	10
	Smoothing, 5 points	0.89		0.323	0.0732	10
	MSC	0.89		0.374	-0.169	10
	Baseline + MSC	0.75		0.537	0.01	8
	SNV	0.44		2.706	-29.091	12
	1 st der, 5 points ^c	0.07		504.6	2750.8	7
	2 nd der, 5 points ^d	0.58		177.6	2669.5	3

^a N = 140; ^b N = 64^cMSC = multiplicative scatter correction^dSNV = standard normal variate^e1st derivative Savitzky-Golay, 2nd polynomial order, 5 points smoothing^f2nd derivative Savitzky-Golay, 3rd polynomial order, 5 points smoothing^gPLS factors = Number of PLS factors* g.100 mL⁻¹: only applies to TA

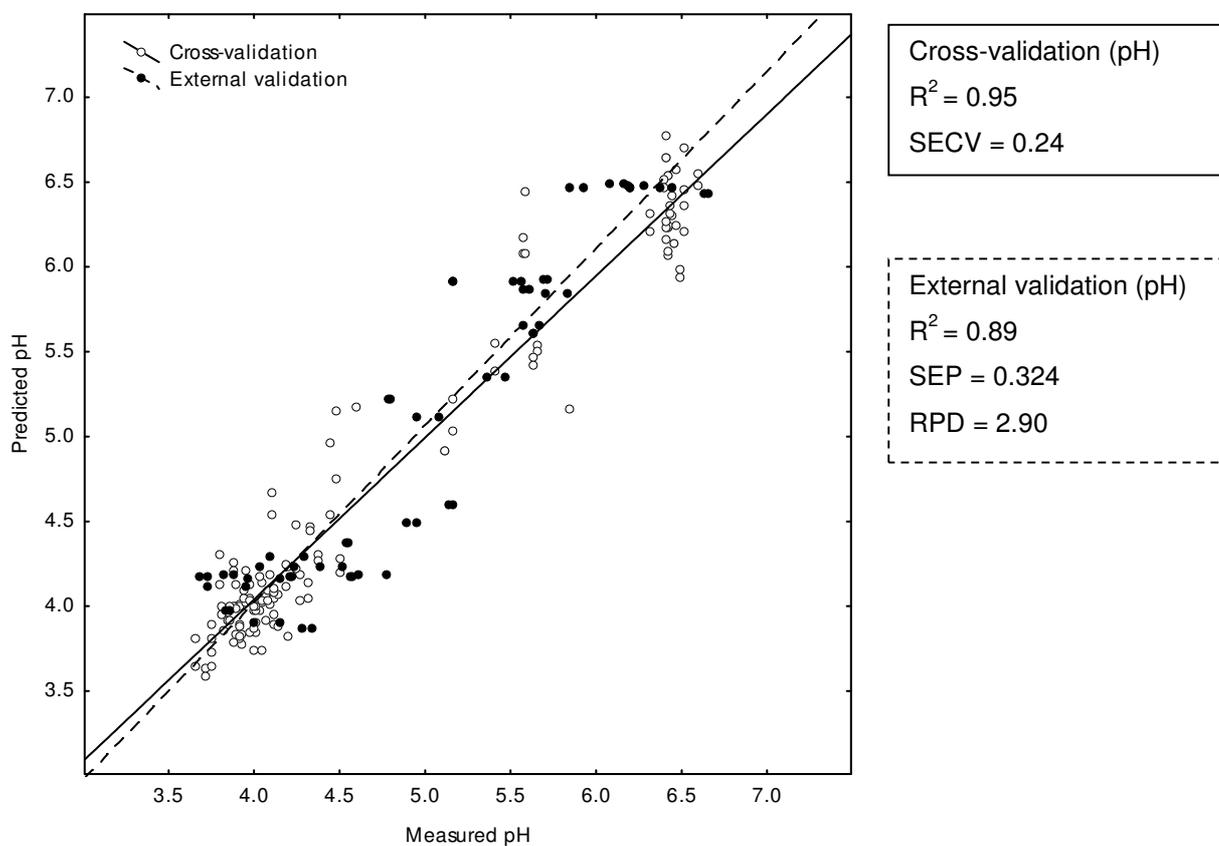


Figure 1. Validation plots and validation results of the measured pH values versus the predicted pH values for (○) the cross-validation ($N = 140$) and (●) the external validation sets ($N = 64$) without pre-treatment. (Cross-validation set (pH), $R^2 = 0.95$, $SECV = 0.24$) and external validation set (pH), $R^2 = 0.89$, $SEP = 0.324$, $RPD = 2.90$).

TA calibration and validation

The Savitzky-Golay smoothing with a segment window of five gave the best results of all the pre-treatments evaluated for the TA calibration (Table 3). The validation plots, as well as the NIR statistical results for TA using cross-validation and an external validation set, are shown in Fig. 2. The R^2 for cross-validation and the SECV obtained for TA were 0.92 and 0.103 g.100 mL⁻¹, respectively. The prediction of TA using an external validation set gave an R^2 of 0.79 and an SEP of 0.137 g.100 mL⁻¹. The RPD value of 2.60 indicates that the model should only be used for screening of samples. Růžičková and Šustová (2006) reported better results ($R^2 = 0.958$, SEP = 0.056 g.100 mL⁻¹ and RPD = 4.93) with yoghurt as the sample material. The robust model obtained compared to this study may originate from the use of a larger sample size (N = 80) and a uniform distribution since the ranges were similar (0.22 – 1.11 g.100 mL⁻¹ vs. 0.22 – 1.35 g.100 mL⁻¹).

Correlation between DL-LA and pH as well as DL-LA and TA

Figures 3 and 4 represent the Spearman's correlation plot between DL-LA vs. TA ($r = 0.86$) and DL-LA vs. pH ($r = 0.89$), respectively. In both cases, the correlations were significant ($p < 0.05$). For DL-LA vs. TA, the TA increased as the concentration in DL-LA increased. On the contrary, for DL-LA vs. pH ($r = 0.89$), the pH decreased as the concentration in DL-LA increased. Both these trends were expected since as pH decreased and TA increased as more acid was produced.

Discussion

Many studies have reported pH and TA NIR calibrations for fruit and vegetables (Pedro & Ferreira, 2005) but few for milk products (Růžičková & Šustová, 2006; Dračková *et al.*, 2008). Based on the definitions of pH and TA, it is clear that they are not directly related to the presence of specific chemical bonds. Since NIR spectroscopy measures the response of the molecular bonds O–H, C–H and N–H, which are subject to vibrational energy changes when irradiated by NIR frequencies (Cen & He, 2007), it should not be possible to measure pH and TA using NIR spectroscopy. However, the estimation of pH and TA using NIR spectroscopy is made possible through presence of specific molecules that would be affected by a change in pH, which would therefore affect their vibrational energy Soller *et al.* (2001). In milk, the molecules that are likely to be affected by a change in pH are the milk proteins.

In Kefir, which is a fermented dairy product, organic acids such as DL-LA would be affected by a change in pH (from dissociated to undissociated form) and it is likely that this compound would allow pH and TA measurements by NIR spectroscopy. DL-LA is the major acid produced in Kefir during lactose degradation in galactose and glucose moieties, with subsequent fermentation of glucose. Other “minor” acids (e.g. acetic, formic and succinic acid) are also produced but in lower quantities than DL-LA (Ntsame Affane *et al.*, 2009).

For pH and TA to be estimated by using NIR spectroscopy, a correlation must exist

Table 3. NIR spectroscopy cross-validation and external validation results for the measurements of TA in Kefir using different pre-treatment techniques.

Compounds	Pre-treatments	Cross-validation				PLS factors ^g
		R ²	SECV*	SEP*	Bias	
TA ^a	No pre-treatment	0.9	0.111		0.0003	10
	Smoothing, 5 points	0.92	0.104		0.0003	10
	MSC ^c	0.87	0.13		-0.0009	8
	Baseline + MSC	0.9	0.115		0.0019	10
	SNV ^d	0.88	0.123		0.0076	10
	1 st der, 5 points ^e	0.8	0.161		0.0003	7
	2 nd der, 5 points ^f	0.62	0.223		0	2
		External validation				
TA ^b	No pre-treatment	0.79		0.139	0.0198	10
	Smoothing, 5 points	0.79		0.137	-0.0137	10
	MSC	0.64		0.181	0.0934	8
	Baseline + MSC	0.55		0.200	-0.2377	11
	SNV ^d	0.52		1.934	21.254	10
	1 st der, 5 points ^e	0.12		296.9	-2128.5	7
	2 nd der, 5 points ^f	0.59		55.17	-847.530	2

^a N = 140; ^b N = 64^cMSC = multiplicative scatter correction^dSNV = standard normal variate^e1st derivative Savitzky-Golay, 2nd polynomial order, 5 points smoothing^f2nd derivative Savitzky-Golay, 3rd polynomial order, 5 points smoothing^gPLS factors = Number of PLS factors*g.100 mL⁻¹

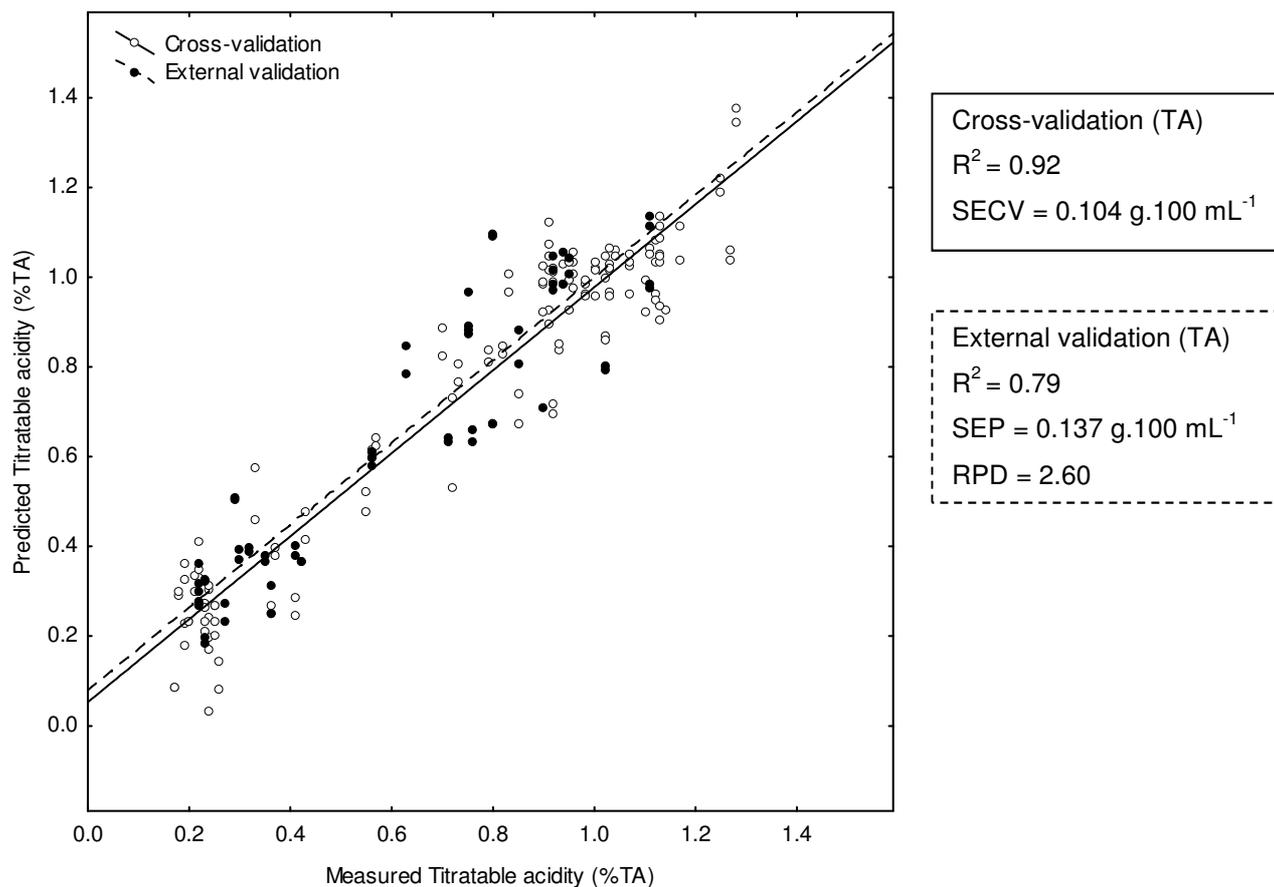


Figure 2. Validation plots and validation results of the measured titratable acidity (TA) values versus predicted TA values for (○) the cross-validation ($N = 140$) and the (●) the external validation sets ($N = 64$) with smoothing with a segment window of five as pre-treatment. (Cross-validation set (TA), $R^2 = 0.92$, $SECV = 0.104 \text{ g.100 mL}^{-1}$) and external validation set (TA), $R^2 = 0.79$, $SEP = 0.137 \text{ g.100 mL}^{-1}$, $RPD = 2.60$).

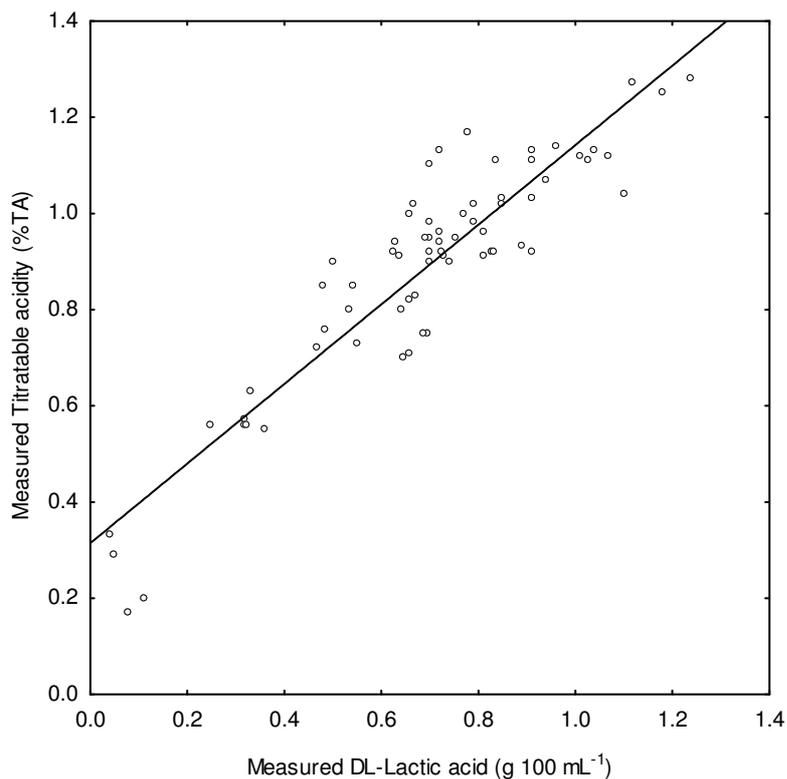


Figure 3. Significant Spearman's correlation ($r = 0.86$; $p = 0.00$) between measured DL-lactic acid ($\text{g} \cdot 100 \text{ mL}^{-1}$) and measured percent titratable acidity (%TA).

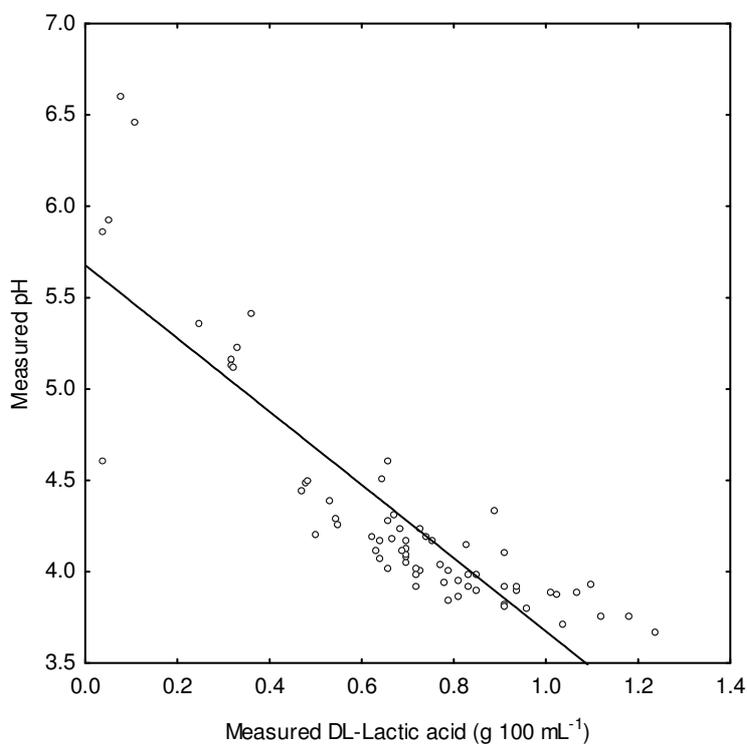


Figure 4. Significant Spearman's correlation ($r = 0.89$; $p = 0.00$) between measured DL-lactic acid ($\text{g} \cdot 100 \text{ mL}^{-1}$) and measured pH values.

between the parameters (pH and TA) and the DL-LA molecules. In this study the relationship between pH, TA and DL-LA was clearly demonstrated by the significant correlations obtained when applying Spearman's correlation to DL-lactic acid vs. TA ($r = 0.86$; $p < 0.05$; Fig. 3) and to DL-lactic acid vs. pH ($r = 0.89$; $p < 0.05$; Fig. 4). The correlations being less than 0.90 could be because DL-LA, although it is the major acid, is not the only acid present in Kefir.

In this study, Kefir was prepared using pasteurised cows' milk. If Kefir had to be prepared with any other type of milk such as sheep, ewe, camel or buffalo milk, it would be advisable to develop new calibrations because the composition of those types of milk differs from cow milk (Wzsolek *et al.*, 2001). In addition some authors (Osborne, 1992; Wüst & Rudzik, 2003) advised that different calibrations must be developed if the processing methods used differ (e.g. traditional vs. technological).

Conclusion

This study clearly showed that NIR spectroscopy can be used to simultaneously estimate pH and TA in Kefir for screening purposes. These two indicators are the key parameters for quality appraisal in the dairy industry worldwide. This correlation is probably only possible due to the use of spiked samples to artificially widen the range of parameters under investigation. Although the NIR spectral response can be correlated to pH and TA changes in this matrix, practical application may be limited by the narrow range of parameters in routine samples. The acquisition of on-line NIR spectrophotometers to monitor acidity parameters could change the face of the dairy industry and would be a very good asset, however, it is important to always be aware of the error associated with NIR spectroscopy analysis and to ensure that the stability of the NIR instrument is regularly checked and the performance of the calibration regularly monitored.

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CHAPTER 6

EVALUATION OF THE METABOLIC PROFILES OF SELECTED LACTIC ACID BACTERIAL STRAINS AND A LACTOSE FERMENTING YEAST

Summary

The metabolic profiles of eight strains of lactic acid bacteria and a lactose fermenting yeast were screened to select the microorganisms to be used for flavour enhancement of Kefir, based on the production of diacetyl and ethyl acetate, respectively responsible for the buttery and fruity note in cultured dairy products. Under these conditions, diacetyl (2.5 – 5.5 mg.L⁻¹) and ethyl acetate (2.13 – 5.07 mg.L⁻¹) were produced, by *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* 318 (*Lc. diacetylactis* 318) and *Candida kefir* 1283 (*C. kefir* 1283), respectively.

The production of more diacetyl (6.92 – 10.4 mg.L⁻¹) was only achieved in milk samples supplemented with citrate and inoculated with *Lc. diacetylactis* 318 whereas addition of ascorbate or citrate in milk samples inoculated with selected microorganisms had no significant effect ($p > 0.05$) on ethyl acetate production.

The effects of citrate and ascorbate on flavour compound production during Kefir fermentation were also determined. The results showed that supplementation with citrate or ascorbate had no stimulatory or inhibitory effect on ethyl acetate production in Kefir samples (LG Kefir and MG Kefir). On the contrary, addition of citrate or ascorbate had a positive impact on diacetyl production, even though the increases observed were not significant ($p > 0.05$). Improvement of the overall flavour of Kefir could be observed as the ratios of diacetyl to acetaldehyde were always found to be higher (0.21 – 0.5) in the samples with added citrate and ascorbate than in the samples not containing these additives (0.12 – 0.17).

Thus, enhancing Kefir flavour could be achieved by the addition of ascorbate or citrate as well as by the microbial enrichment of Kefir grains with diacetyl or ethyl acetate producing microorganisms such as *Lc. diacetylactis* 318 and *C. kefir* 1283.

Introduction

The manufacture of cultured dairy products is made possible through the use of starter cultures containing different lactic acid bacteria (LAB). For the production of Kefir, grains are used as starter and both LAB and yeasts are involved in producing a slightly alcoholic fermented product. Nowadays, Kefir is still traditionally produced and not only viewed as a simple recreational drink but also as a natural probiotic dairy product, with therapeutic attributes and high nutritional value (Ötles & Cagindi, 2003; Sarkar, 2007; Garrote *et al.*, 2010).

A hundred years ago, Kefir was only known by the people from the Caucasus Mountains.

However, Kefir is still unknown in Africa and especially to the Southern African consumer. Such a product could be an affordable replacement for industrial Maas (the locally South African fermented milk beverage) and other probiotic dairy products that are not only expensive but often do not contain all the viable and active probiotic populations labelled on the packaging (Theunissen *et al.*, 2005).

The dominant flavour of Kefir originates from the presence of metabolite compounds such as lactic and acetic acids, acetaldehyde, ethanol, acetone and diacetyl (Beshkova *et al.*, 2003; Magalhães *et al.*, 2011). However, the concentration of these flavour compounds must be balanced with acidity, which in Kefir is mainly imparted by lactic acid and acetic acid to a minor extent. Indeed, as clearly highlighted in Chapter 3, an inadequate concentration of lactic acid or improper pH and TA will affect the flavour and the texture of Kefir or any other fermented dairy products (Vedemuthu, 2006). Thus, since lactic and acetic acids are important contributors to flavour and knowing that the conventional methods of measuring these compounds are time consuming and expensive, a more rapid alternative was investigated – near infrared (NIR) spectroscopy (Chapters 4 and 5). The results showed that the calibration models developed for the prediction of lactic and acetic acids concentration, TA and pH were appropriate for screening purposes ($2 < \text{RPD} < 3$), which differentiates between low, high and normal concentrations. This proof of concept study indicated that an in-line NIR probe installed at a strategic point of the Kefir (or any other fermented dairy product) manufacturing process would alert to any departure from normal acidity levels. These calibration models could, however, not be used for accurate measurement of lactic acid concentration, pH or TA; thus NIR spectroscopy was not used in subsequent chapters to quantify acidity (lactic acid, pH or TA). Substitution of conventional methods with NIR spectroscopic methods would require the calibration models to have a $\text{RPD} > 5$. This could be achieved using the present calibration models by increasing the number of samples representing a broader range of sample traits (e.g. matured Kefir samples, Kefir from various sources) to ensure that all Kefir variation is accounted for.

Currently, the type of mass cultured Kefir grains (MG) (Schoevers & Britz, 2003) that could be sold and/or distributed to South African consumers do not result in a product with the typical Traditional Kefir flavour. Thus, for this study and based on the results obtained in Chapter 3, it is hypothesised that an improper balance between diacetyl and acetaldehyde and/or the absence of ethyl acetate could be the reason for the lack of *traditional* flavour in Kefir prepared with MG. Consequently, overcoming the lack of taste observed in Kefir prepared with MG could be achieved by enhancing the flavour of the product through synthesis of a particular flavour compound.

The aim of the study was thus to evaluate the metabolic profiles of selected LAB strains and a lactose fermenting yeast with emphasis on diacetyl and ethyl acetate production as to select the microorganisms to be used for flavour enhancement of Kefir. Furthermore, effects of citrate

and ascorbate addition on flavour compound production, particularly diacetyl and ethyl acetate during Kefir fermentation will also be determined.

Materials and methods

Bacterial strains and growth conditions

The freeze-dried LAB cultures used in this study are listed in Table 1 and were obtained from the Department of Food Science culture collection (DFSCC), University of Stellenbosch, South Africa. Stock cultures were maintained in MRS (Merck) at 4°C. These cultures were chosen because they have been reported to be present in Kefir (Angulo *et al.*, 1993; Simova *et al.*, 2002; Witthuhn *et al.*, 2005; Zhou *et al.*, 2009). Strain purity was regularly checked by microscopy and Gram staining. Catalase tests were also performed (Harrigan & McCance, 1998).

To plot the standard curves of each organism, all the cultures were grown in MRS (Merck). The respective bacterial counts were determined by dilution and plating in MRS. A growth profile of colony forming units (cfu.mL⁻¹) against absorbance at 540 nm (Spectronic 20, Genesys™), was constructed. These profiles were used to standardise the inoculum size at 10⁶ cfu.mL⁻¹.

Yeast strain growth conditions

A freeze-dried culture of *Candida kefir* 1283 was obtained from the Department of Food Science culture collection (DFSCC), Stellenbosch University, South Africa. A stock culture of *C. kefir* 1283 was chosen because it has been reported to be present in Kefir grains (Angulo *et al.*, 1993; Witthuhn *et al.*, 2005). A stock culture of *C. kefir* 1283 was maintained in yeast extract dextrose peptone broth (YDP) at 4°C. Strain purity was regularly checked by microscopy (Harrigan & McCance, 1998).

To plot a standard curve, *C. kefir* 1283 was grown in YDP. The respective yeast counts were determined by plating on YDP-Agar. A growth profile of colony forming unit (cfu.mL⁻¹) against absorbance at 540 nm (Spectronic 20, Genesys™), was constructed. This standard curve was used to standardise the inoculum size at 10⁵ cfu.mL⁻¹.

Kefir grain activation

Frozen mass cultured Kefir grains (MG) and laboratory Kefir grains (LG) were obtained from the Stellenbosch University, Department of Food Science and defrosted at ambient temperature for 24 h. The grains (20 g) were activated for 24 h at 25°C in 300 mL full cream pasteurised milk, then recovered by sieving and added to a new batch of milk. The grains were considered to be active after the fifth batch of milk.

Table 1. Lactic acid bacteria and lactose fermenting yeast used in the study.

DFSCC strains	Species
1348	<i>Lactobacillus acidophilus</i>
1278	<i>Lactobacillus delbrueckii</i> ssp. <i>delbrueckii</i>
1281	<i>Lactobacillus fermentum</i>
1325	<i>Lactobacillus kefiranofaciens</i>
140	<i>Lactococcus lactis</i> ssp. <i>lactis</i>
318	<i>Lactococcus lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i>
319	<i>Lactococcus lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i>
235	<i>Leuconostoc mesenteroides</i> ssp. <i>dextranicum</i>
1283	<i>Candida kefir</i>

DFSCC: Department of Food Science culture collection

Metabolic profiles

The metabolic profiles were done using headspace gas chromatography (HSGC). A sample (9.75 mL) of full cream milk fermented with a pure culture or a sample of Kefir was placed in a 20 mL glass vial containing 2.5 g of NaCl and 0.25 mL of tetrahydrofuran. The vial was crimp-sealed with a silicone-PTFE seal and aluminium cap and incubated in a waterbath for 50 min at 95°C (Dr. Sigge, G.O, Lecturer, Food Science Department, Stellenbosch, South Africa, Personal Communication, 2004). This temperature was chosen to optimise the volatilisation of the volatile organic compounds (VOCs) in the headspace. The vial's content was mixed several times while in the waterbath. A 1.5 mL aliquot of the headspace gas was withdrawn using a warmed (70°C) Hamilton gas-tight syringe and split-injected into the gas chromatograph at a split ratio of 1:100 (Human, 1998).

The metabolic profiles were identified by comparing the retention time of the unknown compounds to those of the analytical grade standards.

Headspace gas chromatography operating conditions

Separation and identification of volatile organic compounds (VOCs = acetaldehyde, ethanol, acetone, diacetyl, 2-butanone and ethyl acetate) were determined using a Fisons 8000 Series gas chromatograph (Fisons Instruments S.p.A., Milan, Italy) equipped with a flame ionisation detector (FID) and a 60 m DB5 capillary column bonded with a methyl-5% phenyl silicone layer as stationary phase (film thickness 0.25 µm; Quadrex Corporation, Newhaven). Operating parameters were: injector and detector temperatures were set at 150°C and 200°C, respectively; helium was used as carrier gas at a flow rate of 1.1 mL.min⁻¹. The oven heating cycle was programmed at 30°C for 2 min followed by an increase of 5°C per min to 220°C for 10 min.

Quantitative determination of the metabolic compounds was done by integration of the peak areas using an external standard calibration and Borwin Version 1.2 integration software (JMBS Developpements, Le Fontanil, France).

Volatile fatty acids

A standard solution of short chain volatile fatty acids (VFAs = acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids) was prepared by mixing 1 mL of each fatty acid and 0.5 mL of n-hexanol in a 1 000 mL volumetric flask with 250 mL of formic acid and 750 mL of distilled water.

Samples (milk fermented with a pure culture or Kefir grains) were prepared as follows: 10 mL of sample was centrifuged (10 min at 10 000 g) and the supernatant filtered through Whatman No. 1 filter to remove solid particles and obtain a clear supernatant. One millilitre of formic acid (35% v.v⁻¹) and 2 µL of n-hexanol (as internal standard) were added to 3 mL of the filtered supernatant. The GC injection volume was 1 µL and the run time 20 min.

The VFAs were determined using a Varian 3700 GC equipped with a flame ionisation

detector and a 30 m bonded phase Nukol (Supelco, Inc., Belafonte, PA) fused silica capillary column (0.53 mm diameter and 0.5 μm film thickness). The oven heating cycle program was held at 105°C for 2 min followed by an increase of 10°C per min to 190°C for 10 min. Injector and detector temperatures were 150 and 300°C, respectively. The flow rate of nitrogen, the carrier gas, was 6.1 mL.min⁻¹. The VFAs were quantified using the Borwin Version 1.2 integration software (JMBS Developpements, Le Fontanil, France) using the internal standard method (Sigge *et al.*, 2005).

pH and Titratable acidity (TA)

The pH of the Kefir was measured with an Orion pH meter and a glass electrode (Hanna Instruments). The TA was measured in triplicate by the titration of 10 mL sample with 0.11 N sodium hydroxide (NaOH) until the pink phenolphthalein end-point (James, 1999).

In Kefir, the major metabolite is lactic acid but acetic acid, which is also present, might influence the TA. Thus, TA will be expressed as % total acids instead of % lactic acid, using the following formula: TA (as % total acids) = (X mL 0.11 N NaOH used) / 10

Citrate phenotype

Kempler & McKay agar medium (KMK medium) (Kempler & McKay, 1980) was used to differentiate citrate positive (blue colonies) from citrate negative strains (white colonies). The sterile medium was prepared by adding non-fat milk (1 g), milk peptone (0.25 g), dextrose (0.5 g) and agar (1.5 g) to a litre of distilled water and the pH adjusted to 6.6. After cooling to 45°C, 10 mL of potassium ferricyanide (10% m.v⁻¹) and 10 mL of a solution containing 1 g of ferric citrate plus 1 g of sodium citrate in 40 mL of water, were added to the medium. Poured plates were covered in aluminum foil and dried for 24 h at 30°C. Spread plates were prepared and incubated for 48 h either in a hydrogen-carbon dioxide atmosphere (GasPak System, BBL Microbiology Systems) or aerobically at 30°C.

Study 1: Metabolic profiles of species in milk

The purpose of this study was to identify “aroma” forming bacteria (*Lb. acidophilus* 1348, *Lb. delbrueckii* 1278, *Lb. fermentum* 1281, *Lb. kefiranoferiens* 1325, *Ln. mesenteroides* ssp. *dextranicum* 235, *Lc. lactis* 140, *Lc. diacetylactis* 318 and *Lc. diacetylactis* 319) and yeast (*C. kefir* 1283) (Table 1) capable of producing diacetyl and ethyl acetate.

A 100 mL of full cream milk was inoculated with each specific culture at a concentration of 10⁶ cfu.mL⁻¹ for the bacterial strains and 10⁵ cfu.mL⁻¹ for the yeast; and incubated without agitation for 24 h at 22°, 25° and 30°C. At the end of the incubation period, samples were analysed for VOCs, VFAs, TA and pH.

Study 2: Effect of citrate on diacetyl production in milk

This study was undertaken to determine the effects of citrate on diacetyl production by *Lb. acidophilus* 1348, *Lb. kefiranofaciens* 1325 and *Lb. delbrueckii* 1278. These were chosen on the basis that they are part of the normal microbial population of Kefir grains (Santos *et al.*, 2003; Witthuhn *et al.*, 2004; Zhou *et al.*, 2009). *Lactococcus lactis* ssp. *diacetylactis* 318 was also included as it is known to be able to produce diacetyl (García-Quintans *et al.*, 2008).

Filter sterilised citrate (0.22 µm Millipore filter) was added to pasteurised milk (100 mL) to a final concentration of 0.3% (m.v⁻¹). The control contained no added citrate. The cultures (10⁶ cfu.mL⁻¹) were individually inoculated into the milk containing citrate and into the controls and incubated for 24 h at 22°, 25° and 30°C. At the end of the incubation, VOCs, VFAs, TA and pH were determined.

Study 3: Effect of ascorbate on diacetyl production in milk

This study was undertaken to determine the effects of ascorbate addition on diacetyl production. The four bacterial strains used in this study were the same as those used in Study 2.

Filter sterilised ascorbate (0.22 µm Millipore filter) was added (0.25% (m.v⁻¹)) to pasteurised milk. The control contained no added ascorbate. The cultures were individually inoculated (10⁶ cfu.mL⁻¹) into the milk containing ascorbate and into the controls and incubated at 22°, 25° and 30°C for 24 h. At the end of the incubation, VOCs, VFAs, pH and TA were determined.

Study 4: Effect of citrate on diacetyl and ethyl acetate production in Kefir samples

This study was undertaken to evaluate the effects of citrate on the metabolic profiles of Kefir prepared with laboratory Kefir grains (LG Kefir) and Kefir prepared with mass cultured Kefir grains (MG Kefir), especially on the diacetyl and ethyl acetate production.

Kefir grains were added to pasteurised milk (20 g in 300 mL) containing 0.015% (m.v⁻¹) of filter sterilised citrate. The controls contained no citrate. The containers were incubated at 22°C for 24 h and after removing the grains, the beverage was analysed for VOCs, VFAs, TA and pH.

Study 5: Effect of ascorbate on diacetyl and ethyl acetate production in Kefir samples

This study was undertaken to evaluate the effects of ascorbate on the metabolic profile of Kefir prepared with laboratory Kefir grains (LG Kefir) and Kefir prepared with mass cultured Kefir grains (MG Kefir), especially on diacetyl and ethyl acetate production.

Kefir grains were added to pasteurised milk (20 g in 300 mL) containing 0.015% (m.v⁻¹) filter sterilised ascorbate. The controls contained no ascorbate. The containers were incubated at 22°C for 24 h and after removing the grains, the beverage was analysed for VOCs, VFAs, TA and pH.

Statistical analysis

Analysis of variance (ANOVA) using Statistica 9.0 was performed to determine the effects of citrate and ascorbate on the metabolic profile (pH, TA, VOCs and VFAs) of Kefir beverages prepared with two types of Kefir grains. Differences in the metabolic profiles of Kefir and differences between the two Kefirs were compared at the 5% level of significance using the Bonferroni test.

Results and discussion

Study 1: Metabolic profiles of species in milk

The aim of this study was to evaluate diacetyl and ethyl acetate production by eight LAB strains and a lactose fermenting yeast (Table 1). All the data obtained are summarised in Table 2.

pH and TA

The data showed (Table 2) that amongst *Lactococcus lactis* strains, *Lc. diacetylactis* 318 was found to be the stronger acid producer with the TA varying between 0.65 and 1.02% and pH ranging between 4.21 and 4.56; whereas strains 319 and 140 showed lower acid production, with TA ranging between 0.22 and 0.44%. Similar TA results were also found with *Lb. acidophilus* 1348, *Lb. delbrueckii* 1278, *Lb. kefiranofaciens* 1325 and *Lb. fermentum* 1281 (Table 2) and in all cases, TA values were higher at the higher incubation temperatures.

The lowest acid level (0.16 – 0.19%) was found with *Ln. dextranicum* 235 where the pH only dropped from 6.35 to 5.85. However, some strains of *Ln. dextranicum* can decrease the pH to 4.8 as reported by Keenan (1968). According to Bills & Day (1966), low acid concentrations are an indication of a low overall rate of metabolism, which could be attributed to the fact that milk is deficient in specific amino acids, which are essential for *Leuconostoc* growth (Rea *et al.*, 1996; Bellengier *et al.*, 1997).

During the fermentation, the TA of the *C. kefir* 1283 strain was found to increase from 0.28 to 0.60% and the pH decreased from 6.14 to 5.73 as the incubation temperature was increased from 22° to 30°C. This is possible because *C. kefir* is a lactose fermenting yeast (Gadaga *et al.*, 2001; Gadaga *et al.*, 2007; Watanabe *et al.*, 2008).

Diacetyl

Diacetyl was only detected in the *Lc. diacetylactis* 318 samples and at all three incubation temperatures (Table 2), with the concentrations varying between 2.74 and 4.95 mg.L⁻¹ and increased with increasing temperatures. These results differ from those of Bassit *et al.* (1995), who found that diacetyl concentrations decreased with increasing temperatures. The formation of diacetyl was not surprising as the pH was favourable for citrate uptake, which is known to be induced at pH values below 5.5 (García-Quintáns *et al.*, 1998; García-Quintáns *et al.*, 2008). The

Table 2. Metabolite profiles of species inoculated in milk and incubated for 24 h at 22^o, 25^o and 30^oC (n = 3).

Incubation Temperature	Species	*pH	*TA (% total acids)	Acetaldehyde	Ethanol	Acetone	Diacetyl	Ethyl acetate	Acetic acid
				(mg.L ⁻¹)					
22 ^o C	<i>Lc. diacetylactis</i> 318	4.56	0.65	13.00 ± 0.51	36.20 ± 1.70	1.72 ± 0.18	2.74 ± 0.19	–	732.40 ± 34.0
	<i>Lc. diacetylactis</i> 319	6.14	0.22	2.03 ± 0.14	13.40 ± 1.33	3.12 ± 0.23	–	–	263.40 ± 4.11
	<i>Lc. lactis</i> 140	6.39	0.34	1.60 ± 0.11	–	–	–	–	65.00 ± 7.23
	<i>Ln. dextranicum</i> 235	6.34	0.16	–	10.20 ± 0.49	1.53 ± 0.21	–	–	92.10 ± 0.92
	<i>Lb. fermentum</i> 1281	6.28	0.20	–	7.05 ± 0.53	1.50 ± 0.16	–	–	161.80 ± 8.29
	<i>Lb. acidophilus</i> 1348	6.67	0.20	0.44 ± 0.01	–	1.01 ± 0.03	–	–	158.60 ± 8.29
	<i>Lb. delbrueckii</i> 1278	6.29	0.17	2.12 ± 0.27	33.80 ± 2.34	3.87 ± 0.13	–	–	155.50 ± 1.63
	<i>Lb. kefiranofaciens</i> 1325	6.39	0.21	1.80 ± 0.19	18.50 ± 0.99	2.20 ± 0.23	–	–	162.50 ± 24.1
	<i>C. kefir</i> 1283	6.14	0.28	6.65 ± 1.70	378.60 ± 48.9	–	–	2.13 ± 0.09	77.60 ± 12.8
25 ^o C	<i>Lc. diacetylactis</i> 318	4.31	0.81	11.90 ± 0.20	32.40 ± 1.34	1.55 ± 0.16	4.32 ± 0.88	–	619.60 ± 39.7
	<i>Lc. diacetylactis</i> 319	5.67	0.33	–	35.60 ± 1.35	2.37 ± 0.38	–	–	195.60 ± 9.60
	<i>Lc. lactis</i> 140	6.17	0.22	–	5.89 ± 0.86	1.68 ± 0.35	–	–	145.80 ± 1.28
	<i>Ln. dextranicum</i> 235	6.35	0.19	–	17.20 ± 0.95	2.06 ± 0.19	–	–	111.50 ± 4.56
	<i>Lb. fermentum</i> 1281	6.10	0.26	1.77 ± 0.13	88.50 ± 5.85	–	–	–	236.20 ± 7.40
	<i>Lb. acidophilus</i> 1348	6.19	0.24	0.35 ± 0.04	45.60 ± 2.06	3.40 ± 0.76	–	–	127.10 ± 6.48
	<i>Lb. delbrueckii</i> 1278	6.21	0.22	1.99 ± 0.12	43.60 ± 2.06	1.68 ± 0.22	–	–	211.10 ± 30.8
	<i>Lb. kefiranofaciens</i> 1325	6.36	0.21	2.45 ± 0.19	23.70 ± 1.07	2.28 ± 0.15	–	–	221.10 ± 10.1
	<i>C. kefir</i> 1283	6.05	0.42	9.72 ± 2.07	546.40 ± 91.6	–	–	3.87 ± 0.88	153.50 ± 18.4
30 ^o C	<i>Lc. diacetylactis</i> 318	4.21	1.02	9.36 ± 0.67	30.70 ± 2.10	2.21 ± 0.60	4.95 ± 0.42	–	712.40 ± 66.0
	<i>Lc. diacetylactis</i> 319	5.21	0.44	2.27 ± 0.38	32.30 ± 2.22	2.94 ± 0.11	–	–	225.70 ± 22.7
	<i>Lc. lactis</i> 140	5.91	0.35	3.71 ± 0.22	35.20 ± 2.16	2.24 ± 0.18	–	–	165.90 ± 7.64
	<i>Ln. dextranicum</i> 235	6.36	0.21	1.77 ± 0.36	18.90 ± 0.73	2.45 ± 0.43	–	–	140.00 ± 8.57
	<i>Lb. fermentum</i> 1281	5.73	0.34	3.19 ± 0.20	43.90 ± 2.49	1.56 ± 0.19	–	–	191.60 ± 26.5
	<i>Lb. acidophilus</i> 1348	5.55	0.39	2.30 ± 0.50	11.10 ± 0.58	1.95 ± 0.18	–	–	148.40 ± 7.16
	<i>Lb. delbrueckii</i> 1278	6.07	0.26	1.59 ± 0.22	28.90 ± 1.93	2.01 ± 0.03	–	–	233.50 ± 21.1
	<i>Lb. kefiranofaciens</i> 1325	6.15	0.35	–	23.60 ± 1.07	1.81 ± 0.19	–	–	194.30 ± 10.4
	<i>C. kefir</i> 1283	5.73	0.60	12.2 ± 2.09	1387.70 ± 377	–	–	5.07 ± 1.08	101.20 ± 10.5

Data represent the mean ± standard deviation

(*)Standard deviations below 10%;

(–) not detected

presence of a citrate positive phenotype was confirmed using the KMK medium (Photo 1).

Surprisingly, no diacetyl was detected in the *Lc. diacetylactis* 319 samples even when the pH was favourable to initiate citrate degradation (at 30°C, pH 5.21). The present results differ from those reported by Crow (1990), who found that this specific strain (319) produced 18.9 mg.L⁻¹ diacetyl in the presence of lactose and citrate. Thus, several possibilities may have led to the lack of diacetyl formation by *Lc. diacetylactis* 319:

- it is possible that formed diacetyl was irreversibly converted to acetoin by diacetyl reductase.

- it is possible that strain 319 had lost the plasmid CitP that encodes for the synthesis of citrate permease P. To verify, strain 319 was grown on KMK differential agar medium. The plate showed growth of mixed colonies (Photo 2) as if strain 319 was composed of variants (mutants and wild population). This was previously reported with various pure cultures (Kempfer & McKay, 1979).

It was also found that with milk inoculated with *Lc. lactis* 140 and *Ln. dextranicum* 235 did not contain diacetyl. This is not surprising as the pH was also not favourable (> 5.90) to initiate citrate uptake. However, *Ln. dextranicum* 235 culture gave a positive citrate phenotype on the KMK medium (Photo 3). A positive result was also reported by Levata-Javanovic & Sandine (1996) for several strains of *Leuconostoc mesenteroides* ssp. *dextranicum* also grown on the KMK medium.

It is well known that an acidic environment (4.1 < pH < 4.4) is essential for leuconostoc to produce flavour compounds. At neutral pH citrate is consumed without production of diacetyl and acetoin (Levata-Jovanovic & Sandine, 1996). This is why, to obtain the maximum flavour benefits from *Leuconostoc* strains, it has been recommended to combine them with lactose fermenters such as lactococci (Cogan & Jordan, 1994), which would reduce the pH and create a favourable environment for leuconostoc to initiate citrate degradation.

Diacetyl was not present in *Lb. fermentum* 1281 milk samples (Table 2). According to Thornill & Cogan (1984), some heterofermentative bacteria such as *Lb. fermentum* can utilise citrate but without production of diacetyl and/or acetoin. According to Drinan *et al.* (1976), the absence of diacetyl in heterofermentative bacteria is a general characteristic. However, this does not imply that traces of diacetyl cannot be synthesised as shown by Annan *et al.* (2003) who found traces of diacetyl (0.28 mg.L⁻¹) in cereal based substrates inoculated with *Lb. fermentum*. This clearly demonstrates that this bacterium may adjust its metabolic routes according to the substrate availability and enzymatic capacity.

The *Lb. acidophilus* 1348 and *Lb. delbrueckii* 1278 milk samples did not contain diacetyl (Table 2). Both species are thermophilic bacteria, which do not metabolise citrate and produce diacetyl (Hadadji & Bensoltane, 2006). The same result was observed for *C. kefir* 23. In this case, acetoin was detected (2.9 mg.L⁻¹) but not diacetyl suggesting that diacetyl was originally

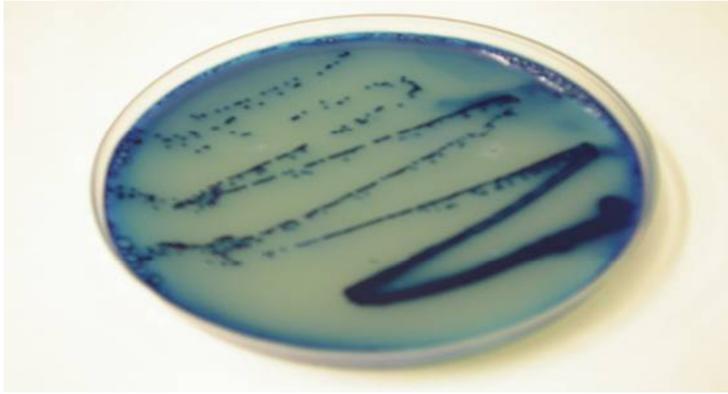


Photo 1. *Lactococcus diacetylactis* 318 grown on KMK medium. The presence of a blue colouration indicates a citrate positive phenotype.



Photo 2. *Lactococcus diacetylactis* 319 grown on KMK medium. The presence of blue colouration indicates a citrate positive phenotype.



Photo 3. *Leuconostoc dextranicum* 235 grown on KMK medium. The presence of blue colouration indicates a citrate positive phenotype.

formed but reduced to acetoin by diacetyl reductase (Gadaga *et al.*, 2007).

Acetaldehyde and ethanol

In the *Lc. diacetylactis* 318 samples, both acetaldehyde (9.36 – 13 mg.L⁻¹) and ethanol concentrations (30.7 – 36.2 mg.L⁻¹) were produced with production decreasing at the higher temperatures (Table 2). This was probably due to optimum activity of the enzymes (alcohol dehydrogenase) involved in acetaldehyde and ethanol formation decreasing as the temperature increased. In other studies (Thornill & Cogan, 1984) where the same strain was used, the concentrations of acetaldehyde and ethanol found were reported to be below 4 mg.L⁻¹ and 1.2 mg.L⁻¹, respectively. In contrast, Schmitt *et al.* (1988) used a strain of *Lc. diacetylactis* which produced up to 180 mg.L⁻¹ of ethanol.

In *Lc. diacetylactis* 319 samples, acetaldehyde (2.00 – 2.30 mg.L⁻¹) and ethanol (13.4 – 35.6 mg.L⁻¹) were detected at all three temperatures. In the *Lc. lactis* 140 samples, acetaldehyde (1.6 – 3.71 mg.L⁻¹) and ethanol (5.89 – 35.2 mg.L⁻¹) concentrations were only detected at 22° and 30°C (Table 2).

In the case of *Ln. dextranicum* 235 samples, acetaldehyde was present at a low concentration (1.77 mg.L⁻¹) and only in the 30°C sample. In the conditions of this study, samples contained 10 to 20 mg.L⁻¹ of ethanol. In the literature, it has been reported that strains of *Ln. dextranicum* produced between 10 and 743 mg.L⁻¹ ethanol at 30°C (Keenan, 1968), suggesting that the ability to produce ethanol depends on the specific strain and not so much the environmental conditions.

The concentrations of acetaldehyde produced by *Lb. fermentum* 1281 were low (1.77 – 3.19 mg.L⁻¹) and increasing with temperatures. The same pattern was observed for ethanol, whose concentrations varied between 7.05 and 88.5 mg.L⁻¹. However, it has been reported that when using other substrates such as modified MRS and cereal-based substrates, the concentration of ethanol produced by *Lb. fermentum* could reach up to 500 mg.L⁻¹ (Annan *et al.*, 2003).

For the *Lb. acidophilus* 1348 (Table 2), acetaldehyde (2.3 mg.L⁻¹) was only present in the 30°C samples. This concentration was probably low because the incubation temperature was not optimum for this thermophilic strain. Østlie *et al.* (2003) found acetaldehyde concentrations of up to 12.6 mg.L⁻¹ in milk samples supplemented with tryptone (0.5% w.v⁻¹) inoculated with *Lb. acidophilus* at 37°C. The *Lb. acidophilus* 1348 strain also produced ethanol at 25° and 30°C (11.8 and 45.6 mg.L⁻¹). This indicates the presence of an alcohol dehydrogenase (ADH) as mentioned by Bonzcar *et al.* (2002). However, Gonzales *et al.* (1994) did not find any ADH activity in the *Lb. acidophilus* strain, suggesting that ethanol synthesis could be strain dependant.

The low concentrations of acetaldehyde (< 2.1 mg.L⁻¹) and high concentrations of ethanol (28.9 – 44.0 mg.L⁻¹), in the *Lb. delbrueckii* 1278 samples suggested that the enzymatic activities

responsible for the acetaldehyde synthesis were low compared to ADH activity as demonstrated by ethanol concentrations reaching 44 mg.L⁻¹.

For *Lb. kefiranofaciens* 1325, the results show that ethanol (18.5 to 23.7 mg.L⁻¹) and acetaldehyde (1.80 to 2.45 mg.L⁻¹) were produced. No previous analytic data on the metabolic profiles of *Lb. kefiranofaciens* could be found in the literature.

Acetone

All the strains examined with the exception of *C. kefir* 1283 produced acetone. The lowest concentration was found at 22°C for *Lb. acidophilus* 1348 and the highest 3.87 mg.L⁻¹ for *Lb. delbrueckii* 1278 (Table 2).

Acetic acid

Data showed that all the strains evaluated produced acetic acid at all three temperatures (Table 2). The highest concentration was produced by *Lc. diacetylactis* 318 (620 to 712 mg.L⁻¹). It is known that this strain actively catabolises citrate with production of diacetyl, acetic and the flavourless compounds acetoin and 2,3-butanediol (Cogan, 1995). In contrast, *Lc. diacetylactis* 319 formed three times lower concentrations (196 to 264 mg.L⁻¹) than strain 318. Among the lactococci, *Lc. lactis* 140 produced the lowest concentrations of acetic acid (92 to 140 mg.L⁻¹). The concentration of acetic acid found in milk inoculated with the other strains was below 270 mg.L⁻¹.

Ethyl acetate

Ethyl acetate was only found in milk samples inoculated with *C. kefir* 1283. The concentration was found to increase (2.13 to 5.07 mg.L⁻¹) with temperature from 22°C to 30°C. The presence of ethyl acetate was also reported in milk inoculated with *C. kefir* 23 (Narvhus & Gadaga, 2003) along with other compounds such as amyl alcohol, isobutanol, 2-methyl-propanal and 3-methyl-propanal.

None of the LAB strains produced ethyl acetate, although “in vitro” studies (Abeijón Mukdsi *et al.*, 2009) showed that *Lactobacillus fermentum* ETC1, *Lactobacillus delbrueckii* ssp. *bulgaricus* ETC2, *Lactobacillus plantarum* Ov156 were able to produce ethyl esters of short chain fatty acids, with ethyl butanoate and ethyl hexanoate being the most abundant esters synthesised.

Conclusions for study 1

Among the eight LAB strains and the lactose fermenting yeast, only *Lc. diacetylactis* 318 and *C. kefir* 1283 were able to synthesise diacetyl and ethyl acetate, respectively. The absence of diacetyl in the metabolic profiles of the other strains may originate from their poor capacity to acidify the growth medium since diacetyl production occurs preferably in an acidic environment (Bassit *et al.*, 1995). As for ethyl acetate, production was independent of the acidity of the medium.

It is, therefore, important to assess whether the lack of diacetyl was caused by the lack of precursors.

Study 2: Effect of citrate on diacetyl production in milk

The metabolic profile of many dairy microbes depends on the ability to metabolise lactose, proteins, fats and other compounds such as citrate. Routing microbial metabolic pathway profiles towards a particular compound can be achieved by enriching the growth environment with specific substrates (Richter *et al.*, 1979; Marshall & Cole, 1983; Levata-Jovanovic & Sandine, 1996). In the dairy industry, milk fortification with citrate is a well known practice and has the advantage of stabilising the level of diacetyl in cultured dairy products as more precursors are available (Vedemuthu, 1994). In the USA, the Code of Federal Regulations (1990) allows the addition of 0.15% citrate, which can be used by, Lactococci and *Leuconostoc* spp. to produce additional flavour compounds (Crow, 1990).

In this study, citrate was added to milk inoculated with *Lc. diacetylactis* 318, *Lb. acidophilus* 1348, *Lb. delbrueckii* 1278 and *Lb. kefiranofaciens* 1325. Those strains were selected because they were previously isolated from Kefir grains. The aim was to determine the impact of citrate as precursor during flavour formation and specifically diacetyl production. All the data are summarised in Table 3.

pH and TA

From the data in Table 3, it can be seen that for all the strains the TA increased in the presence of citrate (0.30 – 0.85%) compared to the controls (0.16 – 0.76%) as the temperature increases. For the pH, in the presence of citrate, the values dropped in most cases, with the exception of *Lc. diacetylactis* 318.

Diacetyl

From all the strains examined, only *Lc. diacetylactis* 318 produced diacetyl (3.99 to 6.74 mg.L⁻¹) with higher amounts in the samples with added citrate (6.92 to 10.4 mg.L⁻¹) (Table 3). This increase may have been a result of the addition of citrate, which probably led to excess pyruvate formation. The values of diacetyl found in the test samples are higher than expected considering that diacetyl concentrations below 5 mg.L⁻¹ have been sufficient to flavour cultured dairy products (Vasavada *et al.*, 1985; Oberman & Libudzisz, 1998).

Acetaldehyde and ethanol

Acetaldehyde and ethanol were produced by the four strains (Table 3) but the concentrations differed at the different incubation temperatures. It was noticed that the addition of citrate inhibited acetaldehyde production, particularly in *Lb. delbrueckii* 1278 and *Lb. kefiranofaciens* 1325 samples whereas in most of the samples, addition of citrate led to a decrease in ethanol production. Similar

Table 3. Effects of citrate on the metabolic profiles of four LAB strains inoculated in milk and incubated for 24 h at 22°, 25° and 30°C (n = 3).

Species	Method	Incubation Temperature	*pH	*TA (% total acids)	Acetaldehyde	Ethanol	Acetone	Diacetyl	Acetic acid
					(mg.L ⁻¹)				
<i>Lc. diacetylactis</i> 318	Control	22°C	4.61	0.61	25.4 ± 0.38	71.10 ± 5.38	2.52 ± 0.22	5.86 ± 0.98	1010.00 ± 39.10
	Control + citrate		4.54	0.74	13.9 ± 0.90	45.50 ± 4.16	1.14 ± 0.12	6.92 ± 0.09	287.00 ± 2.87
<i>Lb. acidophilus</i> 1348	Control	22°C	6.37	0.20	–	8.18 ± 0.28	1.11 ± 0.15	–	145.00 ± 4.48
	Control + citrate		5.34	0.69	–	5.01 ± 0.15	2.96 ± 0.26	–	116.00 ± 11.20
<i>Lb. delbrueckii</i> 1278	Control	22°C	6.23	0.30	2.5 ± 0.04	19.30 ± 1.56	2.64 ± 0.48	–	117.00 ± 8.44
	Control + citrate		5.18	0.57	–	10.20 ± 1.09	3.12 ± 0.24	–	76.30 ± 3.97
<i>Lb. kefiranofaciens</i> 1325	Control	22°C	6.30	0.16	1.52 ± 0.13	16.30 ± 1.63	2.35 ± 0.16	–	150.00 ± 27.20
	Control + citrate		5.14	0.30	–	10.50 ± 0.44	2.18 ± 0.44	–	118.00 ± 17.10
<i>Lc. diacetylactis</i> 318	Control	25°C	4.47	0.66	12.8 ± 0.48	39.90 ± 1.65	1.58 ± 0.31	6.74 ± 0.70	997.00 ± 59.20
	Control + citrate		4.53	0.82	16.4 ± 0.50	51.20 ± 1.29	1.47 ± 0.08	10.4 ± 0.65	350.30 ± 16.90
<i>Lb. acidophilus</i> 1348	Control	25°C	6.26	0.20	–	19.10 ± 1.76	3.07 ± 0.13	–	173.10 ± 18.10
	Control + citrate		5.30	0.76	–	22.30 ± 0.79	8.04 ± 0.27	–	94.10 ± 5.32
<i>Lb. delbrueckii</i> 1278	Control	25°C	5.98	0.30	3.48 ± 0.30	32.70 ± 1.52	2.85 ± 0.20	–	112.20 ± 9.70
	Control + citrate		5.09	0.61	–	21.30 ± 2.03	3.65 ± 0.38	–	76.80 ± 1.29
<i>Lb. kefiranofaciens</i> 1325	Control	25°C	6.21	0.16	2.30 ± 0.15	28.90 ± 0.95	2.05 ± 0.15	–	169.40 ± 15.40
	Control + citrate		5.09	0.40	–	7.09 ± 1.61	2.35 ± 0.39	–	119.30 ± 5.30
<i>Lc. diacetylactis</i> 318	Control	30°C	4.31	0.76	13.8 ± 0.25	34.10 ± 2.79	–	3.99 ± 0.15	1023.00 ± 24.90
	Control + citrate		4.54	0.80	11.7 ± 0.66	36.30 ± 1.29	–	9.52 ± 0.22	326.00 ± 28.10
<i>Lb. acidophilus</i> 1348	Control	30°C	5.80	0.34	2.50 ± 0.29	62.60 ± 1.62	2.45 ± 0.11	–	203.30 ± 3.05
	Control + citrate		4.58	0.85	10.6 ± 0.68	67.50 ± 0.77	3.21 ± 0.09	–	271.10 ± 4.07
<i>Lb. delbrueckii</i> 1278	Control	30°C	5.25	0.30	1.59 ± 0.22	28.90 ± 1.93	2.01 ± 0.03	–	175.10 ± 15.80
	Control + citrate		4.86	0.54	1.56 ± 0.08	2.77 ± 0.11	2.77 ± 0.21	–	102.00 ± 3.09
<i>Lb. kefiranofaciens</i> 1325	Control	30°C	5.68	0.22	–	23.60 ± 1.07	1.81 ± 0.19	–	145.70 ± 7.81
	Control + citrate		5.00	0.51	1.42 ± 0.08	9.92 ± 0.44	2.81 ± 0.21	–	85.30 ± 2.20

Data represent the mean ± standard deviation

(*) Standard deviations below 10%

(–) not detected

results were found by other researchers (Cogan, 1987; Schmitt & Divies, 1991) with *Leuconostoc* and *Lactococcus* strains. They suggested that the enzymes involved in the biosynthesis of ethanol and acetaldehyde might have been slightly inhibited by the presence of citrate (Cogan, 1987).

Acetic acid

Acetic acid was produced by all the strains (Table 3). The highest producer in samples with or without added citrate was *Lc. diacetylactis* 318 and the concentration varied between 997 and 1023 mg.L⁻¹. The addition of citrate led to a sharp decrease in acetic acid (287 – 350 mg.L⁻¹). A possible explanation for the decrease is that acetic acid was used for the synthesis of cell material (Collins & Bruhn, 1970).

Ethyl acetate

None of the samples (with or without added citrate) produced ethyl acetate.

Conclusions for study 2

Addition of citrate was responsible for the production of higher concentrations of diacetyl only in *Lc. diacetylactis* 318, at all incubation temperatures. For the remaining strains, the addition of citrate did not lead to diacetyl production even when the pH was favourable, indicating that the ability to take up and degrade citrate was strongly strain dependant since literature does report production of diacetyl by LAB such as *Lb. acidophilus* and *Lb. delbrueckii*.

Study 3: Effect of ascorbate on diacetyl production

It has been shown in the literature that ascorbate could lead to improved diacetyl formation (Richter *et al.*, 1979). Thus, in this study, ascorbate was added to milk inoculated with *Lc. diacetylactis* 318, *Lb. acidophilus* 1348, *Lb. delbrueckii* 1278 and *Lb. kefiranofaciens* 1325 to evaluate the impact during flavour formation and specifically diacetyl. These cultures were chosen on the basis that they are part of the normal microbial population of Kefir grains (Santos *et al.*, 2003; Witthuhn *et al.*, 2004). All the data obtained are summarised in Table 4.

pH and TA

The presence of ascorbate led to a decrease in pH and an increase in TA in all samples and at all incubation temperatures (Table 4). Similar findings were also reported by Anderson & Elliker (1953).

Diacetyl

Diacetyl was again only detected in the *Lc. diacetylactis* 318 test samples as well as in the controls at all incubation temperatures (Table 4). For the other strains, ascorbate had no effect on diacetyl

Table 4. Effects of ascorbate on the metabolite profiles of incubated in milk for 24 h and at 22^o, 25^o and 30^oC (n = 3).

Incubation Temperature	Species	Method	*pH	*TA (% total acids)	Acetaldehyde	Ethanol	Acetone	Diacetyl	Acetic acid
					(mg.L ⁻¹)				
22 ^o C	<i>Lc. diacetylactis</i> 318	Control	4.58	0.70	15.2 ± 0.72	50.4 ± 4.45	2.00 ± 0.31	4.51 ± 0.52	851.00 ± 42.50
		Control + ascorbate	4.45	0.75	18.4 ± 0.45	46.3 ± 3.39	1.98 ± 0.17	4.40 ± 0.67	837.30 ± 30.50
	<i>Lb. acidophilus</i> 1348	Control	6.45	0.15	1.25 ± 0.11	–	2.08 ± 0.38	–	163.00 ± 5.29
		Control + ascorbate	5.91	0.26	1.27 ± 0.34	–	3.75 ± 1.11	–	112.00 ± 13.90
	<i>Lb. delbrueckii</i> 1278	Control	6.38	0.19	0.63 ± 0.15	–	1.76 ± 0.10	–	151.00 ± 18.70
		Control + ascorbate	5.92	0.32	1.61 ± 0.70	–	4.02 ± 1.35	–	99.00 ± 23.30
	<i>Lb. kefiranofaciens</i> 1325	Control	6.31	0.18	1.11 ± 0.42	11.6 ± 0.10	2.06 ± 0.68	–	151.00 ± 28.00
		Control + ascorbate	5.84	0.31	1.37 ± 0.34	4.94 ± 0.79	3.85 ± 0.81	–	167.00 ± 18.00
25 ^o C	<i>Lc. diacetylactis</i> 318	Control	4.40	0.75	14.3 ± 0.37	30.50 ± 1.41	1.70 ± 0.20	6.00 ± 0.50	700.50 ± 45.10
		Control + ascorbate	4.30	0.82	10.5 ± 0.23	27.40 ± 3.65	1.67 ± 0.36	5.50 ± 0.70	670.60 ± 51.20
	<i>Lb. acidophilus</i> 1348	Control	6.24	0.19	1.36 ± 0.29	29.80 ± 3.91	1.81 ± 0.20	–	162.00 ± 16.6
		Control + ascorbate	5.79	0.27	1.19 ± 0.31	4.77 ± 0.77	3.31 ± 1.22	–	114.00 ± 7.09
	<i>Lb. delbrueckii</i> 1278	Control	6.17	0.21	1.18 ± 0.18	20.20 ± 11.30	2.31 ± 0.57	–	234.00 ± 11.40
		Control + ascorbate	5.68	0.31	1.70 ± 0.19	11.80 ± 5.84	4.56 ± 0.43	–	137.00 ± 3.50
	<i>Lb. kefiranofaciens</i> 1325	Control	6.07	0.20	1.39 ± 0.44	22.00 ± 10.90	2.31 ± 1.06	–	189.00 ± 9.54
		Control + ascorbate	5.66	0.31	1.27 ± 0.32	9.53 ± 3.06	3.45 ± 0.63	–	158.00 ± 8.54
30 ^o C	<i>Lc. diacetylactis</i> 318	Control	4.25	0.90	13.30 ± 0.15	28.80 ± 1.90	–	3.50 ± 0.25	767.30 ± 37.10
		Control + ascorbate	4.15	0.97	9.80 ± 0.30	21.50 ± 2.70	–	3.70 ± 0.31	760.40 ± 40.10
	<i>Lb. acidophilus</i> 1348	Control	5.3	0.42	2.35 ± 0.77	49.90 ± 6.90	2.35 ± 0.49	–	198.00 ± 8.14
		Control + ascorbate	5.14	0.58	2.51 ± 0.41	12.10 ± 2.83	4.68 ± 0.45	–	148.00 ± 23.50
	<i>Lb. delbrueckii</i> 1278	Control	5.23	0.48	1.54 ± 0.42	47.20 ± 20.1	2.00 ± 0.36	–	192.00 ± 10.0
		Control + ascorbate	4.76	0.50	3.36 ± 1.24	26.70 ± 10.2	3.84 ± 0.19	–	169.00 ± 21.0
	<i>Lb. kefiranofaciens</i> 1325	Control	5.72	0.31	1.37 ± 0.84	17.10 ± 11.7	2.77 ± 1.99	–	208.00 ± 10.0
		Control + ascorbate	5.21	0.48	2.25 ± 0.58	39.00 ± 15.5	4.60 ± 0.17	–	171.00 ± 9.50

Data represent the mean ± standard deviation

(*) Standard deviations below 10%

(–) not detected

production since no diacetyl was detected even in the samples where the pH was favourable for diacetyl production. However, presence of diacetyl was reported in mixed strains LAB cultures of *Leuconostoc cremoris* and *Lactococcus lactis* (Richter *et al.*, 1979).

Acetaldehyde and ethanol

Acetaldehyde and ethanol were detected in all samples, except in *Lb. acidophilus* 1348 samples and *Lb. delbrueckii* 1278 samples incubated at 22°C. Ascorbate had a positive effect on acetaldehyde production particularly in samples inoculated with *Lb. delbrueckii* 1278. The concentrations of ethanol generally decreased in the test samples compared to the controls (Table 4).

Acetone

The addition of ascorbate generally caused an increase in acetone concentrations compared to the control, except for *Lc. diacetylactis* 318 where no effect could be observed.

Acetic acid

It is generally observed that for all strains, at all the incubation temperatures, the addition of ascorbate led to a decrease in acetic acid concentrations (Table 4).

Ethyl acetate

None of the samples with or without ascorbate contained ethyl acetate.

Conclusions for study 3

Very little is known on the effects of ascorbate on the production of flavour compounds by pure cultures of LAB compared to citric acid. However, the fact that there is an increase in acidity in presence of ascorbic acid indicate that under optimum culturing conditions some strains may be able to produce diacetyl .

Study 4: Effect of citrate on diacetyl and ethyl acetate production in Kefir samples

Diacetyl plays a major flavour role in Kefir, and it is hypothesised that the addition of citrate to milk would promote the activity of diacetyl producing bacteria. In contrast, ethyl acetate imparts a fruity flavour but Kefir has not been labelled to have a fruity flavour (Wszolek *et al.*, 2001; Irigoyen *et al.*, 2005). Thus, the aim of this study was to evaluate the impact of citrate on the volatile flavour compounds, with emphasis on diacetyl and ethyl acetate, during Kefir production. To achieve this, two types of Kefir grains were used; the laboratory Kefir grains (LG) and mass cultured Kefir grains (MG). All the data are summarised in Table 5.

Table 5. Effects of citrate on the metabolite profiles of LG Kefir and MG Kefir. Kefir samples were incubated for 24 h at 22°C (n = 3).

Kefir grains	Method	*pH	*TA (%)	Acetaldehyde	Ethanol	Acetone	Diacetyl	Ethyl acetate	Acetic acid
LG	without citrate	3.95	0.84	5.18 ± 0.91	915 ± 157.00	0.95 ± 0.21	0.65 ± 0.16	–	357 ± 76.70
LG	with citrate	3.95	0.84	5.41 ± 1.62	898 ± 326.00	1.91 ± 0.54	1.09 ± 0.24	–	468 ± 16.10
MG	without citrate	4.05	0.90	5.31 ± 0.70	1 307 ± 52.10	1.15 ± 0.16	0.92 ± 0.11	0.14 ± 0.018	471 ± 18.70
MG	with citrate	4.03	0.99	3.54 ± 1.90	811 ± 141.00	1.55 ± 0.13	1.77 ± 0.32	0.13 ± 0.005	509 ± 21.70

Data represent the mean ± standard deviation

(*) Standard deviations below 10%

(–) not detected

pH and TA

The addition of citrate did not have a significant impact ($p > 0.05$) on pH or TA of the two types of Kefir (Table 5).

Diacetyl

In both LG Kefir and MG Kefir, the samples containing citrate had higher concentrations of diacetyl than the samples without citrate. However, these increases were not significant ($p > 0.05$).

Ethyl acetate

Traces of ethyl acetate were only found in the MG Kefir (0.14 mg.L^{-1}). Addition of citrate had no significant effect ($p < 0.05$) on ethyl acetate synthesis (0.13 mg.L^{-1}). This suggests that citrate does not have any effect in the biosynthesis of ethyl acetate.

Acetaldehyde and ethanol

In LG Kefir, the concentrations of acetaldehyde were stable in both samples without citrate (5.18 mg.L^{-1}) and with citrate (5.41 mg.L^{-1}). The same pattern was observed for ethanol, where the sample without citrate had 898 mg.L^{-1} of ethanol and the sample with citrate 915 mg.L^{-1} of ethanol.

In MG Kefir, the addition of citrate caused a significant decrease ($p < 0.05$) in the concentrations of acetaldehyde, from 5.31 mg.L^{-1} without citrate to 3.54 mg.L^{-1} in the sample with citrate. The same pattern was observed for ethanol, in which concentrations decreased significantly ($p < 0.05$) from 1310 mg.L^{-1} without citrate to 811 mg.L^{-1} in MG Kefir.

Acetic acid

In both LG Kefir and MG Kefir, addition of citrate led to an increase in the concentration of acetic acid in sample with citrate compared to the samples without citrate. However, those increases were not significant ($p > 0.05$) (Table 5).

Acetone

The effect of citrate addition on acetone production was not significant ($p > 0.05$) in the MG Kefir (Table 5). On the contrary, addition of citrate had a significant effect ($p < 0.05$) on acetone concentration found in LG Kefir, with three times more acetone (1.91 mg.L^{-1}) found than in the sample without citrate (0.63 mg.L^{-1}).

Discussion for study 4

The values obtained for acetaldehyde, ethanol, acetone, diacetyl, ethyl acetate, acetic acid, pH and TA in the samples without citrate and with citrate were within the range found in the literature. This indirectly suggested that addition of citrate did not cause any 'metabolic imbalance' which

could have given values outside the range usually found in the literature. In addition, it was highlighted in Chapter 3 that in most good quality cultured dairy products, the ratio of diacetyl to acetaldehyde varies between 3 and 5 (Sandine *et al.*, 1972). However, in Chapter 3, it was shown that a lower ratio, ranging from 0.10 to 1.75, might be more applicable to Kefir. In this study, the ratio found for LG Kefir (without citrate) was 0.12 and the ratio found after addition of citrate was 0.21. For MG Kefir, the ratios found were 0.17 and 0.5 for the samples without and with citrate, respectively. In both LG Kefir and MG Kefir, the ratios obtained in the samples with citrate were higher than the ratios obtained in the samples without. Thus, these results give a clear indication that addition of citrate did contribute to the improvement of the flavour of LG Kefir since the diacetyl to acetaldehyde ratio improved.

Conclusion for study 4

Citrate can be used as an additive to improve diacetyl production in Kefir, with a positive effect on the overall flavour of Kefir, by improving the ratio of diacetyl to acetaldehyde. This is particularly important because an excess acetaldehyde is responsible for the 'grassy' odour whereas excess diacetyl can cause cultured dairy products to have a harsh and pungent flavour. In addition, an insufficient quantity of acetaldehyde cannot smooth out the astringent diacetyl after-flavour (Sandine *et al.*, 1972; Cais-Sokolinska *et al.*, 2008).

Study 5: Effects of ascorbate on diacetyl and ethyl acetate production in Kefir samples

Richter *et al.* (1979) reported that the addition of ascorbate to milk stimulated the production of diacetyl in mixed cultures of lactic acid bacteria. However, the effects of ascorbate on ester production are unknown. Kefir grains are a consortium of microorganisms, and thus the effects of ascorbate on diacetyl and ethyl acetate production in Kefir were evaluated in this study. All the data are summarised in Table 6.

pH and TA

The addition of ascorbate did not have a significant effect ($p > 0.05$) on the pH or TA values on both types of Kefir (Table 6).

Diacetyl

In LG Kefir, no diacetyl was detected in both samples with and without ascorbate, whereas in the previous study, diacetyl was present. The lack of diacetyl may originate from its transformation into acetoin, which was not measured in this study.

No diacetyl was detected in the MG Kefir sample without ascorbate, whereas 1.05 mg.L^{-1} of diacetyl was found in the MG Kefir, with added ascorbate. The presence of diacetyl in the sample with ascorbate could be ascribed to the stimulatory effect that ascorbate might have had in some species, particularly *Leuconostoc* sp. and *Lactococcus lactis* (Richter *et al.*, 1979).

Table 6. Effects of ascorbate on the metabolite profiles of LG Kefir and MG Kefir. Kefir samples were incubated for 24 h at 22°C (n = 3).

Kefir grains	Method	*pH	*TA (%)	Acetaldehyde	Ethanol	Acetone	Diacetyl	Ethyl acetate	Acetic acid
LG	without ascorbate	4.33	0.76	7.35 ± 1.07	1 491 ± 320.00	–	–	–	348.9 ± 27.80
LG	with ascorbate	4.41	0.77	12.2 ± 5.41	1 926 ± 302.00	3.70 ± 0.27	–	–	324.0 ± 20.40
MG	without ascorbate	4.53	0.74	3.23 ± 0.99	1 070 ± 358.00	1.38 ± 0.30	–	0.385 ± 0.10	444.9 ± 12.10
MG	with ascorbate	4.43	0.74	4.93 ± 1.10	744 ± 162	2.69 ± 0.15	1.05 ± 0.32	0.303 ± 0.07	462.2 ± 61.90

Data represent the mean ± standard deviation

(*) Standard deviations below 10%

(–) not detected

Ethyl acetate

Similarly to results found in study 4, ethyl acetate was only found in MG Kefir (0.38 mg.L^{-1}) and addition of ascorbate did not have a significant effect ($p > 0.05$) on ethyl acetate synthesis (0.30 mg.L^{-1}).

Acetaldehyde and ethanol

Acetaldehyde and ethanol were detected in both Kefirs. For LG Kefir, the results showed that ascorbate had a positive effect on acetaldehyde production in the LG Kefir (Table 6), since the concentration increased from 7.35 mg.L^{-1} to 12.2 mg.L^{-1} . A similar increase ($p > 0.05$) was observed for ethanol (from 1491 to 1926 mg.L^{-1}). However, for MG Kefir, addition of ascorbate led to a decrease in ethanol from 1070 mg.L^{-1} to 744 mg.L^{-1} ; whereas a slight increase in acetaldehyde was observed (from 3.23 to 4.93 mg.L^{-1}). These variations were not significant ($p > 0.05$).

Acetic acid and acetone

The addition of ascorbate did not have a significant effect ($p > 0.05$) on acetic acid formation in both LG and MG Kefir (Table 6).

As for acetone, 3.70 mg.L^{-1} of acetone was found in LG Kefir with ascorbate while none was detected in the sample without ascorbate. In MG Kefir, acetone concentration in the sample with ascorbate was almost double (2.69 mg.L^{-1}) than in the sample without ascorbate (1.38 mg.L^{-1}) ($p < 0.05$). The impact of acetone on Kefir's flavour is negligible, unless concentration rises above 5 mg.L^{-1} (Bodyfelt *et al.*, 1988; Liu *et al.*, 2002; Beshkova *et al.*, 2003).

Discussion for study 5

The metabolic profile of LG Kefir and MG Kefir obtained in study 4 differed from the metabolic profile obtained in this study. However, it must be remembered that with cultured dairy products such as Kefir, the balance between flavour compounds is the factor that determines organoleptic quality.

Similarly to the observation made in study 4, the values obtained for each flavour compound as well as the pH and the TA in the samples with and without ascorbate were also within the range found in the literature; indicating that ascorbate did not have a disruptive effect on bacterial metabolism.

For LG Kefir, conclusions based on diacetyl to acetaldehyde ratio cannot be drawn because diacetyl could not be detected in all samples. Güzel-Seydim *et al.* (2000) also reported that Kefir prepared from Kefir grains did not contain diacetyl. However, results clearly showed that the concentrations of acetaldehyde were higher than those found in study 4, in both samples with and without ascorbate. Such concentrations probably 'compensated' for the lack of diacetyl. Some researchers believe that in yoghurt, diacetyl plays a major role only when acetaldehyde concentrations are low (Güler & Park, 2011). This highlights the fact that in Kefir, even when

diacetyl is absent, an organoleptically acceptable Kefir can still be obtained.

The increase in ethanol observed in the LG Kefir sample with ascorbate contributed to increase the yeasty flavour whereas the increase in acetone had a negligible effect on flavour because concentration was below 5 mg.L⁻¹ (Bodyfelt *et al.*, 1988; Liu *et al.*, 2002; Beshkova *et al.*, 2003).

For MG Kefir, the production of diacetyl in the sample with ascorbate, brought forward the buttery flavour of Kefir whereas ascorbate had no stimulatory or inhibitory effect on ethyl acetate production.

Conclusion for study 5

Ascorbate can be used as an additive to improve diacetyl production in Kefir, with a positive effect on the overall flavour of Kefir, as the ratio of diacetyl to acetaldehyde increase. In addition, even in absence of diacetyl, ascorbate promoted acetaldehyde and ethanol production, which are also important contributors in the flavour of Kefir (Beshkova *et al.*, 2003; Zajšek & Goršek, 2010).

Conclusions

In the eventuality that mass cultured Kefir grains (MG) are distributed or sold as such to South African consumers, addition of the food additives citrate or ascorbate during Kefir production could be recommended depending on the consumers taste preferences. However, providing consumers with microbially stabilised MG that give an acceptable product is certainly a more viable and the cheapest option in the long term. Since MG Kefir lacks the buttery flavour, microbial stabilisation of MG could be achieved with the diacetyl producer *Lc. diacetylactis* 318 or alternatively with *C. kefir* 1283, producer of ethyl acetate.

From an industrial point of view, the improvement of the flavour of Kefir with the addition of fruit flavours (strawberry, raspberry, blackberry and peach) or specific starter culture (yoghurt starter, buttermilk starter) during the fermentation process is currently common practice (Marshall & Cole, 1985; Duitschaever *et al.*, 1991; Muir *et al.*, 1999; Yilmaz *et al.*, 2006; Merrett, 2008) since only 40% of non regular consumers of Kefir give a positive score when tasting unflavoured Kefir for the first time (Duitschaever *et al.*, 1987). For this study, the addition of citrate and ascorbate led to a particularly interesting outcome for the dairy industry, since it showed that these food additives could be used to improve the production of compounds associated with the buttery flavour of Kefir, with no impact on the acidity. To our knowledge, this is the first report to investigate the impact of citrate and ascorbate on the compounds associated with the buttery flavour of Kefir prepared with Kefir grains. However, it must be highlighted that depending on the microbial composition of Kefir grains, results may differ. In addition, further studies need to be done, to evaluate the effects of

citrate and ascorbate on the flavour profile of Kefir prepared with pure cultures instead of Kefir grains.

Finally, although ethyl acetate was detected in MG Kefir for the first time, the fruity flavour that this compound imparts was 'masked', probably because the concentrations of ethyl acetate were too low. Therefore, investigating the conditions under which production of this compound is favoured could be of great value for Kefir's flavour improvement.

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CHAPTER 7

PRODUCTION OF ETHYL ACETATE IN KEFIR AND ITS IMPACT ON THE FLAVOUR OF KEFIR DURING STORAGE

Summary

The odour active ester, ethyl acetate, was identified in Kefir prepared with MG Kefir grains (MG Kefir) at a very low concentration (0.36 mg.L^{-1}). Promoting ethyl acetate production in MG Kefir was achieved by combining the effects of length of incubation (6 to 18 h at 22°C) with higher concentrations of the substrates (ethanol and acetic acid at $0.79\% \text{ m.v}^{-1}$), provided that a strong ester forming microorganism such as *C. kefir* 1283 was present. However, the highest yield ($p < 0.05$) in ethyl acetate concentration was not found in the samples where both substrates were added (6.10 mg.L^{-1}) but rather in the samples containing only added ethanol (9.22 mg.L^{-1}) indicating that ethanol is an important factor in ethyl acetate production by Kefir starter.

The impact of ethyl acetate on the organoleptic quality of Kefir was studied through the monitoring of the metabolic profile of MG Kefir and LG Kefir during 6 days of storage at refrigerated (4°C) and room temperatures (25°C). Under these conditions, both Kefir produced acetaldehyde, ethanol, acetone, ethyl acetate and acetic acid. Samples of Kefir stored at refrigerated temperature (4°C) had a stable metabolic profile and were judged acceptable although containing between 14 and 40 mg.L^{-1} of ethyl acetate throughout the storage period. On the contrary samples of Kefir stored at room temperature (25°C) quickly degraded because over acidification took place within two days.

The results indicate that ethyl acetate at the concentrations found in this study is a positive contributor to Kefir's flavour.

Introduction

Kefir is a self-carbonated and slightly alcoholic fermented milk, which is produced by the addition of Kefir grains to milk. Kefir grains are a microbial consortium mostly composed of lactic acid bacteria (LAB) and yeasts. Moulds and acetic acid bacteria (Pedrozo Miguel *et al.*, 2010) may also be found, although some authors consider them as contaminants (Angulo *et al.*, 1993).

The relationship between yeasts and LAB is symbiotic. Both groups of microorganisms are involved in the biosynthesis of compounds that contribute to the overall flavour of the Kefir beverage. Yeasts, through alcoholic fermentation, produce ethanol and CO_2 , which are considered to give an exotic and yeasty flavour to Kefir (Beshkova *et al.*, 2003; Magalhães *et al.*, 2011a) whereas LAB produce flavour compounds such as aldehydes (acetaldehyde, propionaldehyde), ketones (diacetyl), organic acids (lactic and acetic acids) and esters (ethyl

acetate). The yeasts are also able to produce secondary metabolites such as aldehydes and esters. This latter class of compounds is scarcely mentioned in the literature of Kefir, related to flavour (Beshkova *et al.*, 2003; Magalhães *et al.*, 2011b). Yet, esters are very important flavour compounds because of their two intrinsic attributes. Firstly, their perception threshold is very low (ppb). Secondly, esters contribute to the development of a fruity flavour, which can be considered a positive contributor or even a defect in the overall flavour of different types of fermented products such as cheese (Nogueira *et al.*, 2005; Thierry *et al.*, 2006), beers and wines (Christiani & Monnet, 2001; Verstrepen *et al.*, 2003). In addition, esters can mask the off-flavour caused by excessive concentrations of short chain fatty acids (Abeijón Mukdsi *et al.*, 2009).

It has been previously mentioned that Kefir prepared with mass cultured Kefir grains (MG Kefir) gives a product with an atypical taste, when compared to the taste of Traditional Kefir. In the previous chapter, traces of ethyl acetate were found in MG Kefir and it was considered that the fruity flavour imparted by esters might be an attribute that could be used to improve the unacceptable flavour of MG Kefir, since Traditional Kefir often exhibit a subtle fruity flavour. Thus, this study was undertaken to identify the environmental parameters that promote ethyl acetate production in Kefir. In addition, the impact of ethyl acetate on the organoleptic quality of Kefir at refrigerated and room temperatures were also considered.

Material and methods

Kefir grain activation and preparation

Frozen mass cultured Kefir grains (MG) and laboratory Kefir grains (LG) were obtained from the University of Stellenbosch, Department of Food Science and defrosted at ambient temperature for 24 h. The grains (20 g) were activated for 24 h at 25°C in 300 mL full cream pasteurised milk, then recovered by sieving and added to a new batch of milk. The grains were considered to be active after the fifth batch of milk.

For the preparation of Kefir, 20 g of Kefir grains (LG and MG) were inoculated into 300 mL of pasteurised milk and incubated at 22°C for 24 h. After the incubation period, the grains were removed by sieving and the Kefir beverage obtained was used in the different studies.

Metabolic profiles

The metabolic profiles were done using headspace gas chromatography (HSGC). A sample (9.75 mL) of full cream milk fermented with a pure culture or a sample of Kefir was placed in a 20 mL glass vial containing 2.5 g of NaCl and 0.25 mL of tetrahydrofuran. The vial was crimp-sealed with a silicone-PTFE seal and aluminium cap and incubated in a waterbath for 50 min at 95°C (Dr. Sigge, G.O, Lecturer, Food Science Department, Stellenbosch, South Africa, Personal

Communication, 2004). This temperature was chosen to optimise the volatilisation of the VOC in the headspace. The vial's content was mixed several times while in the waterbath. A 1.5 mL aliquot of the headspace gas was withdrawn using a warmed (70°C) Hamilton gas-tight syringe and split-injected into the gas chromatograph at a split ratio of 1:100 (Human, 1998). The metabolic profiles were identified by comparing the retention time of the unknown compounds to those of the analytical grade standards.

Headspace gas chromatography operating conditions

Separation and identification of volatile organic compounds (VOCs = acetaldehyde, ethanol, acetone, diacetyl, 2-butanone and ethyl acetate) were determined using a Fisons 8000 Series gas chromatograph (Fisons Instruments S.p.A., Milan, Italy) equipped with a flame ionisation detector (FID) and a 60 m DB5 capillary column bonded with a methyl-5% phenyl silicone layer as stationary phase (film thickness 0.25 µm; Quadrex Corporation, Newhaven). Operating parameters were: injector and detector temperatures were set at 150°C and 200°C, respectively; helium was used as carrier gas at a flow rate of 1.1 mL.min⁻¹. The oven heating cycle was programmed at 30°C for 2 min followed by an increase of 5°C per min to 220°C for 10 min.

Quantitative determination of the metabolites compounds was done by integration of the peak areas using an external standard calibration and Borwin Version 1.2 integration software (JMBS Developpements, Le Fontanil, France).

Volatile fatty acids

A standard solution of short chain volatile fatty acids (VFAs = acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids) was prepared by mixing 1 mL of each fatty acid and 0.5 mL of n-hexanol in a 1 000 mL volumetric flask with 250 mL of formic acid and 750 mL of distilled water.

Samples (milk fermented with a pure culture or Kefir grains) were prepared as follows: 10 mL of sample was centrifuged (10 min at 10 000 g) and the supernatant filtered through Whatman No 1 filter to remove solid particles and obtain a clear supernatant. One millilitre of formic acid (35% v.v⁻¹) and 2 µL of n-hexanol (as internal standard) were added to 3 mL of the filtered supernatant. The GC injection volume was 1 µL and the run time 20 min.

The VFAs were determined using a Varian 3700 GC equipped with a flame ionisation detector and a 30 m bonded phase Nukol (Supelco, Inc., Belafonte, PA) fused silica capillary column (0.53 mm diameter and 0.5 µm film thickness). The oven heating cycle program was held at 105°C for 2 min followed by an increase of 10°C per min to 190°C for 10 min. Injector and detector temperatures were 150 and 300°C, respectively. The flow rate of nitrogen, the carrier gas, was 6.1 mL.min⁻¹. The VFAs were quantified using the Borwin Version 1.2 integration software (JMBS Developpements, Le Fontanil France) using the internal standard method (Sigge *et al.*, 2005).

pH and Titratable acidity (TA)

The pH of the Kefir was measured with an Orion pH meter and a glass electrode (Hanna Instruments). The TA was measured in triplicate by the titration of 10 mL sample with 0.11 N sodium hydroxide (NaOH) until the pink phenolphthalein end-point (James, 1999).

In Kefir, the major metabolite is lactic acid but acetic acid, which is also present, might influence the TA. Thus, TA will be expressed as % total acids instead of % lactic acid, using the following formula: TA (as % total acids) = (X mL 0.11 N NaOH used) / 10

Strains and growth conditions

Freeze-dried cultures of *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* 318 and *Candida kefir* 1283 were obtained from the Department of Food Science, Stellenbosch University. Stock cultures were maintained in MRS (Merck) and in yeast extract peptone dextrose broths (YPD) at 4°C, for *Lc. diacetylactis* 318 and *C. kefir* 1283, respectively. Strain purity was regularly checked by microscopy and Gram staining (Harrigan & McCance, 1998).

To construct growth curves, the bacterial and yeast counts were determined by dilution in MRS and YPD broths; and plating in MRS-Agar and YPD-Agar. Growth profiles of colony forming units (cfu.mL⁻¹) against absorbance at 500 nm (Spectronic 20 Genesys, Spectronic Instruments, Cape Town), were constructed. These profiles were used to standardise the inoculum size at 10⁶ cfu.mL⁻¹ and at 10⁵ cfu.mL⁻¹, for the *Lc. diacetylactis* 318 and *C. kefir* 1283 strains, respectively.

Preparation of substrates

The reactions contributing to the production of esters are esterification, alcoholysis, acidolysis or transesterification. These reactions are catalysed by esterase, lipase, alcohol acyl transferase or alcohol acetyl transferase (Liu *et al.*, 2004; Thierry *et al.*, 2006).

In the case of this study, the ester of interest was ethyl acetate, which production requires the presence of an alcohol (ethanol) and an acid (acetic acid) (Abeijón Mukdsi *et al.*, 2009). Thus for the production of ethyl acetate in Kefir, these chemicals were added to the different aliquots of Kefir as to reach a concentration of 0.79% m.v⁻¹ for ethanol and for acetic acid. It was assumed that enzymes involved in ethyl acetate biosynthesis were present in Kefir beverage. Both ethanol and acetic acid were of analytical reagent grade (Merck).

Study 1: Factors promoting ethyl acetate synthesis in Kefir

The aim of this study was to determine the environmental parameters that promote the production of ethyl acetate in Kefir prepared with MG Kefir grains (MG Kefir). For that purpose, MG Kefir was enriched with either *Candida kefir* 1283 or *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* 318, and/or substrates (acetic acid and/or ethanol). Figure 1 shows the diagram of the experimental design of Study 1. To facilitate the discussion, the following abbreviations were used: K for MG

Kefir, E for ethanol, A for acetic acid, AE for acetic acid plus ethanol, D for *Lc diacetylactis* 318 and C for *C. kefir* 1283.

Preparation of MG Kefir

MG Kefir was prepared as described and then divided into four batches namely Control (K24h), MG Kefir 1, MG Kefir 2 and MG Kefir 3 (Fig. 1).

Control

The control K24h (MG Kefir incubated for 24 h at 22°C) was used to determine the content in VOCs, VFAs, pH and TA. This sample was further incubated for 6 h at 22°C (K6h), after which, the determination of pH, TA, VOCs and VFAs was done.

MG Kefir 1

MG Kefir 1 was subdivided in three aliquots. These aliquots were supplemented with ethanol (sample KE), acetic acid (sample KA) and ethanol plus acetic acid (sample KAE), respectively. Then, these aliquots were further incubated 6 h at 22°C, after which VOCs, VFAs, pH and TA were determined.

MG Kefir 2

MG Kefir 2 was subdivided in four aliquots, which were further incubated 6 h at 22°C, after addition of (Fig. 1):

- *Lc. diacetylactis* 318 in the first aliquot (sample KD),
- *Lc. diacetylactis* 318 and ethanol in the second aliquot (sample KDE),
- *Lc. diacetylactis* 318 and acetic acid in the third aliquot (sample KDA) and
- *Lc. diacetylactis* 318, ethanol and acetic acid in the fourth aliquot (sample KDAE).

At the end of the incubation period, VOCs, VFAs, pH and TA were determined.

MG Kefir 3

MG Kefir 3 was divided in four aliquots. The four aliquots were matured for 6 h at 22°C, after addition of (Fig. 1):

- *C. kefir* 1283 in the first aliquot (sample KC),
- *C. kefir* 1283 and ethanol in the second aliquot (sample KCE) ,
- *C. kefir* 1283 and acetic acid in the third aliquot (sample KCA) and;
- *C. kefir* 1283, ethanol and acetic acid in the fourth aliquot (sample KCAE).

At the end of the incubation period, VOCs, VFAs, pH and TA were determined.

These samples along with K6h were again incubated for 18 h at 22°C after which pH, TA,

VOCs and VFAs were measured.

Statistical analyses

Statistica 9.0 was used for the statistical evaluation of the results and a multifactorial variance analysis (ANOVA) was applied to determine the differences between each treatment for the variations in pH, TA, VOCs and VFAs. Differences were compared at 5% level of significance using the LSD test.

King Test

The King test was performed to qualitatively detect the presence of diacetyl plus acetoin in samples. Two solutions A (potassium hydroxide 30% m.v⁻¹) and B (0.1 g dicyandiamide, 4 g α -naphthol, 10 mL amyl alcohol and 40 mL ethyl alcohol) were prepared. Solutions A (1 mL) and B (1 mL) were added to 2 mL of the sample to be tested and the test tube was incubated in a water bath at 30°C for 30 min. A result is positive when a pink to red colouration develops whereas a result is considered negative when the medium remain yellowish (King, 1948).

Study 2: Stability of Kefir during storage

The impact of ethyl acetate on the organoleptic quality of Kefir was studied through the monitoring of the metabolic profile of MG Kefir and LG Kefir during 6 days of storage at refrigerated (4°C) and room temperatures (25°C).

Metabolic profiles during storage

Kefir was prepared as previously described. The Kefir beverage obtained was divided into 3 aliquots. The first aliquot was used to measure the pH, TA, VOCs and VFAs and the other two aliquots were stored at 4° and 25°C, respectively. Every second day during a 6 day period, 50 mL of each aliquot was withdrawn for analysis of the VOCs, VFAs, pH and TA.

Statistical analyses

Statistica 9.0 was used for the statistical study and a multifactorial variance analysis (ANOVA) was applied to determine the differences between types of Kefir grains and the storage temperatures regarding the variations in pH, TA, VOCs and VFAs. Differences were compared at 5% level of significance using LSD test.

Sensory evaluation

The sensory evaluation of Kefir beverages produced was done by five panellists familiar with Kefir and similar fermented dairy products.

Results and discussion

Study 1: Environmental parameters promoting ethyl acetate in Kefir

The aim was to identify the environmental parameters that promote ethyl acetate synthesis in Kefir prepared with mass cultured Kefir grains (MG Kefir) by supplementing it with microorganisms (*Lc. diacetylactis* 318 or *C. kefir* 1283) and/or substrates (ethanol and/or acetic acid).

The plot in Fig. 2 represents the concentrations of ethyl acetate found in the Control K24h, K6h and the different aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3.

Ethyl acetate concentration in Control (K24h)

The control K24h contained 0.36 mg.L^{-1} of ethyl acetate. After further incubation, the ethyl acetate concentration increased to 0.61 mg.L^{-1} (sample K6h). The difference in the concentrations of ethyl acetate of Control K24h (0.36 mg.L^{-1}) and sample K6h (0.61 mg.L^{-1}) was found to be significant ($p < 0.05$). This indicates that the MG Kefir grains contained microorganisms that have the enzymatic capacity to produce ethyl acetate (esterases and /or acyl alcohol transferases). In addition, this result suggests that a longer incubation period is necessary to achieve higher concentrations of ethyl acetate.

Ethyl acetate concentrations in MG Kefir 1

The concentration of ethyl acetate found in the Control K24h differed significantly ($p < 0.05$) from that found in samples K6h (0.61 mg.L^{-1}), KA (0.61 mg.L^{-1}), KE (0.67 mg.L^{-1}) and KAE (0.75 mg.L^{-1}) (Fig. 2). Even though the concentrations of ethyl acetate found in these samples did not differ significantly ($p > 0.05$) from one another, an increase in the concentration of ethyl acetate was observed in samples with added ethanol; the highest concentration being found in the sample containing both substrates (KAE = 0.75 mg.L^{-1}) (Fig. 2). These results suggest that the presence of both substrates play a role in the production of ethyl acetate.

Ethyl acetate concentrations in MG Kefir 2

The concentrations of ethyl acetate found in Control K24h (0.36 mg.L^{-1}) significantly differed ($p < 0.05$) from samples K6h (0.61 mg.L^{-1}), KD (0.70 mg.L^{-1}), KDA (0.97 mg.L^{-1}), KDE (0.87 mg.L^{-1}) and KDAE (1.04 mg.L^{-1}) (Fig. 2). As previously mentioned for MG Kefir 1, the addition of the substrates contributed to more ethyl acetate being produced, with the highest concentration found in sample containing both substrates (KDAE = 1.04 mg.L^{-1}). In addition, no significant difference ($p > 0.05$) in the concentration of ethyl acetate was found between sample K6h (0.61 mg.L^{-1}) and sample KD (0.70 mg.L^{-1}), suggesting that the addition of *Lc. diacetylactis* 318 did not have an effect (stimulate or inhibit)

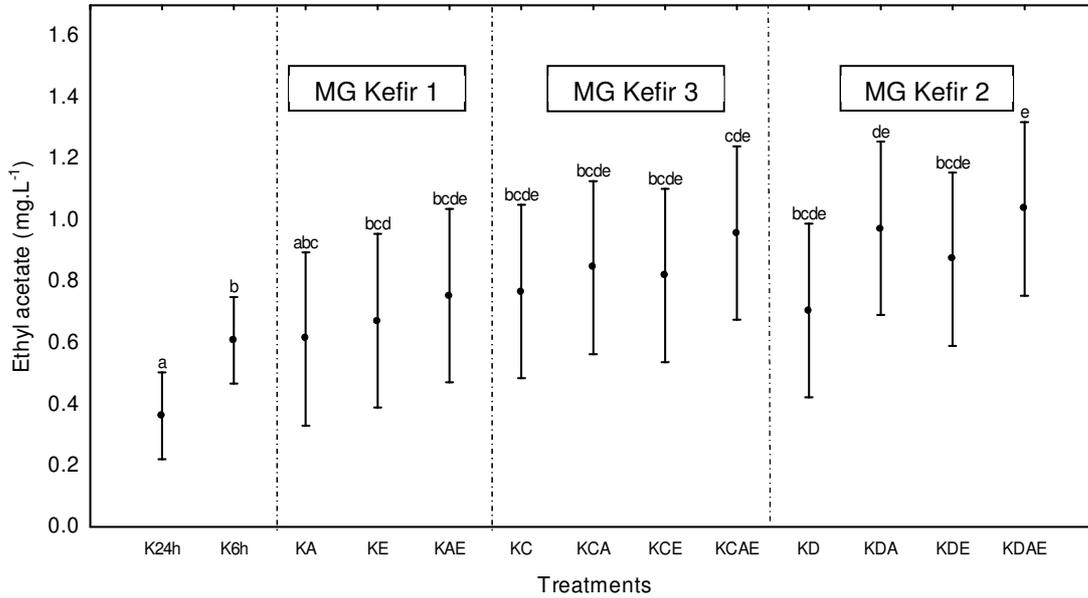


Figure 2. Concentrations of ethyl acetate in the Control incubated for 24 h at 22°C (K24h) and in sample K6h, aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3, further incubated for 6 h at 22°C. ^{a, b, c} Means with different superscripts differ ($p < 0.05$).

on ethyl acetate production under the environmental conditions of this study.

Ethyl acetate concentrations in MG Kefir 3

Ethyl acetate content of Control K24h (0.36 mg.L⁻¹) significantly differed ($p < 0.05$) from samples K6h (0.61 mg.L⁻¹), KC (0.77 mg.L⁻¹), KCA (0.84 mg.L⁻¹), KCE (0.82 mg.L⁻¹) and KCAE (0.96 mg.L⁻¹). Similarly to MG Kefir 2, more ethyl acetate was produced in samples with added substrates, particularly, in sample containing both substrates (KCAE = 0.96 mg.L⁻¹). In addition, the concentration in ethyl acetate found in sample K6h (0.61 mg.L⁻¹) did not differ significantly ($p > 0.05$) from sample KC (0.77 mg.L⁻¹) suggesting that the addition of *C. kefyri* 1283 did not have a significant stimulating effect under the conditions of this study. However, it must be highlighted that even though statistically no significant difference were observed between sample K6h (0.61 mg.L⁻¹) and samples containing microorganisms (KD = 1.04 mg.L⁻¹; KC = 0.96 mg.L⁻¹), it does not imply that the difference would not be picked up if the products had to be tasted.

The data in Fig. 2 shows that the highest concentrations of ethyl acetate were found in samples KDE (0.87 mg.L⁻¹), KCAE (0.96 mg.L⁻¹), KDA (0.97 mg.L⁻¹) and KDAE (1.04 mg.L⁻¹). However, the concentrations of ethyl acetate found in these samples did not differ significantly from one another ($p > 0.05$) but did differ significantly ($p < 0.05$) from the concentrations in ethyl acetate found in Control K24h (0.36 mg.L⁻¹) and sample K6h (0.61 mg.L⁻¹). Thus identifying which parameter(s) between 'substrates' and 'length of incubation' are mostly significant in promoting ethyl acetate synthesis in MG Kefir, it was decided to further incubate K6h, KC, KCA, KCE and KCAE for 18 h at 22°C. The reason why samples with added *C. kefyri* 1283 were used was because it was found in Chapter 6 that *C. kefyri* 1283 produced ethyl acetate at all incubation temperatures whereas *Lc. diacetylactis* 318 did not. In addition, Plata *et al.* (2003) reported that *Candida* yielded up to 100 times more ethyl acetate in the presence of ethanol.

Ethyl acetate in MG Kefir 3 after 18 h at 22°C

The aliquots of MG Kefir 3, incubated for 30 h at 22°C (24 h + 6 h) were further incubated for 18 h at 22°C. The results obtained after this additional incubation (Fig. 3) show that ethanol had the most significant effect on ethyl acetate synthesis when compared to acetic acid. Indeed, the concentration of ethyl acetate found in sample KCE18 (9.22 mg.L⁻¹) was the highest and differed significantly from samples KCA18 (1.46 mg.L⁻¹) and KCAE18 (6.10 mg.L⁻¹). In addition, sample KCE contained nine times more ethyl acetate than the sample without added ethanol (KC18 = 0.98 mg.L⁻¹), which suggests that the concentration of ethanol is the determining parameter in producing substantial amount of ethyl acetate. Similar findings were reported by Thierry *et al.* (2006). These authors

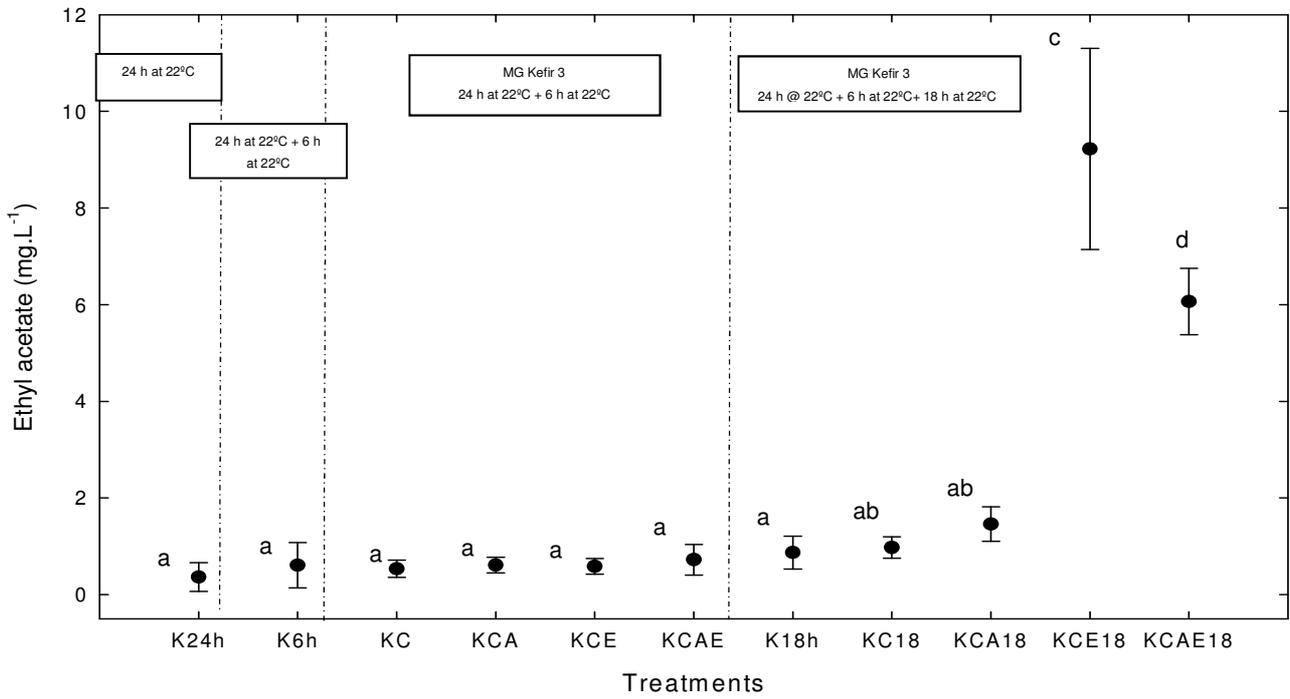


Figure 3. Concentrations of ethyl acetate in the Control incubated for 24 h at 22°C (K24h), K6h, aliquots of MG Kefir 3 further incubated for 6 h at 22°C and 18 h at 22°C. ^{a, b, c} Means with different superscripts differ ($p < 0.05$).

reported that 'pilot cheeses' contained five times less ethyl acetate than test cheeses prepared with added ethanol or than cheeses where in situ production of ethanol occurred. However, a closer look at Fig. 3 show that the length of incubation is also a determining factor since sample KCE (0.82 mg.L⁻¹) and KCAE (0.96 mg.L⁻¹) contained ten to fifteen times less ethyl acetate than the counterparts KCE18 (9.22 mg.L⁻¹) and KCAE18 (6.10 mg.L⁻¹). The possible explanation to why the length of incubation is also determinant for ethyl acetate synthesis in Kefir could be linked to the pH, which was more acidic after 18 h (3.71 – 3.81) than after 6 h (3.94 – 4.05) (Table 1). Indeed, in such acidic environment, lactic acid bacteria cease to grow, start decline whereas yeasts activity can still proceed with the synthesis of secondary metabolites such as ethyl acetate.

Furthermore, the presence of *C. kefir* 1283 alone was not effective in producing a higher ethyl acetate concentration (KC = 0.87 mg.L⁻¹; KC18 = 0.98 mg.L⁻¹) (Fig. 3). This suggests that the presence of a strong ethyl acetate producing strain is not the sole requirement for production of ethyl acetate. The presence of substantial amount of substrates, particularly ethanol is important for a good yield of ethyl acetate. It can be hypothesised that enzymes involved in ethyl acetate production are activated when ethanol reached a specific concentration. Below that concentration, the activity of those enzymes is minimal.

In this study, production of ethyl acetate was not strongly influenced by the concentration of acetic acid in the medium, which contradicts the report of Antonelli *et al.* (1999). However, it must be pointed out that the mechanisms of ethyl acetate and esters synthesis in general are not clearly understood or even predictable, since other parameters (enzyme activities, strain, water activity) can also affect the formation (Liu *et al.*, 2004; Oliszewski *et al.*, 2007).

The samples KC, KCA, KCE, KCAE, KC18, KCA18, KCE18, and KCAE18 were tasted by five trained panellists and the most preferred samples were samples KCA, KCAE, KCE18 and KCAE18, which contains 0.84 mg.L⁻¹, 0.96 mg.L⁻¹, 6.10 mg.L⁻¹ and 9.22 mg.L⁻¹ of ethyl acetate, respectively. It is interesting to note that the samples containing 6.10 mg.L⁻¹ and 9.22 mg.L⁻¹ were found acceptable. This indicates that the fruity flavour imparted by ethyl acetate is positively influencing the flavour of Kefir. Consequently, samples of Kefir containing only ethyl acetate (along with acetaldehyde, ethanol and acetic acid) but no diacetyl are not to be seen as defects. This finding was recently confirmed by Magalhães *et al.* (2011c) who reported that the sample of Kefir containing 8.18 mg.L⁻¹ ethyl acetate was found acceptable by consumers (N = 25).

Acetaldehyde and ethanol concentrations in various treatments of MG Kefir

The profiles of acetaldehyde and ethanol concentrations found in the Control K24h, K6h and the aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3 are illustrated in Fig. 4 and Fig. 5, respectively.

The results in Fig. 4 showed that all samples containing added ethanol had concentrations in

Table 1. pH and TA found in the aliquots of MG Kefir 3 incubated for a further 6 h and 18 h at 22°C.

Incubation	Samples	pH	TA (% total acids)
24 h at 22°C	K24h (Control)	4.12	0.87
+ 6 h at 22°C	K6h	3.95	1.03
	KC	4.01	1.01
	KCA	3.97	1.10
	KCE	4.05	0.93
	KCAE	4.04	0.96
+ 18 h at 22°C	K18h	3.75	1.21
	KC18	3.76	1.08
	KCA18	3.71	1.23
	KCE18	3.81	1.08
	KCAE18	3.77	1.15

(*) The data represent the mean

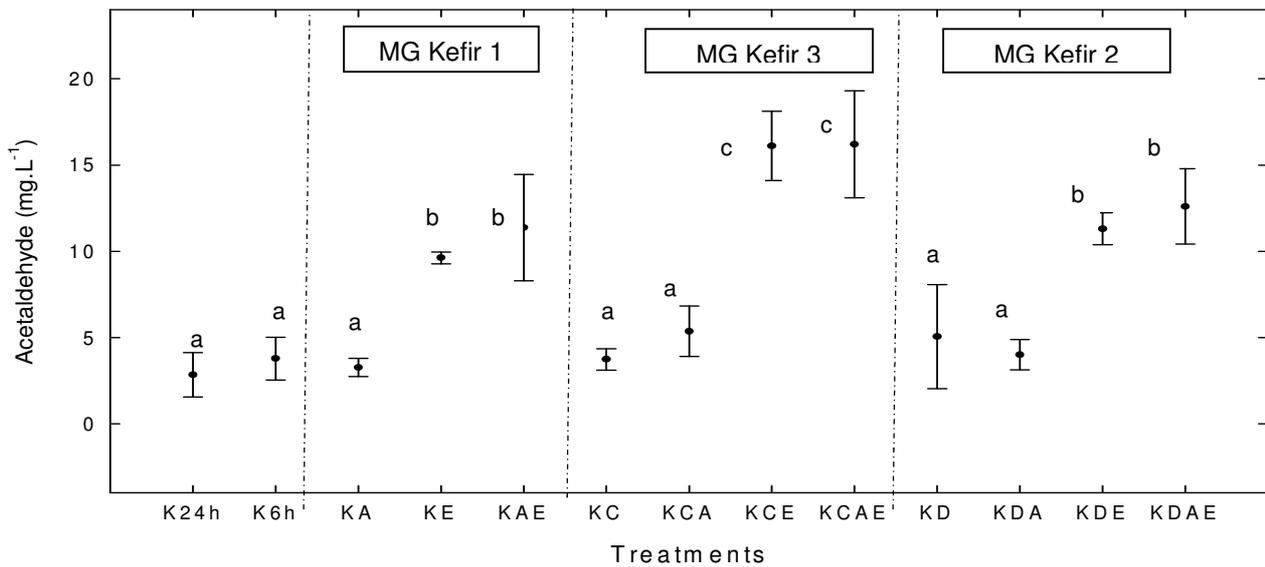


Figure 4. Concentrations of acetaldehyde in the Control incubated for 24 h at 22°C (K24h), K6h and aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3, further incubated for 6 h at 22°C. ^{a, b, c} Means with different superscripts differ ($p < 0.05$).

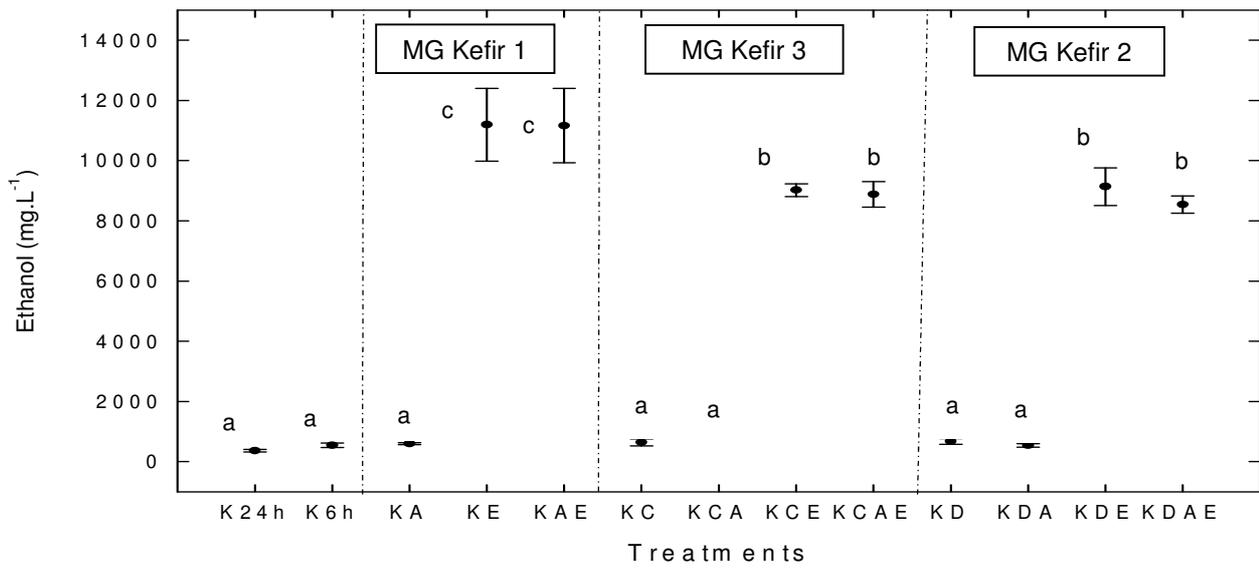


Figure 5. Concentrations of ethanol in the Control incubated for 24 h at 22°C (K24h), K6h, aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3, further incubated for 6 h at 22°C. ^{a, b, c} Means with different superscripts differ ($p < 0.05$).

acetaldehyde significantly higher ($p < 0.05$) (KE = 9.62 mg.L⁻¹; KAE = 11.4 mg.L⁻¹; KCE = 16.1 mg.L⁻¹; KCAE = 16.2 mg.L⁻¹; KDE = 11.3 mg.L⁻¹; KDAE = 12.6 mg.L⁻¹) than the samples without (K24h = 2.84 mg.L⁻¹, K6h = 3.78 mg.L⁻¹, KC = 3.73 mg.L⁻¹, KA = 3.26 mg.L⁻¹; KCA = 5.36 mg.L⁻¹; KD = 5.05 mg.L⁻¹ and KDA = 4.00 mg.L⁻¹). These results suggested that the presence of a higher ethanol concentration must have turned the activity of alcohol dehydrogenase (ADH) towards the synthesis of acetaldehyde.

The concentrations of ethanol in samples without added ethanol (Fig. 5), varied between 363 and 738 mg.L⁻¹. The data showed that the addition of microorganism and/or acetic acid did not impact ethanol production (K6h = 541 mg.L⁻¹, KA = 598 mg.L⁻¹, KC = 639 mg.L⁻¹, KCA = 738 mg.L⁻¹, KD = 669 mg.L⁻¹ and KDA = 537 mg.L⁻¹). The higher concentrations of ethanol in samples with added ethanol (KE = 11 195 mg.L⁻¹, KAE = 11 164 mg.L⁻¹, KCE = 9 021 mg.L⁻¹, KCAE = 8 881 mg.L⁻¹, KDE = 9 139 mg.L⁻¹ and KDAE = 8 541 mg.L⁻¹) may originate from the added ethanol (7 900 mg.L⁻¹) as well as the formation or conversion of volatile compounds to other compounds by microbial enzymes such as alcohol dehydrogenase (Ott *et al.*, 2000; Grønnevik *et al.*, 2011).

Acetic acid concentrations in various treatments of MG Kefir

The concentrations of acetic acid found in the Control K24h, K6h and the aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3 are illustrated in Fig. 6. No particular pattern was discernable. The concentrations in acetic acid varied between 437 mg.L⁻¹ and 769.5 mg.L⁻¹. It was also found that in general, the length of incubation did not affect acetic acid formation. The added acetic acid was not recovered probably because it has been converted into other compounds by microbial enzymes and/or because of losses due to volatilisation (Collins & Bruhn, 1970; Pronck *et al.*, 1996).

pH in various treatments of MG Kefir

The pH of the Control K24h, K6h and the aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3 are illustrated in Fig. 7. The pH of Control K24h was 4.12 after incubation at 22°C for 24 h and differed significantly ($p < 0.05$) from the pH of other samples, except for samples KCE (pH = 4.05) and KCAE (pH = 4.04).

The results clearly show that the additional incubation of 6 h at 22°C contributed to lower the pH due to the microbial activity of Kefir microbial population. It can be suggested that the population responsible for the decrease in pH is the lactobacilli, whose growth is favoured at low pH and since it is generally admitted that in the microbial consortium of Kefir, lactococci decrease the pH during the first hours of the fermentation (Farnworth, 2005).

The pH of the samples with added *C. kefir* 1283 did not differ from the pH of sample K6h (pH = 3.95) ($p > 0.05$). In addition, samples with added *C. kefir* 1238 had a slightly higher pH compared to the remaining samples (except for K24h). This could be explained by the fact that *C. kefir* has the

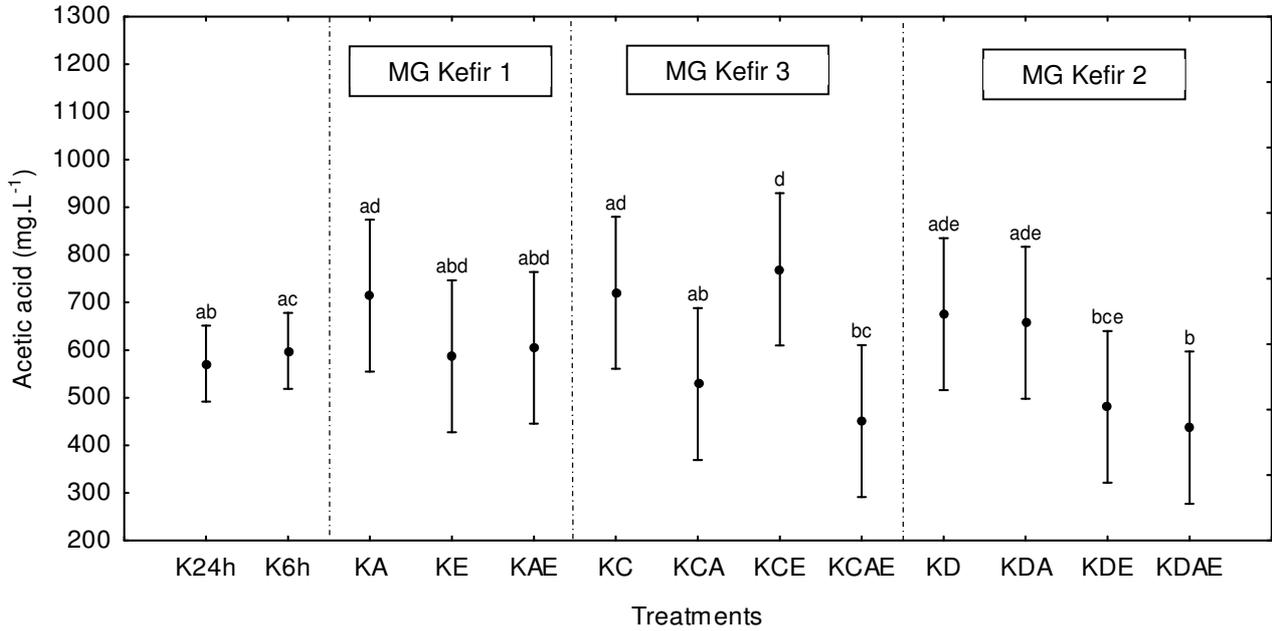


Figure 6. Concentrations of acetic acid in the Control incubated for 24 h at 22°C (K24h), K6h, aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3, further incubated for 6 h at 22°C. ^{a, b, c} Means with different superscripts differ ($p < 0.05$).

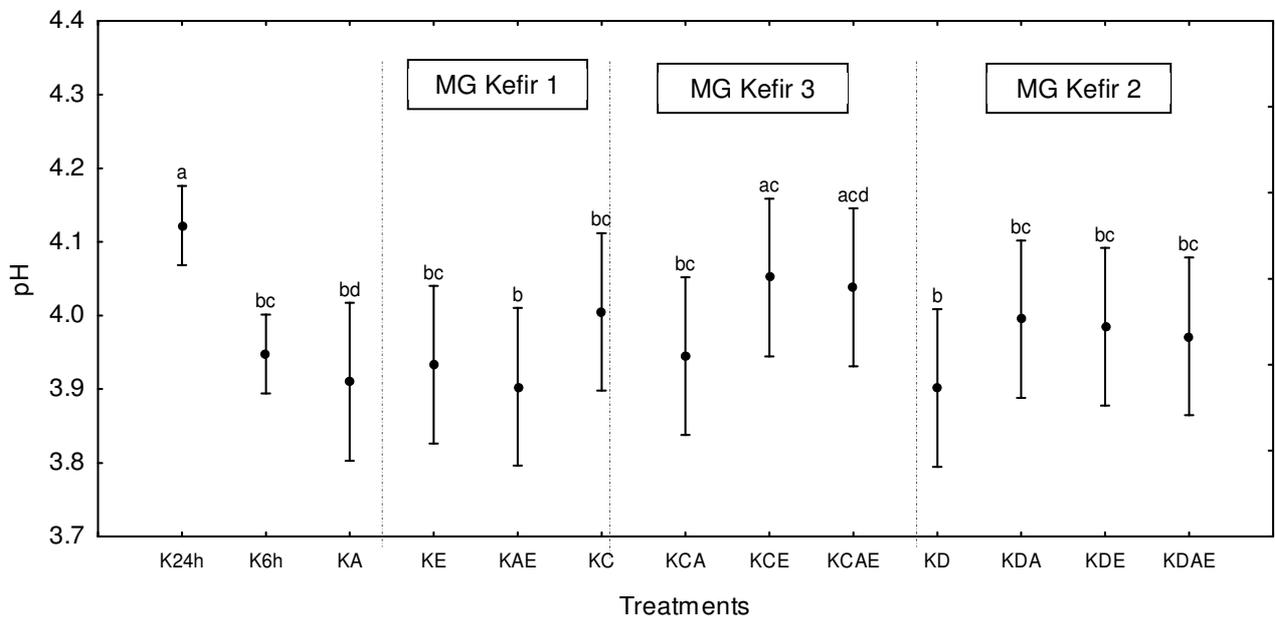


Figure 7. pH in the Control incubated for 24 h at 22°C (K24h), K6h, aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3 further incubated for 6 h at 22°C. ^{a, b, c} Means with different superscripts differ ($p < 0.05$).

capacity to metabolise lactic acid. This ability was used by Leclercq-Perlat *et al.* (2004) to deacidify a medium (water homogenised cheese, sterilised to kill lactic acid bacteria) from pH 4.8 to 5.8 after incubation at 25°C.

Similarly to the samples with added *C. kefir* 1283, the pH of the samples with added *Lc. diacetylactis* 318 (KD = 3.90, KDA = 3.99, KDE = 3.98 and KDAE = 3.97) did not differ ($p > 0.05$) from sample K6h (pH = 3.95). It must be mentioned that *Lc. diacetylactis* 318 was added in Kefir which already had a low pH (3.94). Usually, lactococcus species do not grow in acidic pH (Koroleva, 1988); this could be the reason why the sample containing added of *Lc. diacetylactis* 318 did not lead to a further decrease of pH.

Titrateable acidity (TA) variations in various treatments of MG Kefir

The variations in TA of Control K24h, K6h, MG Kefir 1, MG Kefir 2 and MG Kefir 3 are illustrated in Fig. 8. After 24 h incubation at 22°C, the TA of the Control K24h was 0.88%. The additional incubation period of 6 h at 22°C significantly contributed ($p < 0.05$) to increasing the TA to 1.05% in sample K6h. The TA of this sample did not differ ($p > 0.05$) from the other samples (KA = 1.07%, KE = 1.06%, KAE = 1.04%, KC = 1.01%, KCA = 1.10%, KCE = 0.93%, KCAE = 0.96%, KD = 1.01%, KDA = 0.99%, KDE = 0.97% and KDAE = 0.98%).

It must be mentioned that except for sample KCA, the TA of samples enriched with *C. kefir* 1283 were generally lower than the TA of the other samples (Fig. 8). This is probably due to the ability of *C. kefir* to deacidify the medium by assimilating lactic acid (Leclercq-Perlat *et al.*, 2004).

The presence of *Lc. diacetylactis* 318 did not lead to an increase in acidity level probably because the acidic environment (pH = 3.95) was not favourable for growth although some other metabolic activities such as citrate metabolism can proceed (Cogan, 1975; Levata-Jovanovic & Sandine, 1996).

Diacetyl variations in various treatments of MG Kefir

Diacetyl was detected at concentrations varying between 0.5 and 2.2 mg.L⁻¹ but could not be detected in some samples, probably because diacetyl had been converted to acetoin (not measured in this study). However, to confirm that diacetyl and/or acetoin were present in the samples enriched with *C. kefir* 1283 and *Lc. diacetylactis* 318, the King test was done and a pink colouration, characterising the presence of diacetyl and acetoin was observed (Photos 1a & 1b).

Conclusion for study 1

Under the conditions of this study, it was shown that up to 9.22 mg.L⁻¹ of ethyl acetate could be produced in MG Kefir by lengthening the usual incubation period (24 h at 22°C) by 6 to 18 h at 22°C,

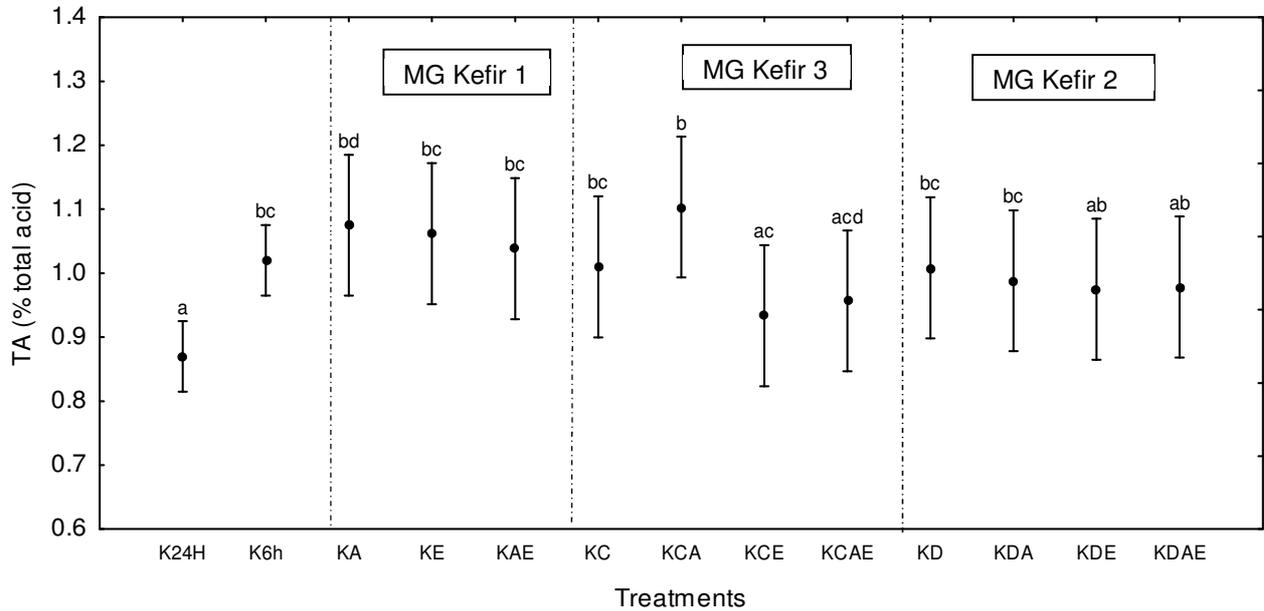


Figure 8. Titrateable acidity (TA) in the Control incubated for 24 h at 22°C (K24h), K6h, aliquots of MG Kefir 1, MG Kefir 2 and MG Kefir 3, further incubated for 6 h at 22°C. ^{a, b, c} Means with different superscripts differ ($p < 0.05$).

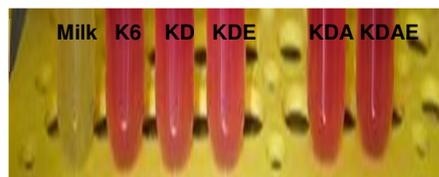


Photo 1a. Qualitative determination of diacetyl plus acetoin (King test) in milk, K6h and aliquots of MG Kefir 2.

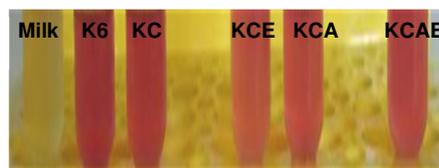


Photo 1b. Qualitative determination of diacetyl plus acetoin (King test) in milk, K6h and aliquots of MG Kefir 3.

addition of a strong ethyl acetate producer (*C. kefir* 1283) as well as addition of substrates (ethanol and acetic acid at 0.79% m.v⁻¹).

The samples containing no added *C. kefir* 1283 did not lead to a significant increase in ethyl acetate production, suggesting that the lack or absence of ethyl acetate observed in Kefir is an indication that Kefir grains, the starter culture, do not contain a yeast strain capable of producing substantial amounts of ethyl acetate. In addition, yeasts are often mentioned as being responsible for the yeasty flavour of Kefir, through the production of ethanol and carbon dioxide. However, we can see that yeasts may play a critical role by imparting a fruity flavour to Kefir through synthesis of ethyl acetate.

Kefir may often exhibit a subtle fruity flavour, which may go unnoticed depending on the perceived acidity (Wszolek *et al*, 2001). But more generally, would not be perceptible because ethyl acetate is often not present or present in traces in Kefir (Beshkova *et al*, 2003). However, when present in substantial amount as found in this study and by Magalhães *et al*. (2011c), the level at which ethyl acetate would cause an off-flavour to Kefir, under storage conditions, is still unknown. This was thus investigated.

Study 2: Metabolite profiles of Kefir during storage

The aim of this section was to study the impact of ethyl acetate on the organoleptic quality of Kefir by monitoring the metabolic profile of MG Kefir and LG Kefir during 6 days of storage at refrigerated (4°C) and room temperatures (25°C).

MG Kefir and LG Kefir were prepared by adding 20 g of mass cultured Kefir grains and 20 g of laboratory Kefir grains, respectively, into 300 mL of milk.

Metabolite profiles of LG and MG Kefir on day 0

Kefir was prepared using LG Kefir grains and MG Kefir grains; and samples were incubated 30 h at 22°C to favourise ethyl acetate production. Under these conditions, it was found that MG Kefir and LG Kefir contained 20.1 and 14.8 mg.L⁻¹ ethyl acetate, respectively. Other compounds usually found in Kefir, were also detected in MG Kefir and LG Kefir, respectively, namely: acetaldehyde (6.60 and 21.8 mg.L⁻¹), ethanol (3 368 and 3 785 mg.L⁻¹), acetone (0.20 and 2.00 mg.L⁻¹), diacetyl (0.40 and 0.85 mg.L⁻¹) and acetic acid (524 and 569 mg.L⁻¹). The TA content was found to be 1.05 and 1.12% and the pH 3.91 and 4.07 for the MG Kefir and LG Kefir, respectively. In addition, it was observed that the initial concentrations, pH and TA (data obtained after 24 h at 22°C on day 0) found in the MG Kefir and LG Kefir did not significantly differ ($p > 0.05$), suggesting that both types of Kefir grains had similar microbial populations.

Metabolic profiles of LG and MG Kefir during storage

Ethyl acetate

The concentrations of ethyl acetate in LG and MG Kefir stored at 4°C and 25°C are illustrated in Fig. 9. At refrigerated temperature (4°C), the concentrations of ethyl acetate did not significantly vary ($p > 0.05$) from day 0 (20.1 mg.L⁻¹ and 14.8 mg.L⁻¹) to day 4 (23.9 mg.L⁻¹ and 22.2 mg.L⁻¹), respectively for the MG and LG Kefir. However, ethyl acetate concentrations significantly ($p < 0.05$) increased from day 4 to day 6, reaching 37 mg.L⁻¹ and 39.5 mg.L⁻¹, respectively for the MG and LG Kefir.

At room temperature (25°C), the concentrations of ethyl acetate increased sharply from day 0 to day 6, in both MG and LG Kefir. The final concentrations of ethyl acetate (199.5 and 194 mg.L⁻¹) on day 6 were 10 and 43 times higher than on day 0 (20.1 mg.L⁻¹ and 14.8 mg.L⁻¹), respectively for MG and LG Kefir. This sharp increase observed during storage at room temperature (25°C) may have originated from a multiplication of the yeast population compared to other groups of microorganisms or from spoilage by acetic acid bacteria (Magalhães *et al.*, 2011c). In addition, these results suggest that enzymes involved in ethyl acetate formation were more active at 25°C than at 4°C. This result is in accordance with Daudt & Ough (1973) who reported that volatiles esters synthesis increased over a temperature range of 15°C to 28°C, with 21°C being optimum for ethyl acetate production.

Acetaldehyde

During refrigerated storage (4°C), acetaldehyde concentrations were generally stable in both Kefirs (Fig. 10), with no significant differences ($p > 0.05$) observed. The concentrations varied between 6.6 mg.L⁻¹ and 16.7 mg.L⁻¹ for MG Kefir and between 18.8 mg.L⁻¹ and 21.8 mg.L⁻¹ for LG Kefir. These results are similar to those reported by Ertekin & Güzel-Seydim (2010) who found that the acetaldehyde concentration in Kefir remained stable over 7 days period of storage at 4°C.

At room temperature (25°C), the profiles of acetaldehyde were similar for both Kefirs. A sharp increase occurred from day 0 to day 6 (Fig. 10). At the end of the storage period, the final concentrations of acetaldehyde were 113 mg.L⁻¹ and 119 mg.L⁻¹ for LG Kefir and MG Kefir, respectively. The increase in acetaldehyde could be ascribed to the combined activities of threonine aldolase and alcohol dehydrogenase (ADH) present in LAB and yeasts (Güzel-Seydim *et al.*, 2000; Grønnevik *et al.*, 2011).

Ethanol

At refrigerated temperature (4°C), no significant difference ($p > 0.05$) was found between the initial concentrations (3 668 and 3 300 mg.L⁻¹) and the concentrations found on day 6 (3 584 and 5 532 mg.L⁻¹), respectively for MG and LG Kefir (Fig. 11). Ertekin *et al.* (2010) also reported that ethanol

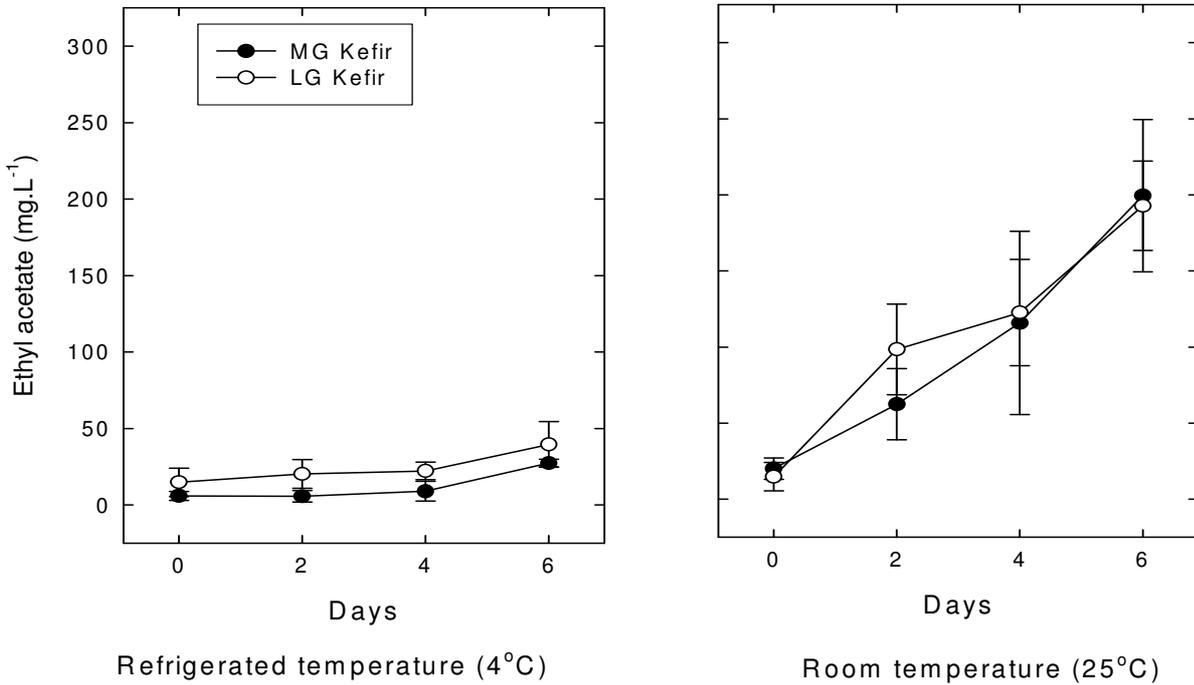


Figure 9. Profiles of ethyl acetate concentrations found in MG Kefir and LG Kefir stored at 4° and 25°C for 6 days. Vertical bars indicate 0.95 confidence interval.

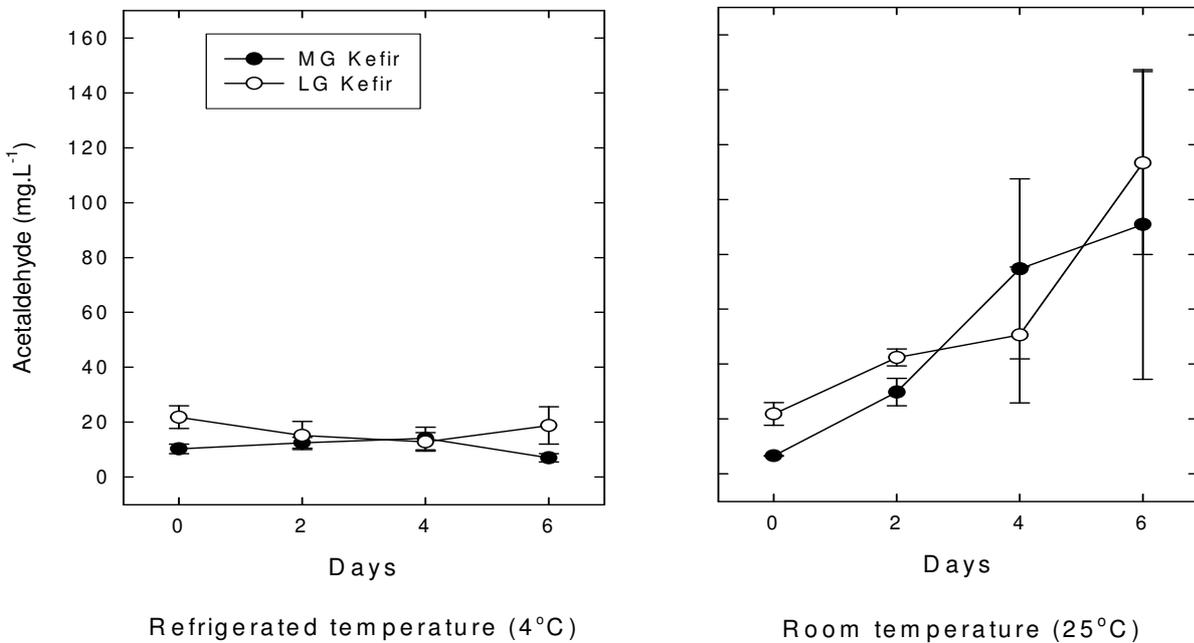


Figure 10. Profiles of acetaldehyde concentrations found in MG Kefir and LG Kefir stored at 4° and 25°C for 6 days. Vertical bars indicate 0.95 confidence interval.

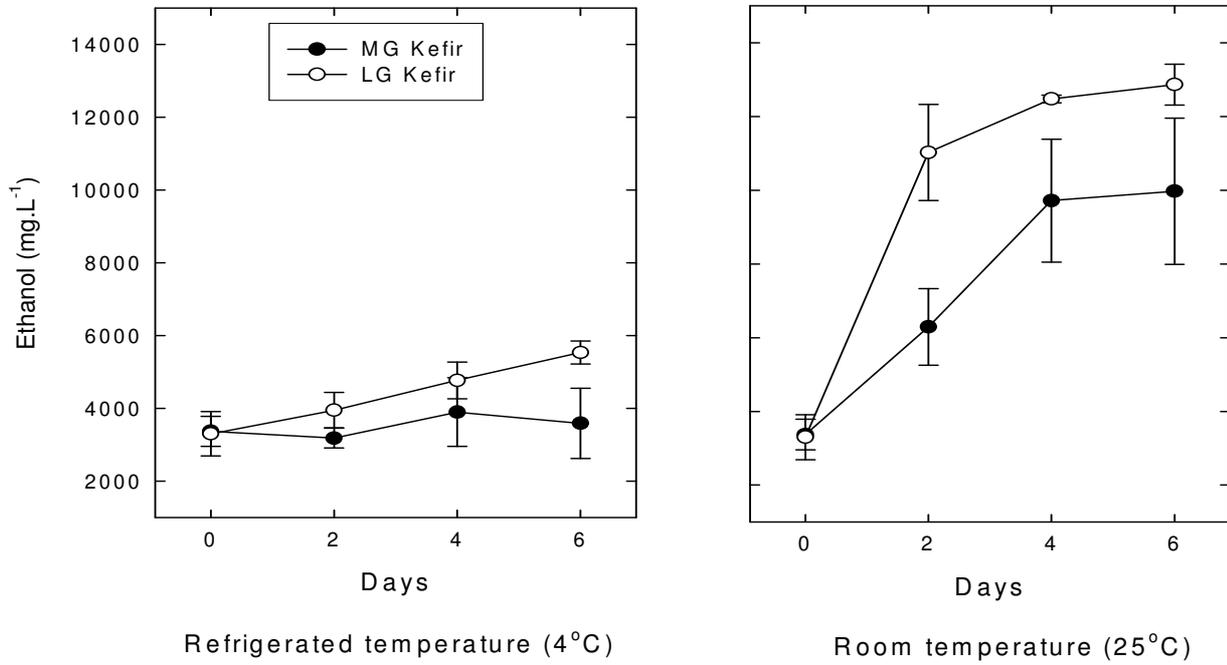


Figure 11. Profiles of ethanol concentrations found in MG Kefir and LG Kefir stored at 4° and 25°C for 6 days. Vertical bars indicate 0.95 confidence interval.

concentration remained constant over a 7 days storage period.

At room temperature (25°C), the ethanol concentration significantly increased ($p < 0.05$) from day 0 to day 6 for both Kefir (Fig. 11). At the end of the storage period, ethanol concentrations (9 969 and 12 861 mg.L⁻¹) were two to four times higher than on day 0, respectively for MG and LG Kefir. The increase in ethanol could be attributed to an increase in yeast population, which produce ethanol more efficiently than lactic acid bacteria (LAB) (Rea *et al.*, 1996; Viljoen *et al.*, 2003). A stabilisation in ethanol concentration was only observed from day 4 to day 6, in both Kefirs, indicating that yeast growth had stabilised. The same pattern was observed in the study of García Fontán *et al.* (2006).

Acetic acid

The concentration of acetic acid did not significantly vary ($p > 0.05$) in both Kefir samples incubated at refrigerated temperature (4°C) (Fig. 12); varying from 524 mg.L⁻¹ to 625 mg.L⁻¹ (MG Kefir) and from 560 mg.L⁻¹ to 569 mg.L⁻¹ (LG Kefir).

At room temperature (25°C), an increase in the production of acetic acid was observed, especially in LG Kefir, where a sharp increase ($p < 0.05$) occurred from by day 2 (849.5 mg.L⁻¹), after which the concentrations gradually increased to 938 mg.L⁻¹ by day 6. For the MG Kefir, the increase was more gradual, reaching 782 mg.L⁻¹ on day 6. Increase in acetic acid may originate from citrate metabolism and/or from the oxidation of ethanol by Kefir's microorganisms (Rea *et al.*, 1996).

pH

The pH profiles at 4°C and 25°C are illustrated in Fig. 13. At 4°C, the pH was stable throughout the storage period whereas at 25°C, the pH dropped significantly ($p < 0.05$) from day 0 to day 2 after which the pH stabilised. On day 6 of room temperature (25°C) storage, the pH was 3.29 and 3.50 for MG and LG Kefir, respectively (Fig. 13). The stabilisation of the pH cannot be explained by the exhaustion of lactose but can likely be ascribed to an inhibition of glucose and/or galactose catabolism due to the acidic environment (García Fontán *et al.*, 2006; Grønnevik *et al.*, 2011).

TA

At refrigerated temperature (4°C), the TA remained fairly constant over the study period for both Kefirs (Fig. 14) whereas at room temperature (25°C), a sharp increase ($p < 0.05$) in the TA was observed from day 0 to day 6 for the MG Kefir (Fig. 14). This increase is probably due to the production of lactic acid by the lactobacilli population, which remained viable due to the production of growth factors by the yeast population (Viljoen *et al.*, 2003; García Fontán *et al.*, 2006)

The pattern was different for the LG Kefir where a sharp increase occurred from day 0 to day 4 after which the TA stabilised. The stabilisation suggests that very little pyruvate was converted into

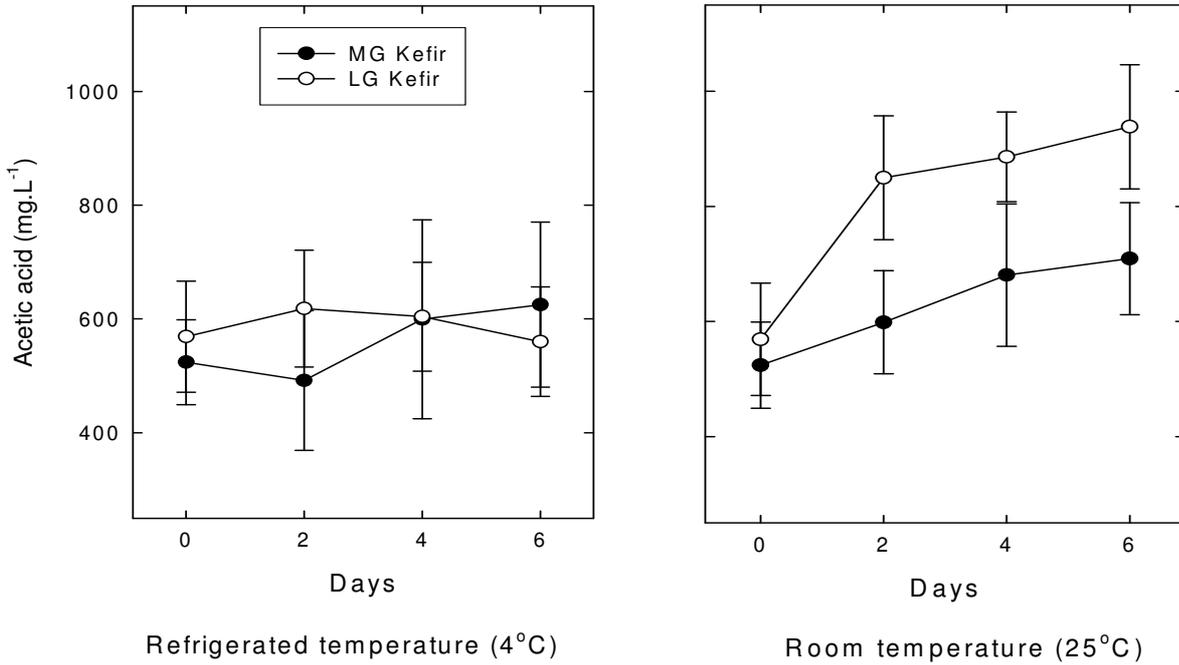


Figure 12. Profiles of acetic acid concentrations found in MG Kefir and LG Kefir stored at 4° and 25°C for 6 days. Vertical bars indicate 0.95 confidence interval.

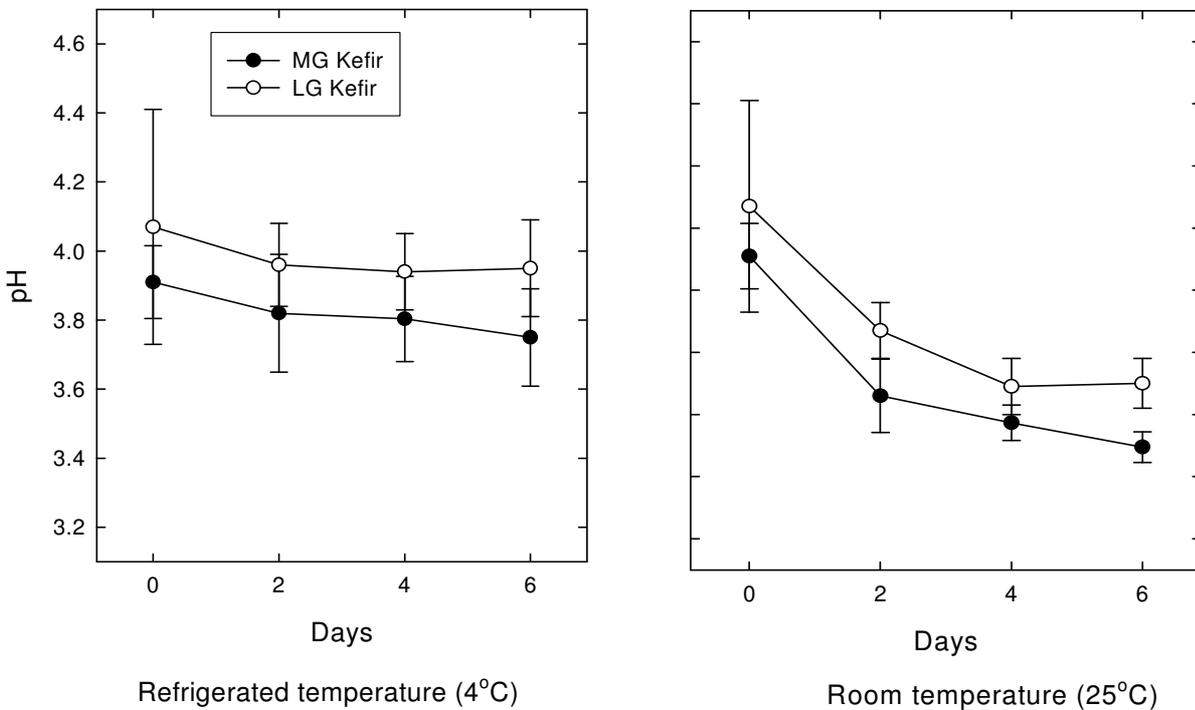


Figure 13. pH profiles of MG Kefir and LG Kefir stored at 4° and 25°C for 6 days. Vertical bars indicate 0.95 confidence interval.

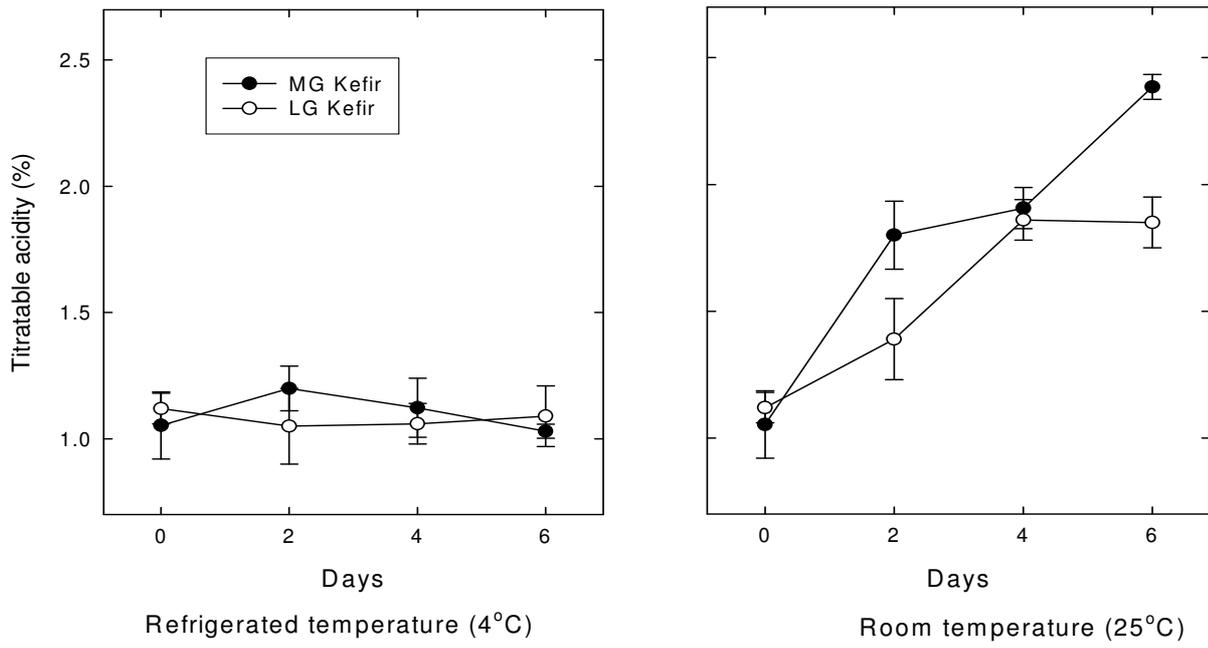


Figure 14. TA profiles of MG Kefir and LG Kefir stored at 4° and 25°C for 6 days. Vertical bars indicate 0.95 confidence interval.

lactic acid or a stabilisation in the growth of lactic acid bacteria, particularly the lactobacilli, which are the most resistant to acidic environment (Gran *et al.*, 2003; García Fontán *et al.*, 2006).

Thus, while MG Kefir showed gradual increase in TA, LG Kefir showed a stabilisation. The difference in both profiles indicates that different type of interactions took place during the storage at room temperature.

Diacetyl

Very low or no diacetyl was found ($< 1 \text{ mg.L}^{-1}$) in both Kefirs. The absence of diacetyl in some samples, particularly those incubated at 25°C, could be ascribed to the conversion of diacetyl to acetoin by diacetyl reductase, since this enzyme is more active at room than at refrigerated temperatures (Bassit *et al.*, 1995).

Discussion for study 2

At room temperature (25°C), the conditions were found to be more favourable to initiate various metabolic activities by LAB and yeasts present in Kefir grains (Seiler, 2003). This probably resulted in the increase in the concentration of flavour compounds observed throughout the storage period at room temperature. However, in terms of acidity, within two days of storage at room temperature, over acidification took place, with the pH dropping down to 3.8 and the TA increasing to 1.4%. This confirms the results obtained in the preliminary study done in Chapter 3, which showed that over acidification occurred at 25°C. The informal tasting done with Kefir samples stored for two days showed that it was not acceptable because over acidification negatively affected the texture and the taste. Since the proper acidification level is essential for a balanced flavour and texture, these results suggest again that storage of Kefir at room temperature can only be short.

At refrigerated temperature (4°C), the metabolic profiles of both Kefirs remained stable throughout the storage period, which was not surprising because previous studies have shown that the microbial counts of yeasts, lactic acid bacteria (lactobacilli and lactococci) in Kefir remained stable under refrigerated storage (Beshkova *et al.*, 2002; Güzel-Seydim *et al.*, 2005). In addition, it is well known that at refrigerated temperatures, microbial metabolism is slower due to the temperature, which is not optimum for LAB and yeasts. Indeed, under colder conditions, the metabolism is shifted towards the synthesis of cold induced proteins such as cold shock proteins (Csp), which helps in maintaining membrane fluidity, DNA supercoiling, transcription and translation of the molecules necessary for cellular adaptation to cold (Van de Guchte *et al.*, 2002).

The informal sensory tasting done on the refrigerated samples of Kefir showed that Kefir was judged acceptable throughout the storage period and that consequently, the amount of ethyl acetate ($14 - 40 \text{ mg.L}^{-1}$) were not found to cause any defects.

Conclusion to study 2

Ethyl acetate is an ester responsible for fruitiness in fermented products (e.g. wines, cheeses, beers). This attribute may turn out to become a defect if ethyl acetate exceeds certain predefined limits. For example, in beers, the acceptable levels of ethyl acetate vary between 8 and 32 mg.L⁻¹; whereas for wines, concentrations of ethyl acetate below 80 mg.L⁻¹ are required for a positive effect on sensory quality (Plata *et al.*, 2003; Verstrepen *et al.*, 2003). In the conditions of this study, Kefir containing up to 40 mg.L⁻¹ of ethyl acetate was found acceptable, although lacking diacetyl. It is well known that both diacetyl and ethyl acetate are potent flavours below 5 mg.L⁻¹ (Abeijón Mukdsi *et al.*, 2009). In addition, a ratio between 3 and 5 are required to obtain good cultured products. We have seen in the case of Kefir that this ratio is lower. However for ethyl acetate such details were not available in the literature of Kefir. This study has provided some answers; thus contributing to extend the knowledge on the flavour of Kefir.

Since the storage period was relatively short, further work using Kefir prepared with Kefir grains and Kefir prepared with pure culture, should be done, by extending the storage period to 4 weeks, as this could be of value for the dairy industry.

It has previously been hypothesised that the lack of taste of MG Kefir compared to Traditional Kefir could be caused by an improper ratio of diacetyl to acetaldehyde or by the lack of ethyl acetate. In this study, 4 panellists out of 5 preferred LG Kefir to MG Kefir. This again, confirms that MG do not give a well balanced product. Indirect improvement (food additives, addition of microorganisms, lengthening of incubation period) of MG Kefir as done in this study and the previous chapter gave promising results and would be sustainable options on a commercial scale; but stabilised Kefir grains not requiring any additions would still be the ultimate solution. Thus, an alternative worth investigating would be to stabilise MG Kefir through microbial enrichment of MG, i.e. incorporation of flavour forming microorganisms such as *Lc. diacetylactis* 318 and *C. kefir* 1283, responsible for diacetyl and ethyl acetate production. This process would further enhance the flavour of MG Kefir and produce good mass cultured Kefir grains that could be distributed and/or sold in South Africa.

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CHAPTER 8

METABOLIC PROFILES AND CONSUMER PREFERENCE OF TRADITIONAL KEFIR AND THREE VARIANTS OF KEFIR PREPARED WITH MASS CULTURED KEFIR GRAINS

Summary

This study was undertaken to evaluate the metabolite profile of four types of Kefir (Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir), and establish the consumer degree of liking of these Kefirs. It was established that all Kefir samples contained acetaldehyde (7 – 45 mg.L⁻¹), ethanol (186 – 1774 mg.L⁻¹), diacetyl (5 – 12 mg.L⁻¹), acetone (4.5 – 14 mg.L⁻¹), 2-butanone (1 – 4 mg.L⁻¹), ethyl acetate (1.2 – 30 mg.L⁻¹) and acetic acid (892 – 4 490 mg.L⁻¹). Titratable acidity (0.85% – 0.96%) and pH (4.13 – 4.25) were within the range found in the literature. Multivariate plots showed that Trad-Kefir was mainly associated with TA, ethanol, ethyl acetate and 2-butanone. Lacto-Kefir associated strongly with acetic acid whereas Candi-Kefir and MG Kefir associated with pH, diacetyl and acetone.

The consumer results indicated that pH ($r = 0.978$; $p < 0.05$) was a significant driver of liking flavour, especially for female consumers ($r = 0.982$; $p < 0.05$). Although there was a strong association between liking and pH for the male consumers, this association was not significant ($r = 0.939$; $p > 0.05$). For the female and male consumers, Trad-Kefir was the least liked (mean hedonic scores between 3.94 and 5.37) and Candi-Kefir was the most preferred (6.17 to 6.54) indicating a possibly gender effect. Application of Ward's clustering technique revealed the existence of three clusters of consumer liking. Cluster I, cluster II and cluster III represented 36%, 20% and 44% of the consumer panel, respectively. Results showed that all Kefir products were generally disliked by cluster I consumers. For cluster II, ethanol resulting in a yeasty flavour was the driver of liking ($r = 0.963$; $p < 0.05$). Thus, Trad-Kefir with the highest ethanol concentration was scored best (7.5) and MG Kefir with the lowest ethanol concentration obtained the lowest liking score (4.86). However, for cluster III, pH was the driver of liking ($r = 0.999$; $p < 0.05$), with Trad-Kefir being the least liked (4.91) and Candi-Kefir the most liked (7.63).

The microbial enrichment of Kefir grains led to products that were acceptable by the majority of the consumers. The outcome of this study can be used by the South African dairy industry to expand the current market in many ways.

Introduction

Maas is the commercial name of the South African sour milk. It is also known as *Amasi* in Zulu and

Xhosa, *Mafi* in Sotho and *Dikmelk* or *Kalbasmelk* in Afrikaans. Traditionally, this product is made from raw milk in calabash or clay pots by the rural communities in South Africa. The commercial version of Maas, although containing a preservative, is highly appreciated especially among the younger generation (Van Wyk *et al.*, 2002; Burger, 2010). Apart from Maas, Bulgarian and Greek yoghurts are other fermented dairy products well known to South African consumers.

The compounds responsible for the typical flavour of Maas are a blend of acetaldehyde, ethanol and diacetyl, as well as other minor compounds. Acetaldehyde is the main compound responsible for the typical flavour of yoghurt (Gran *et al.*, 2003; Ertekin & Güzel-Seydim, 2010). Buttermilk, which is also sold in the South African dairy market, is in contrast mainly characterised by its buttery flavour originating from the presence of diacetyl (Vedemuthu, 2006).

The price of many fermented dairy products has steadily increased over the past recent years making many of these products 'suddenly' unaffordable. This highlights a need among the South African consumers for low-cost but good quality fermented dairy products similar to Maas or yoghurt. This was recently confirmed by figures that show an increase in the demand for Maas (3.2%) and yoghurt (8.5%) by South African consumers (Coetzee, 2011). Kefir could be such a product since it is in many aspects similar to Maas and according to Burger (2010), "Kefir would taste similar to the Maas connoisseur".

In terms of flavour, Kefir is an acidic, slightly alcoholic and fizzing fermented dairy beverage. The buttery flavour is prominent when the balance with the other flavour compounds is achieved. However, a subtle fruity flavour may also be present. The presence of macro- and micro-nutrients makes it nutritionally beneficial. Thus, Kefir presents numerous advantages that would make it a great asset for South Africans.

The starter used to make Kefir is called Kefir grains. These grains are microbially-rich, cauliflower-like structures normally consisting of three groups of microorganisms (lactic acid bacteria, yeasts and acetic acid bacteria) living as part of a symbiotic association (Loretan *et al.*, 2003; Chen *et al.*, 2008). However, natural Kefir grains grow slowly, so a supply of Kefir grains can only be obtained through mass culturing (Libudzisz & Piatkiewicz, 1990; Schoevers & Britz, 2003). The drawback is that Kefir prepared with mass cultured Kefir grains (MG Kefir) has an unacceptable flavour compared to Traditional Kefir. In the previous chapters, improvements of the buttery and fruity flavours of MG Kefir were achieved by using indirect means such as addition of food additives, enrichment with microorganisms and/or lengthening of incubation period.

The purpose of this study was to evaluate the metabolic profiles and establish the consumer preference for MG Kefir, Kefir prepared with enriched mass cultured Kefir grains as well as Traditional Kefir. Mass cultured Kefir grains were enriched with the flavour forming microorganisms *Candida kefyr* 1283 and *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* 318.

Material and methods

Strains and growth conditions

Freeze-dried cultures of *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* 318 and *Candida kefir* 1283 were obtained from the Department of Food Science, Stellenbosch University. Stock cultures were maintained in MRS (Merck) and in yeast extract peptone dextrose broths (YPD) at 4°C, for *Lc. diacetylactis* 318 and *C. kefir* 1283, respectively. Strain purity was regularly checked by microscopy and Gram staining (Harrigan & McCance, 1998).

To construct growth curves, the bacterial and yeast counts were determined by dilution in MRS and YPD broths; and plating in MRS-Agar and YPD-Agar. Growth profiles of colony forming units (cfu.mL⁻¹) against absorbance at 500 nm (Spectronic 20 Genesys, Spectronic Instruments, Cape Town), were constructed. These profiles were used to standardise the inoculum size at 10⁶ cfu.mL⁻¹ and at 10⁵ cfu.mL⁻¹, for the *Lc. diacetylactis* 318 and *C. kefir* 1283 strains, respectively.

Mass culturing, enrichment and Kefir preparation

The following Kefir grains were used for the preparation of Kefir beverages:

- Mass cultured Kefir grains (= MG)
- MG enriched with *C. kefir* 1283 (= MGC)
- MG enriched with *Lc. diacetylactis* 318 (= MGL)
- Traditional Kefir grains (= TG)

Mass cultured Kefir grains (MG) were obtained as described by Schoevers & Britz (2003). The MGC and MGL grains were also obtained through mass cultivation, with the difference that microbial cultures *C. kefir* 1283 (10⁵ cfu.mL⁻¹) and *Lc. diacetylactis* 318 (10⁶ cfu.mL⁻¹) were added during the mass cultivation. The Kefir beverages prepared using the MG, MGL and MGC grains were named MG Kefir, Lacto-Kefir and Candi-Kefir, respectively.

Activated traditional grains (TG) were obtained from Professor T.J. Britz (Department of Food Science – University of Stellenbosch) and served to prepare Traditional Kefir (Trad-Kefir), which served as control. It was assumed that the microbial population of TG had stabilised since these grains had been used at least ten years. Therefore, it was assumed that the microbial population had stabilised (Prof. T.J. Britz, Food Science Department, Stellenbosch, South Africa, Personal Communication, 2008).

For the preparation of the Kefir beverages, 20 g of Kefir grains (MG, MGC, MGL, TG) were inoculated into 300 mL pasteurised milk and incubated at 22°C for 24 h. The Trad-Kefir was prepared under uncontrolled home-style conditions. After the incubation period, the grains were removed and Kefir beverages were used to identify the metabolic profiles and consumer tasting.

Metabolic profiles of Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir

Volatile organic compounds

The volatile organic compounds (acetaldehyde, ethanol, acetone, diacetyl, 2-butanone and ethyl acetate) were determined as described in Chapter 3.

Short chain volatile fatty acids determination

The content in short chain volatile fatty acids (acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid and iso-valeric acid) was determined as described in Chapter 3.

pH and Titratable acidity (TA)

The pH and titratable acidity (TA) were determined as described in Chapter 3.

Consumer preference analysis

The consumer preference test was conducted with a group of 85 consumers. Consumers were recruited on the basis that they regularly consume fermented dairy products such as Maas, Greek and Bulgarian yoghurts. On the day of tasting, consumers were asked to complete a questionnaire. The first page contained socio-demographic information (gender, age, ethnic group, income, education), as well as questions regarding the consumption habits of various fermented dairy products available on the South African market (Addendum 1). The degree of liking of the samples was asked in the second page (Addendum 2).

Consumers were presented with a set of four samples (Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir) served according to a randomised complete block design. Samples (15 mL) were served on white trays, directly from the refrigerator (4°C) in polystyrene cups coded with three-digit codes. The tasting took place in a room with standardised artificial daylight lighting and temperature control (ca. 21°C).

Consumers rated each sample for liking on a 9-point hedonic scale ranging from *Like extremely (9) to Dislike extremely (1)*. In this test, consumers were asked to indicate which term best describe their attitude towards the products being tested (Lawless & Heymann, 2010). Consumers were firstly instructed to indicate their preference for the overall aroma of the product. This was done orthonasally. Then consumers were requested to indicate their preference for the overall flavour of the product by tasting the product. Finally the consumers were asked to describe the specific aroma and flavour of the different products.

Statistical analysis

Consumer sensory data were analysed using SAS[®] software (Version 9; SAS Institute Inc, Cary, USA)

and subjected to the Shapiro-Wilk test for non-normality of the residuals (Shapiro & Wilk, 1965). If non-normality was found to be significant ($p \leq 0.05$) and caused by skewness, the outliers were identified and removed until the data were normal or symmetrically distributed (Glass *et al.*, 1972). Then, analysis of variance (ANOVA) was performed and student's t-least significant difference (LSD) was calculated at the 5 % significance level to compare treatment means.

Ward's clustering was performed to cluster individual judges in terms of their liking of the four products. The purpose of this algorithm is to join together objects into successively larger clusters, using some measure of similarity or distance XLStat (Version 7.5.2, Addinsoft, New York, USA). To determine the differences in preference patterns between clusters, an ANOVA was performed with cluster as factor to test for Cluster*Sample interaction as well as separately for each cluster.

Principal component analysis (PCA) using the correlation matrix was conducted using XLStat (Version 7.5.2, Addinsoft, New York, USA) to visualise and elucidate the relationships between the samples and their attributes.

Results and discussion

Chemical attributes

The results in Table 1 clearly show that Trad-Kefir was the most acidic product, with a pH of 4.13 and TA of 0.96%. For the other Kefir types, the pH varied between 4.23 and 4.25; and TA between 0.85% and 0.90%. These values of pH and TA were within the range reported in the literature (Simova *et al.*, 2002; Chen *et al.*, 2009; Magalhães *et al.*, 2011). In addition, the results reported in Table 1 show that Candi-Kefir and MG Kefir had the highest concentration in diacetyl and acetone whereas Lacto-Kefir had the highest concentration of acetic acid.

The concentration of diacetyl ranged from 5.1 to 12.6 mg.L⁻¹. According to Sandine *et al.* (1972), it is important that the balance between acetaldehyde and diacetyl is achieved to obtain cultured dairy products of optimum quality. An excess acetaldehyde is responsible for the 'grassy' odour whereas excess diacetyl causes cultured dairy products to have a harsh and pungent flavour. In addition, insufficient quantities of acetaldehyde cannot smooth out the astringent diacetyl after-taste (Cais-Sokolinska *et al.*, 2008). Thus, in most good quality cultured dairy products, a ratio of diacetyl to acetaldehyde varies between 3 and 5 (Sandine *et al.*, 1972). However, in this study, the ratios found for the four types of Kefir variants varied between 0.12 and 2.2. The latter ratio is wider than the ratio reported in literature (0.10 – 1.76) (Wszolek *et al.*, 2001; Beshkova *et al.*, 2003; Grønnevik *et al.*, 2011) and in Chapter 3. However, it must be highlighted that the sensory balance of Kefir cannot only rely on diacetyl and acetaldehyde, especially in instances where diacetyl is absent in Kefir. This was

Table 1. Chemical attributes (metabolic profiles) of the four types of Kefir.

Kefir types	pH*	TA*	mg.L ⁻¹						
			Acetaldehyde	Ethanol	Acetone	2-butanone	Diacetyl	Ethyl acetate	Acetic acid
Trad-Kefir	4.13	0.96	45 ± 20.3	1774 ± 252.1	10 ± 0.1	4 ± 1.0	5.5 ± 1.9	30 ± 7.2	1 382 ± 302.1
Candi-Kefir	4.25	0.89	16 ± 4.3	721 ± 362.3	12 ± 1.6	1 ± 0.3	6.7	1.5 ± 0.4	2 431 ± 178.3
Lacto-Kefir	4.23	0.9	7 ± 1.1	531 ± 150.0	4.5 ± 1.7	1.1 ± 0.3	5.1 ± 2.1	1.2 ± 0.3	4 490 ± 560.0
MG Kefir	4.24	0.85	9 ± 0.7	186 ± 68.4	14 ± 1.4	1.7 ± 0.1	9.6 ± 4.4	11 ± 1.9	892 ± 57.7

The results represent the mean the standard deviation (SD)

(*) Standard deviations are not shown because they are below 10%.

verified in the previous chapter, where it was found that samples without diacetyl, but containing ethyl acetate along with acetaldehyde, ethanol, acetone and acetic acid, were found to be acceptable. Ethyl acetate is responsible for imparting a fruity flavour to fermented products. In this study, the Kefir variants contained between 1.1 and 30 mg.L⁻¹ of ethyl acetate (Table 1). These values were within the range found in the previous chapter (Chapter 7) as well as reported by Magalhães *et al.* (2011).

The PCA bi-plot (Fig. 1) showed that Trad-Kefir, which was the control sample for this study, was associated with titratable acidity (TA), ethanol, ethyl acetate, acetaldehyde and 2-butanone, whereas both Mass cultured Kefir and Candi-Kefir associated strongly with diacetyl and acetone and Lacto-Kefir with acetic acid, respectively.

Consumer acceptability

Consumer socio-demographic information

The consumer panel consisted of 85 individuals, 52% were older than 30 and the remaining younger than 30. The male consumers represented 38% of the consumer panel and the female consumers 65%. The majority of the consumer panel (87%) indicated that they associate culturally with the White or Coloured groupings whereas the remaining associate culturally with black South Africans. Only 16% of the total consumers group were regular Kefir consumers, the remaining had never heard of Kefir or have heard of it but have never tasted it. The breakdown was as follows: 39% were Maas, 39% Bulgarian yoghurt, 31% Greek yoghurt and 57% Buttermilk consumers.

General consumer acceptability

Figure 2 is a PCA bi-plot indicating the drivers of liking for aroma and flavour of the respective products. The first two principal components explained 93.6% of the variance. According to Factor 1, degree of liking of aroma and flavour associated strongly with Mass cultured, as well as Candi-Kefir. The liking of Candi-Kefir and MG Kefir (Fig. 2; Fig. 3) is most probably as a result of the pH and the high content of diacetyl and acetone (Table 1). The correlation values indicated that degree of liking of flavour and aroma associated with pH ($r = 0.978$; $p < 0.05$), diacetyl ($r = 0.750$; $p > 0.05$) and to a lesser extent with acetone ($r = 0.206$; $p > 0.05$). The extremely strong significant correlation of liking of pH thus indicates that the acidity of the product could be regarded as an important driver of liking. This is in accordance with the data in the literature, which reported that a too high or too low acidity can influence degree of liking negatively (Güler & Park, 2011). Although not significant, the correlation values also indicate the importance of an optimum concentration of diacetyl, which is one of the most important flavour compounds in fermented dairy products and results in a buttery flavour (Oberman &

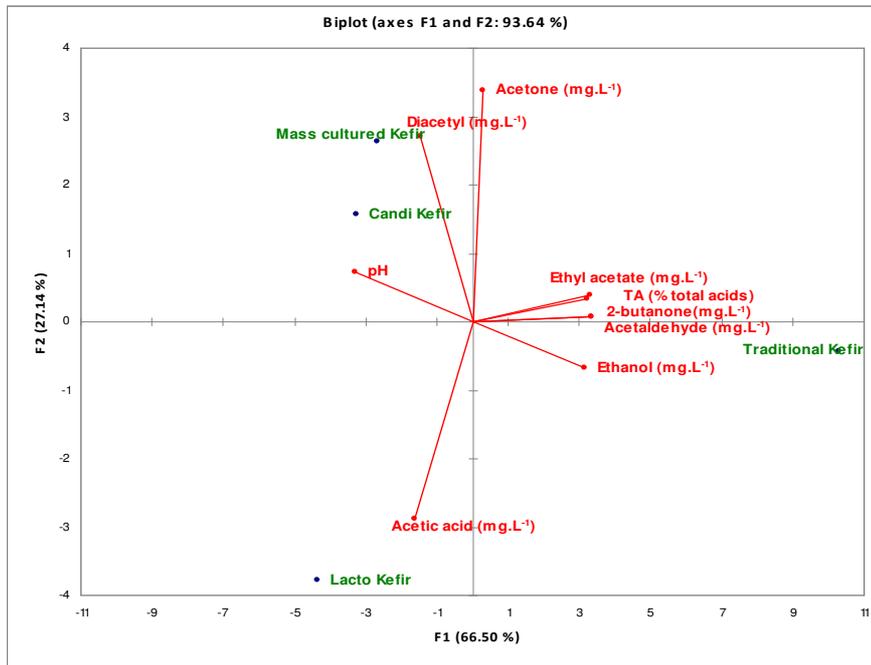


Figure 1. PCA bi-plot indicating the association of chemical constituents in relation to Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir.

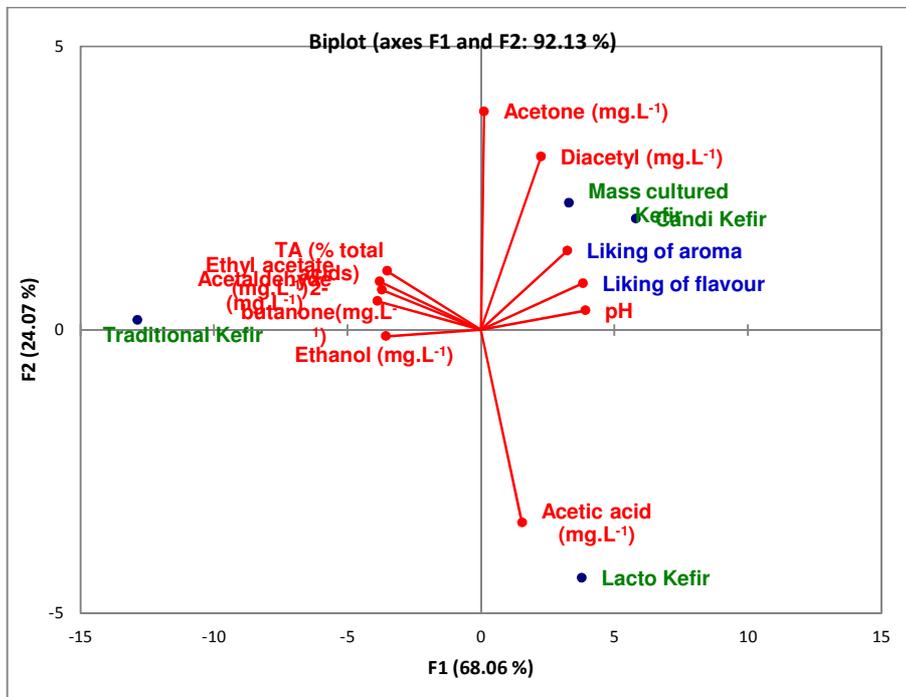


Figure 2. PCA bi-plot indicating the position of chemical compounds in relation to liking of aroma and flavour. The first two principal components explained 92.1% of the variance.

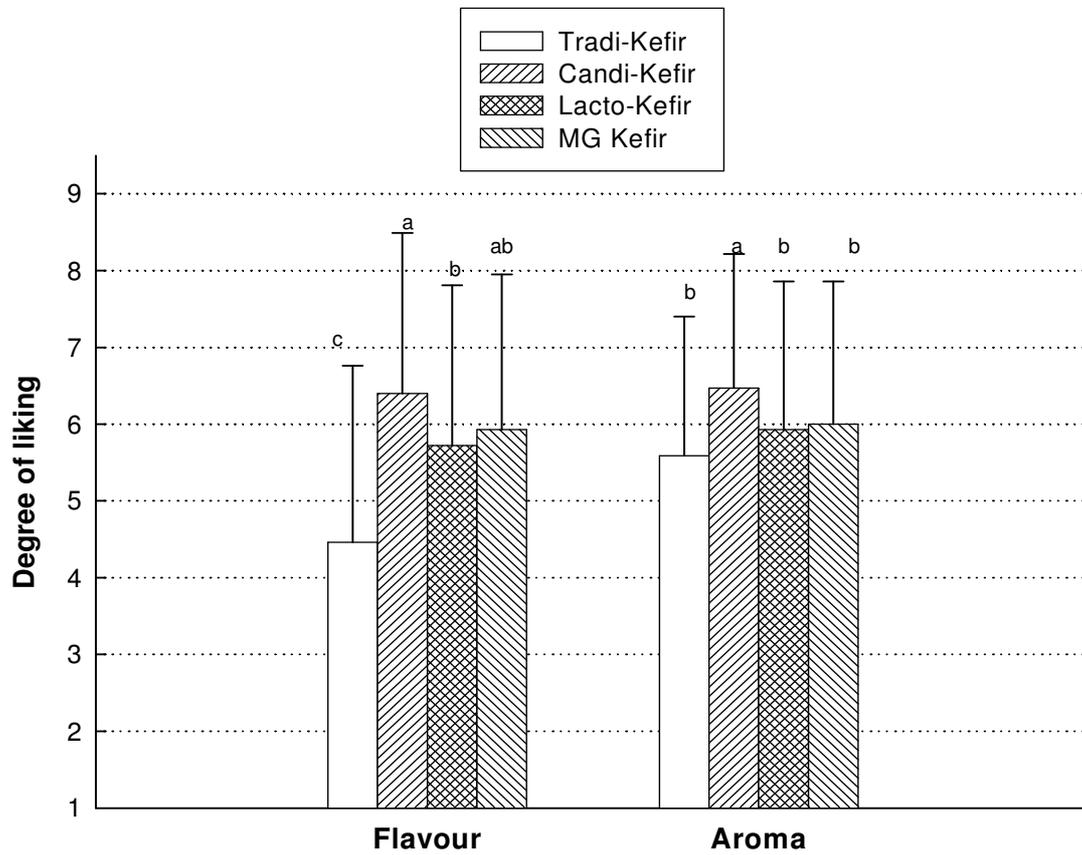


Figure 3. Degree of liking of flavour and aroma for Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir by the entire consumer panel. Means (+SD) with different alphabetical letters differ significantly.

Libudzisz, 1998).

The degree of liking of the flavour and aroma of Lacto-Kefir (Fig. 2; Fig. 3) was slightly less than that of Candi-Kefir and MG-Kefir, possibly as a result of the presence of a high concentration in acetic acid in this product (Table 1). According to Wszolek *et al.* (2006), high concentrations of acetic acid may impart a 'vinegary' taste that can be regarded as undesirable to consumers.

The aroma and flavour of the Trad-Kefir (Figs. 2 and 3) was least liked by this consumer group (mean liking score for flavour was 4.6), most probably as a result of the presence of a significant amount of ethanol, ethyl acetate, acetaldehyde, 2-butanone, as well as a high acidity (Table 1). According to the data in Table 1 this variant had the lowest pH (4.13) and the highest TA (0.96%).

Figures 4a and 4b indicate the degree of liking of flavour and aroma of the respective users of Kefir and other fermented products. It is clear from Fig. 4a that Candi-Kefir was considered the best product by the different groups of fermented milk consumers with mean scores for liking of flavour ranging from 6.37 to 7.57. In Fig. 4a, we can see that Trad-Kefir, which was the most acidic product, was scored low by Greek yoghurt consumers (4.31), Bulgarian yoghurt consumers (4.21) and Buttermilk consumers (4.54) whereas it was scored higher by regular Maas consumers (5.31) and Kefir consumers (6.29). The latter high score for Trad-Kefir was expected: sixteen percent (16%) of this group of consumers were regular consumers of Kefir and as neither grains nor beverage is commercially available in South Africa, one can make the assumption that regular Kefir consumers have access to Trad-Kefir, probably because they possess the grains (Loretan *et al.*, 2003). This shows that regular Kefir consumers are accustomed to the unique, moderately acidic flavour of Trad-Kefir. Conversely, Bulgarian yoghurt, Greek yoghurt and Buttermilk have a rather slightly acidic flavour (Table 2). It is thus unlikely that regular consumers of the latter products would like a moderate acidic product such as Trad-Kefir. Reports indicate that fermented milk products tend to be found less acceptable (low scores) than yoghurt products (Muir *et al.*, 1999; Bayarri *et al.*, 2011). Again such results must be interpreted with caution, especially if the panel used was mainly constituted by users of slightly acidic products.

Maas consumers represented 39% of the consumer panel. The score given by Maas consumers for Trad-Kefir (5.31) was high compared to the mean scores given by Greek yoghurt consumers (4.31), Bulgarian yoghurt consumers (4.21) and Buttermilk consumers (4.54) (Fig. 4a). Thus, from the results it appears that the potential target market for Kefir would be Maas consumers, which confirm the results reported by Van Wyk *et al.* (2002) where Kefir and Maas were equally preferred. In addition, Kefir and Maas would have a similar taste for the Maas connoisseur Burger (2010).

From Fig. 4a, it appears that Greek yoghurt consumers, Bulgarian yoghurt consumers and Buttermilk consumers were responsible for the *trend* in the degree of liking of flavour for the entire consumer panel as indicated in Fig. 3. These consumers represented a large portion of the consumer

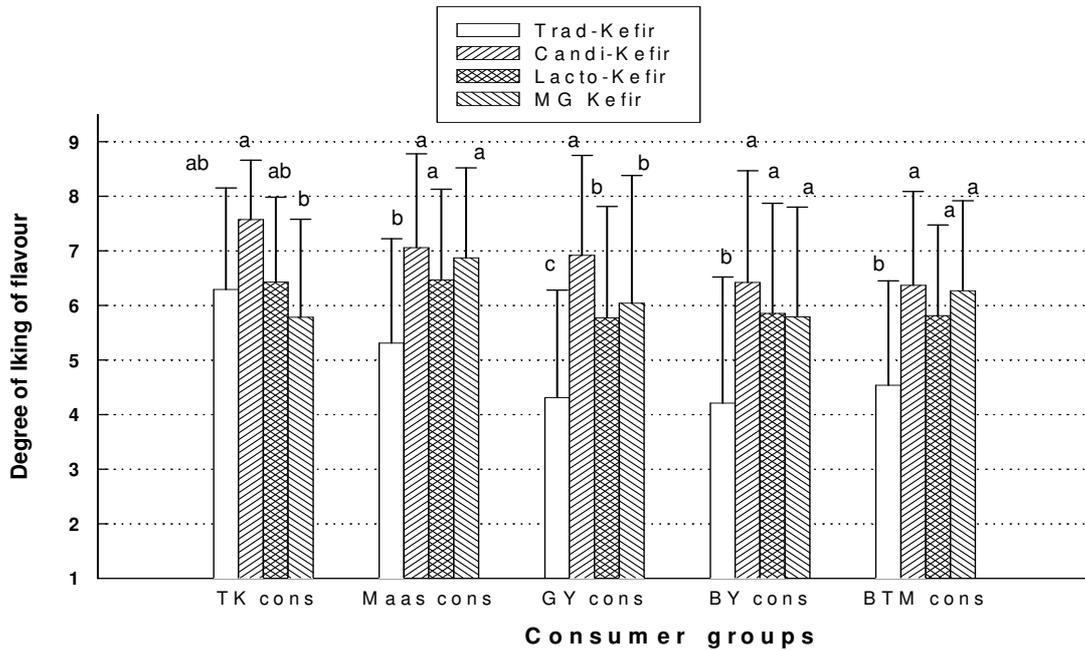


Figure 4a. Degree of liking of flavour for Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir by consumer categories (Trad-Kefir consumers, Maas consumers, Greek yoghurt consumers, Bulgarian Yoghurt)

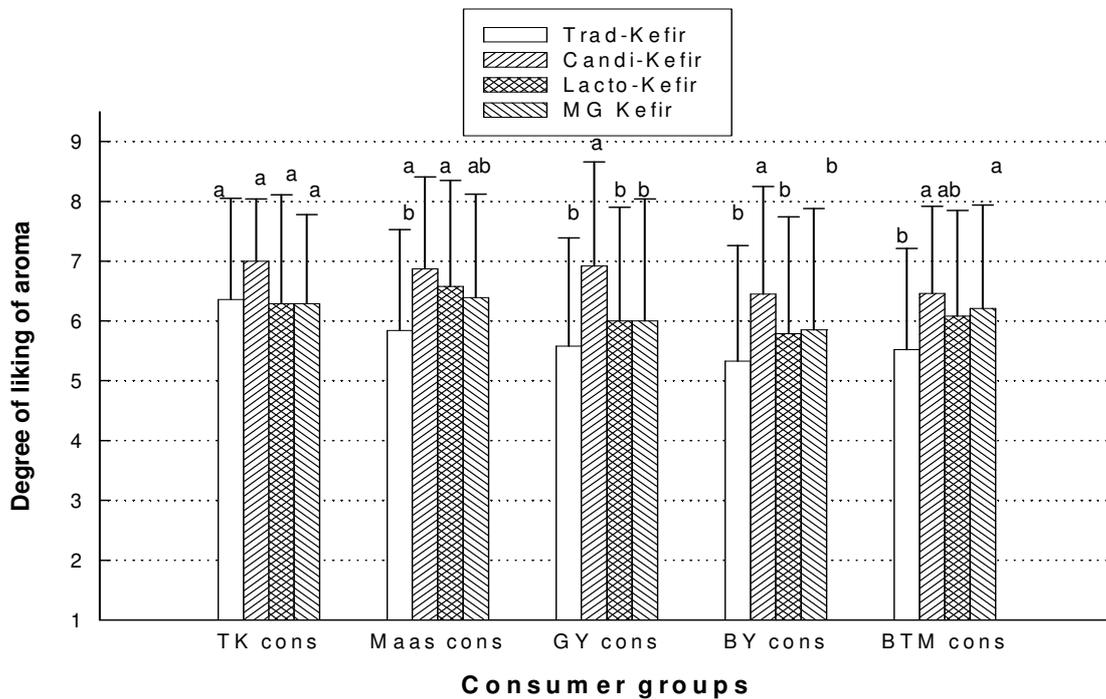


Figure 4b. Degree of liking of aroma for Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir by consumer categories (Trad-Kefir consumers, Maas consumers, Greek yoghurt consumers, Bulgarian Yoghurt consumers and Buttermilk consumers). Means (+SD) with different alphabetical letters differ significantly.

panel, thus their scores 'weighed' more in the final results as depicted in Fig. 3.

According to Fig. 4b, Candi-Kefir obtained the best score for aroma and, although the flavour of Trad-Kefir was generally not liked, the aroma was found acceptable with mean score ranging from 5.33 to 6.36.

Role of gender in consumer acceptability

The PCA bi-plot (Fig. 5a) indicates that pH was a significant driver of liking of flavour for the female ($r = 0.982$; $p < 0.05$), but not a significant driver of liking of flavour for the male ($r = 0.939$; $p > 0.05$) consumers. This was confirmed by the results shown in Fig. 5b, where it can be seen that female consumers differed significantly in their preference pattern (3.94 – 6.54), whereas the male consumers did not; and thus gave reasonably similar scores (5.37 – 6.17) for all four types of Kefir. A reasonably similar result is found in Fig. 5c for the liking of aroma.

Segmentation of consumer acceptability

Ward's cluster analysis identified three different clusters or groups of consumers (Fig. 6) based on their degree of liking of the different types of Kefir and the socio-demographic information of the different clusters is presented in Table 3. From this Table, it is clear that the regular consumption of Kefir, Maas and Buttermilk played a significant role in the clustering of the consumers. After conducting the cluster analysis, the liking scores of each cluster for the flavour of the respective products were superimposed on the chemical data in a PCA analysis (Fig. 7).

Cluster I

Cluster I comprised the second largest group of consumers (36%) (Table 3). The socio-demographic composition of this cluster showed that none of the consumers were regular consumers of Kefir, 21% were regular consumers of Maas, whereas 39% to 52% of this group of consumers were regular consumers of Greek yoghurt, Bulgarian yoghurt and Buttermilk.

This group of consumers gave low scores (2.41 to 5.30) to all the types of Kefir (Fig. 8), with the lowest score obtained by Trad-Kefir. Many reasons can be given to explain why this group of consumers gave such low scores; the most probable being that this group of consumers have never consumed any Kefir products, furthermore less than 25% of this group of consumer drink or use Maas regularly. They are thus unfamiliar with the natural acidic taste of Kefir.

Cluster II

Consumers from cluster II represent 20% of the consumer panel and have a completely different

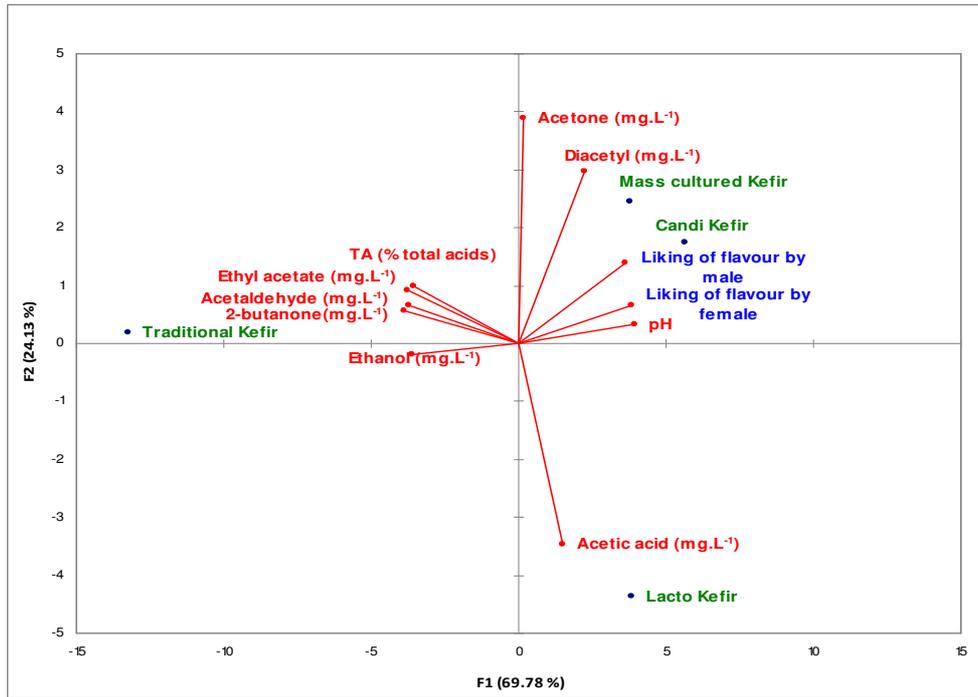


Figure 5a. PCA bi-plot indicating the position of chemical compounds in relation to the liking of aroma and flavour by male and female consumers. The first two principal components explained 93.9% of the variance.

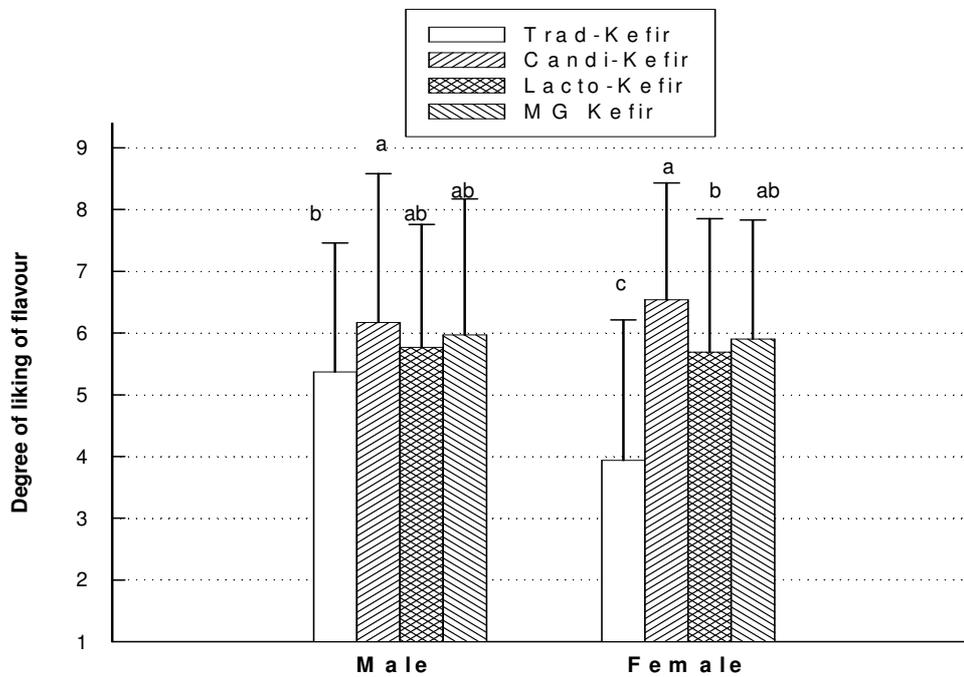


Figure 5b. Degree of liking of flavour for Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir by male and female. Means (+SD) with different alphabetical letters differ significantly.

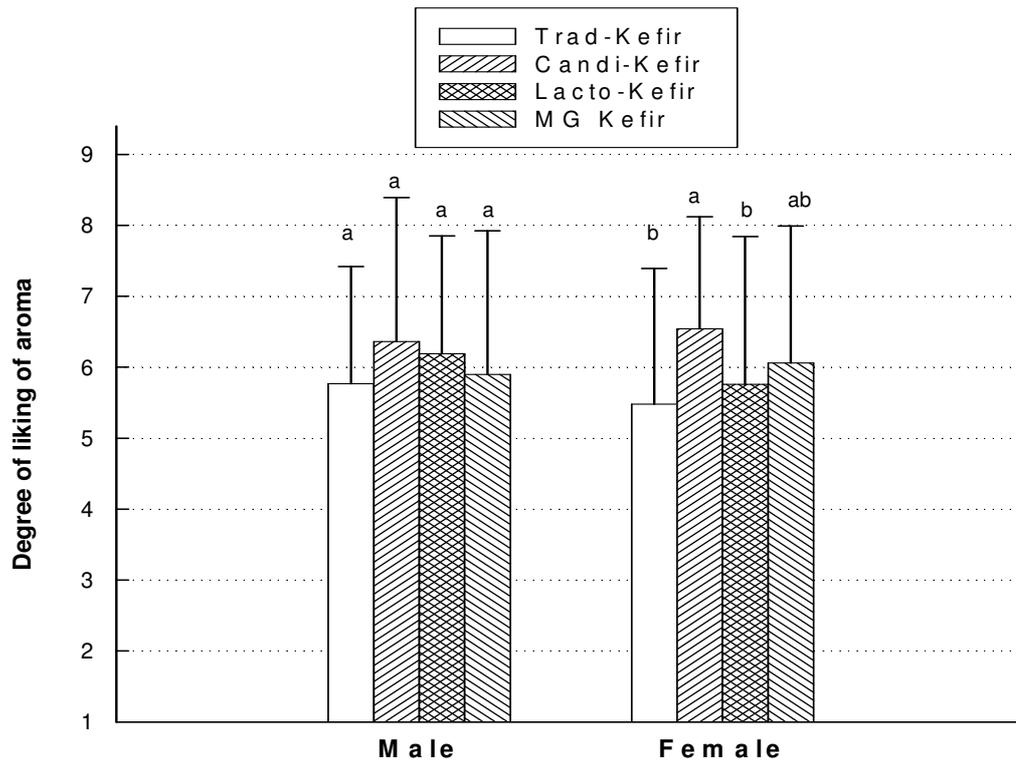


Figure 5c. Degree of liking of aroma for Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir by male and female. Means (+SD) with different alphabetical letters differ significantly.

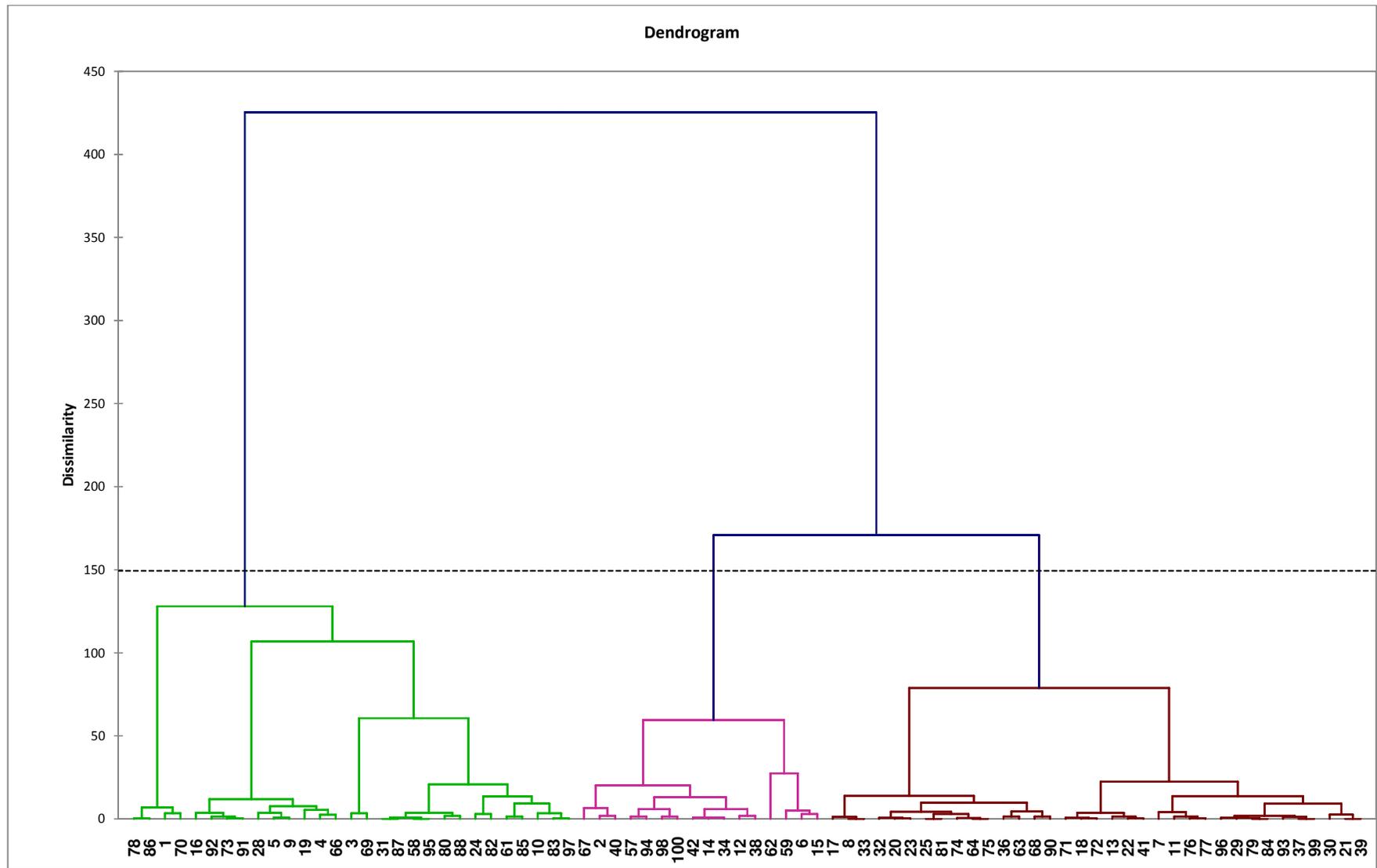


Figure 6. Consumer segmentation using Ward's cluster analysis of degree of liking of flavour.

Table 3. Socio-demographic information and characteristics of each cluster expressed as percentage.

Socio-demographics	Cluster I (%) N = 36%	Cluster II (%) N = 20%	Cluster III (%) N = 44%
Female	72	56	60
Male	28	44	40
< 30 years of age	59	56	46
> 30 years of age	41	44	54
Kefir consumption			
Not regular	100	50	83
Regular	0	50	17
Greek Yoghurt consumption			
Not regular	61	75	66
Regular	39	25	34
Bulgarian Yoghurt consumption			
Not regular	55	69	63
Regular	45	31	37
Maas consumption			
Not regular	79	62	43
Regular	21	38	57
Buttermilk consumption			
Not regular	48	56	29
Regular	52	44	71

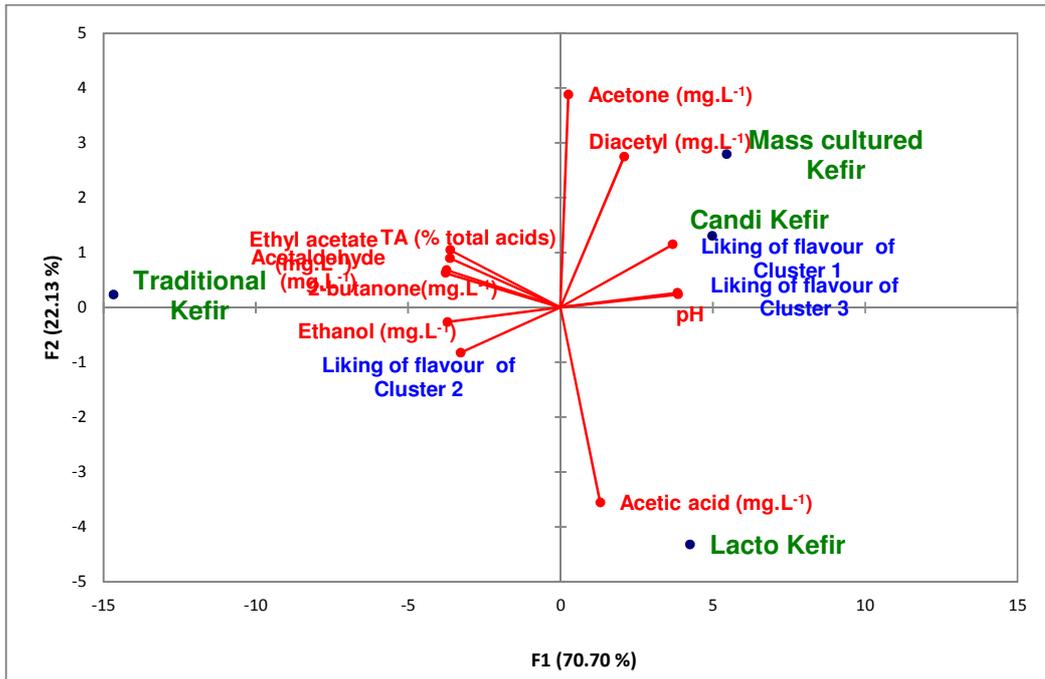


Figure 7. PCA bi-plot indicating the degree of liking of flavour of each cluster in relation to the four Kefir samples. The first two principal components explained 92.8% of the variance.

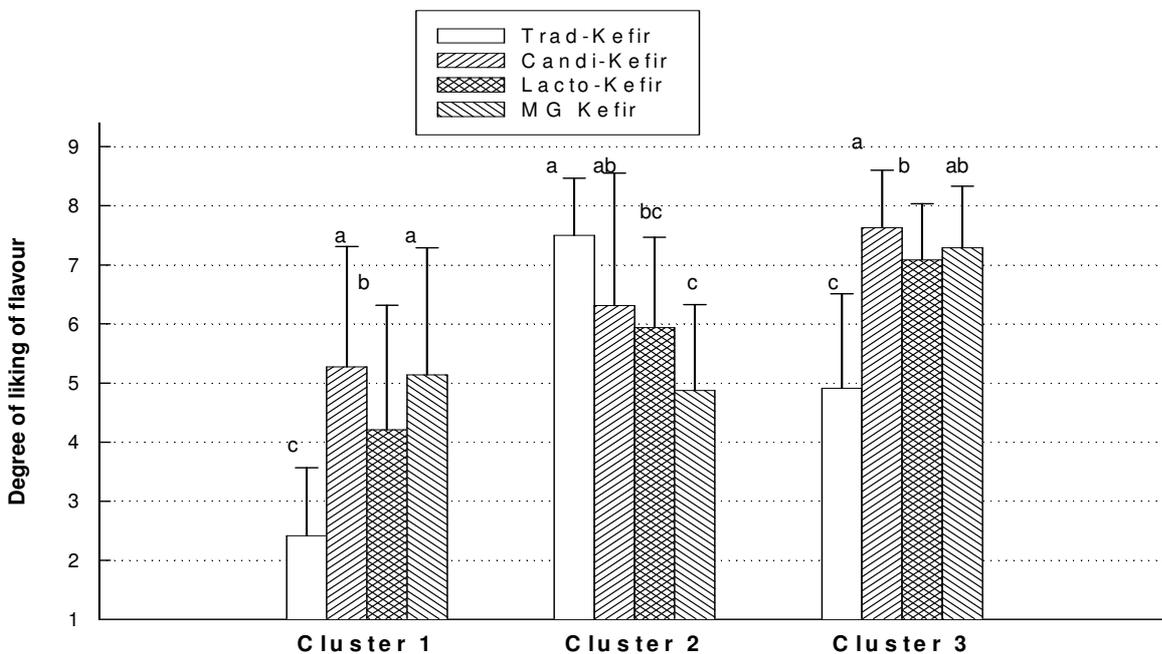


Figure 8. Degree of liking of flavour for Trad-Kefir, Candi-Kefir, Lacto-Kefir and MG Kefir by cluster I, cluster II and cluster III. Means (+SD) with different alphabetical letters differ significantly.

preference pattern when compared to consumers from cluster I. As opposed to consumers from cluster I not liking acidic products, the preference liking of consumers from cluster II was significantly driven by ethanol content ($r = 0.963$; $p < 0.05$) (Fig. 7). Ethanol is responsible for the yeasty flavour in Kefir, which is an essential character of Kefir. Thus, it can be suggested that consumers of cluster II liked the 'yeasty flavour' of the four types of Kefir. This was confirmed by the results (Fig. 8), which indicated that the degree of liking of Kefir types decreased as the ethanol content decreased. For this cluster of consumers, Trad-Kefir obtained the best score (7.5), followed by Candi-Kefir (6.31), Lacto-Kefir (5.94) and MG Kefir (4.87). The concentrations of ethanol in these products were 1 774 mg.L⁻¹, 720.5 mg.L⁻¹; 531 mg.L⁻¹ and 186 mg.L⁻¹, respectively. In addition, the highest percentage regular consumers of Kefir were found in this cluster (Table 3), which explains why Trad-Kefir obtained the best score (7.5). This group of consumers also gave the lowest score to MG Kefir (4.87). As stated in the introduction, regular Kefir consumers would find the taste of MG Kefir unatypical.

Cluster III

Cluster III comprised the largest group of consumers (44%) and their preference pattern appeared to be similar to that of cluster I. For this group pH was the driver of liking ($r = 0.999$; $p < 0.05$) and likewise to cluster I, Candi-Kefir obtained the highest preference rating (7.63), whereas Trad-Kefir obtained the lowest mean score (4.91) (Fig. 8). However, it must be highlighted that this group of consumers gave considerably higher scores to all these products (4.91 – 7.63) when compared to consumers from cluster I (2.41 – 5.27) (Fig. 8).

The high scores for liking of the flavour (7.09 – 7.63) obtained for Candi-Kefir, Lacto-Kefir and MG Kefir, may originate from the fact that these types of Kefir were mildly acidic products. However, it is interesting to note that the liking scores obtained by Candi-Kefir (7.63), MG Kefir (7.29) and Lacto-Kefir (7.09) decreased as the content in diacetyl, 12 mg.L⁻¹, 9.6 mg.L⁻¹ and 5 mg.L⁻¹, decreased respectively. Though, the driver of liking of flavour for cluster III was not strongly driven by diacetyl ($r = 0.646$; $p > 0.05$), it can be speculated that as an extremely high proportion of cluster III consumers drink Buttermilk regularly (71%), the buttery flavour of Kefir types could have prompted the preference ratings of these consumers, especially that the buttery flavour imparted by diacetyl is usually the prominent flavour found in Buttermilk (Vedemuthu, 2006).

Description of sensory attributes

No formal descriptive sensory analysis was conducted in this study. In view of this, the consumers were asked during the consumer preference testing to describe in their own words how they perceive the aroma and flavour of the four types of Kefir.

It was expected from the microbial enrichment that Candi-Kefir would exhibit a stronger

fruity flavour and Lacto-Kefir a stronger buttery flavour compared to Mass cultured Kefir. According to this group of consumers both Candi-Kefir and Lacto-Kefir were described as having a rather '*buttery and buttermilk-like flavour and odour*'. Two consumers mentioned that Trad-Kefir had some fruity notes ('*grape like/ banana*'), which could be explained by the fact that Traditional Kefir had the highest content in ethyl acetate (30 mg.L⁻¹).

The fact that Candi-Kefir and Lacto-Kefir were described as having a rather '*buttery flavour*' clearly indicate the importance of diacetyl in Kefir. However, the fact that Trad-Kefir was labelled as 'fruity', does confirm that ethyl acetate may impart a perceptible and positive fruity flavour to Kefir. This corroborates the findings of the previous chapter and clearly indicates that ethyl acetate can also be a major contributing aroma compound in the flavour of Kefir.

Conclusions

Four types of Kefir were tasted by 85 consumers. Three clusters of consumers were identified based on their liking of flavour of the four types of Kefir. Cluster I did not particularly like products with a high degree of acidity as indicated by the reasonably low scores given (2.41 – 5.30) given for liking of flavour. For cluster III, liking scores for flavour of the different Kefir types were similarly guided by the acidity of the products. Thus, less acidic Kefir products obtained better consumer liking scores (7.09 – 7.63) than Trad-Kefir which was the most acidic product (4.91). One can thus come to the conclusion that acidity is a vital driver of liking for a specific segment of consumers. As for Cluster II, ethanol was the main driver of liking of flavour and consequently, Trad-Kefir, the most 'yeasty' product obtained the best score (7.5).

This study has provided some understanding on the preference liking of Kefir, an unknown fermented dairy product to South African consumers. It is clear from the results that microbial enrichment of mass cultured Kefir grains with *C. kefir* 1283 and *Lc. diacetylactis* 318 would supply grains that would give Kefir beverages (Candi-Kefir and Lacto-Kefir) acceptable to approximately 40% of consumers. Thus these enriched grains have the potential to be marketed to South African consumers.

A further positive is that Trad-Kefir grains result in a product with a moderately acidic, alcoholic slightly fruity profile, a variant that is also highly acceptable as indicated by a specific segment of the consumers. Moreover, production of sweet flavoured types of Kefir would probably attract a larger group of consumers.

The study also highlighted the complexity of microbial interaction within the Kefir grain that originated from the microbial enrichment. However, since no microbial analyses were done to confirm

that *C. kefir* 1283 and *Lc. diacetylactis* 318 did integrate into the grains, further studies could be done to confirm this. Also, additional work should also be done to identify which sensory attributes, i.e. sweetness, saltiness, bitterness, astringency, acidity, fruitiness, creaminess, etc drive the liking or disliking of the consumers within specific consumer segments of the South African population.

The outcome of this study can be used by the South African dairy industry to expand the current market in many ways. The fact that there is a strong indication of the drivers of consumer liking can be regarded as positive for future research and development endeavours of the industry.

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CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

Background

Cheese, yoghurt, buttermilk and commercial Maas are some of the popular fermented dairy products consumed in South Africa. The consumption of traditional Maas, a South African traditional fermented milk made from raw milk, is declining as raw milk is not readily available; and even when it is, the microbiological quality is questionable. However, although commercial Maas is sold, many consumers find it of lower sensory value compared to traditional Maas. In addition, the retail price of commercial Maas, yoghurt and fermented dairy derivatives has steadily increased over the years. Even though several fermented dairy products have been developed as improvements to existing formula or range expansions, the South African consumers are silently expectant of a 'new' low-cost fermented or self-preparatory dairy product that would be as good as Maas or yoghurt.

Previously, Van Wyk *et al.* (2002) identified Kefir as an excellent product to be introduced in the South African market for several reasons. It is healthy; suitable for lactose intolerant persons; can be home-prepared and has a flavour and acidity that is similar to Maas (Burger, 2010). Furthermore, the main advantage of Kefir resides in the Kefir grain (starter culture), which is endlessly reusable, making Kefir a healthy acceptable and cost saving solution once Kefir grains have been acquired. This is such an unusual characteristic for a starter culture that both Kefir grains and Kefir can be qualified as a '*lifetime opportunity not to be missed*'.

Production of large volumes of mass cultured Kefir grains ('MG') has been shown to be feasible (Schoevers & Britz, 2003) but the drawback is that Kefir made with mass cultured Kefir grains has an unacceptable flavour compared to the flavour of Traditional Kefir.

The overall objective of this dissertation was thus to determine the impact of environmental factors on the metabolic profiles of Kefir produced using different Kefir grains. This was followed by the subsequent enrichment and evaluation of Kefir prepared with mass cultured grains so as to obtain a Kefir beverage that has improved organoleptic qualities.

Flavour profiles

The objective in this study was to determine the impact of controlled (laboratory made) and uncontrolled (home-made) incubation conditions. The results of this study showed that production of a good quality Kefir could be obtained under uncontrolled conditions. In addition, under controlled conditions, incubation at 22°C was preferable to incubation at 25°C, since over acidification occurred at this temperature. This shows the importance of proper acidity as inadequate pH or titratable acidity

(TA) would affect the texture and the flavour of Kefir.

The metabolic profiles of MG Kefir (MG22 Kefir) and Traditional Kefir revealed that the pH and TA were similar and both products contained acetaldehyde, ethanol, acetone, diacetyl and acetic acid. However, the MG22 Kefir still lacked the original, pronounced buttery and subtle fruity flavour of Traditional Kefir. It was hypothesised that the lack of flavour was caused by an inadequate ratio of diacetyl to acetaldehyde and/or the absence of ethyl acetate. Since metabolites are synthesised by the symbiotic activity of the Kefir grain consortium, the difference in the MPs could only be ascribed to an intrinsic microbial imbalance within the MG or the absence of a significant number of flavour forming microbes. This would thus require addition of food additives, microbes and/or substrates during Kefir fermentation to stimulate flavour production of specific compounds (Chapter 6 & 7) or the microbial enrichment of MG Kefir grains during mass cultivation with diacetyl and ethyl acetate producing microbes to make up for the lack of buttery and fruity flavours (Chapter 8).

Acidity

One of the outcomes of Chapter 3 was also that acidity was important for proper flavour and texture. pH and TA are the common parameters used to monitor acidity, whereas quantification of lactic acid, which is the major acid formed in Kefir, is not routinely used, since the available methods are both expensive and time consuming. Proper acidity in terms of lactic acid is vital to bring forth the organoleptic balance and to impart a good texture to Kefir. However, data on a quick and non-destructive method to quantify DL-lactic acid in fermented dairy products could not be found. Thus, the use of Near Infrared Reflectance (NIR) Spectroscopy as a rapid method to quantify DL-lactic acid and other acidity parameters, including acetic acid, pH and titratable acidity (TA) was explored (Chapters 4 & 5). As part of the study, models were successfully developed for lactic acid (RPD = 2.57), pH (RPD = 2.90) and TA (RPD = 2.60) but not for acetic acid (RPD = 1.17). The RPD values obtained for lactic acid, pH and TA indicated that the models could successfully be used for screening. However, for routine analysis, RPD values higher than 5 are needed (Williams, 2001). Thus, these models will have to be improved. This can be accomplished as more samples become available from different sources of Kefir production.

This work was a proof of concept that NIR spectroscopy can be used to estimate DL-LA, pH and TA in Kefir in particular and fermented dairy products in general. The acquisition of on-line NIR spectrophotometers to monitor acidity parameters could change the face of the dairy industry and would be a very good asset since NIRS is time saving and cost effective in the long run.

Buttery flavour

One of the negative characters of MG Kefir was that it lacked the buttery flavour imparted by diacetyl.

According to literature, an inadequate ratio between diacetyl and acetaldehyde is usually responsible for this flavour defect. Thus, it was argued that by balancing the ratio of diacetyl to acetaldehyde through production of more diacetyl, it might lead to the stabilisation of the flavour of MG Kefir (Chapter 6).

It is well known that flavour improvement of Kefir can be done by using synthetic flavours or by addition of a specific culture during the fermentation process. However, no data on the effects of additives like citrate and ascorbate, on the production of diacetyl, which is associated with buttery flavour in Kefir, could be found in the literature and this was thus, investigated. Results showed that it was possible to increase the concentration of diacetyl ($p > 0.05$) in both MG Kefir (Kefir prepared with mass cultured Kefir grains) and LG Kefir (Kefir prepared with laboratory Kefir grains) by adding citrate or ascorbate during the fermentation process. This highlighted that citrate and ascorbate have the potential to improve the production of diacetyl, the compound associated with the buttery flavour of Kefir. This could be particularly of value to manufacturers of commercial Kefir, which are usually found to be less appealing than Traditional Kefir. In addition, “Kefir grain owners”, may also benefit from the findings of this study. Indeed, the flavour of home-made Kefir may vary depending on the temperature and other environmental factors. Thus by using the food additives, when necessary, the organoleptic quality of Kefir may be kept constant.

Another alternative investigated the enhancement of the buttery flavour in MG Kefir by the addition of *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* 318 (*Lc. diacetylactis* 318) in Kefir (Chapter 7) during Kefir fermentation. However, this was not successful in producing higher concentrations of diacetyl probably because the acidity of MG Kefir was not favourable for the organism to initiate the necessary metabolic activity. Also, it could have been possible that diacetyl was converted into acetoin, a flavourless compound, which was not measured in this study. This finding highlighted the fact that microbial enrichment of MG Kefir grains during mass cultivation would probably be a better alternative in achieving sustainable production of diacetyl in MG Kefir.

Fruity flavour

Ethyl acetate is responsible for imparting a fruity flavour to fermented products. At the time of this work, and to our knowledge, the presence of ethyl acetate has only been reported in Kefir at concentrations of 0.02 and 2.77 mg.L⁻¹ (Beshkova *et al.*, 2002; Aghlara *et al.*, 2009) whereas the Kefir produced in this study contained between 0.00 and 0.40 mg.L⁻¹ (Chapters 6 & 7). Very little is known about the impact of ethyl acetate as a metabolite in the flavour of Kefir. The fruity flavour is often unnoticed due to acidity. In the literature, it is only stated that ethyl acetate imparts a fruity flavour to Italian cheeses but on the other hand in presence of excess concentrations ethyl acetate causes off-flavour (Liu *et al.*, 2004). In light of this, it was hypothesised that Kefir could exhibit a perceptible and

acceptable fruity flavour in presence of significant amounts of ethyl acetate. It was, thus, decided to study the environmental conditions under which significant amounts of ethyl acetate could be produced in MG Kefir (Chapter 7).

It was found that by extending the incubation period (6 to 18 h at 22°C), adding the substrates ethanol (0.79% m.v⁻¹) and acetic acid (0.79% m.v⁻¹), and the culture *Candida kefyr* 1283 (10⁵ cfu.mL⁻¹), significantly higher concentrations (p < 0.05) of ethyl acetate (0.61 to 9.22 mg.L⁻¹) were produced in the MG Kefir compared to the control (0.36 mg.L⁻¹). This finding suggested that the lack of ethyl acetate in Kefir primarily originates from the lack of a strong ester forming microorganism. In addition, the presence of appropriate concentrations of the correct substrates seems also to be important.

An informal tasting revealed that MG Kefir samples, containing 6.10 and 9.22 mg.L⁻¹ of ethyl acetate, were still found to be acceptable. In light of this and since no background was available on the long term impact of ethyl acetate on the flavour of Kefir, it was also decided to investigate the impact of ethyl acetate on the organoleptic quality of Kefir, incubated 30 h at 22°C and stored at refrigerated (4°C) and room (25°C) temperatures over 6 days (Chapter 7). The shelf-life study showed that by day 6 at 4°C, both MG Kefir and LG Kefir contained up to 40 mg.L⁻¹ of ethyl acetate (and traces of diacetyl) and did not exhibit any flavour defects. This was not the case for the samples stored at 25°C. Indeed, by day 2 at 25°C, the Kefir was already organoleptically unacceptable mainly because of over-acidification.

This work has broadened the knowledge on Kefir's flavour by providing additional information. Indeed, although esters are potent flavours below 5 mg.L⁻¹, it is now known that up to 40 mg.L⁻¹ of ethyl acetate in Kefir will not negatively affect the flavour of Kefir. For comparison, in beers, 8 to 32 mg.L⁻¹ of ethyl acetate are acceptable for quality flavour. It is also known that absence of diacetyl in Kefir does not imply 'bad quality' (Chapter 7).

Furthermore, this study has provided data on the flavour profiles of Kefir prepared with different types of Kefir grains. However, throughout this study, it was observed that the metabolic profiles varied. For example, Traditional Kefir would generally exhibit a buttery flavour. However, it could at times also exhibit a fruity flavour. Thus, to have a better understanding of Kefir grains metabolism and flavour variation, simultaneous study of the metabolic profile of Kefir and the microbial composition of Kefir and Kefir grains over a year is recommended.

Microbial enrichment of MG and sensory evaluation

In the two previous headings, indirect ways of flavour improvement of MG Kefir were 'explored' by using food additives or by inoculation with selected microorganisms and/or substrates into Kefir. Although these indirect flavouring methods led to some improvements in the flavour of the final products, they would only be of value for the dairy industry and to Kefir grain owners. However, it

would be cheaper in the long run, for consumers to acquire microbially stable (MG) Kefir grains.

Thus, it was hypothesised that stabilisation of MG Kefir could be achieved through microbial enrichment with flavour forming microorganisms such as *Lc. diacetylactis* 318 and *C. kefir* 1283. In doing so, it was expected that the products obtained would exhibit a perceptible stronger buttery or fruity flavour and be as acceptable as Traditional Kefir (Chapter 8). Microbial enrichment took place using the mass cultivation method of Schoevers & Britz, 2003. The enriched MG grains were used to prepare Candi Kefir and Lacto Kefir obtained from MG enriched with *Lc. diacetylactis* 318 and *C. kefir* 1283, respectively.

A sensory tasting was done, where Traditional Kefir, MG Kefir, Candi Kefir and Lacto Kefir were evaluated. The results obtained showed that regular consumers of Maas generally liked Traditional Kefir compared to consumers of Plain yoghurt, Greek yoghurt and Buttermilk. This is understandable since Maas is a product 'closer' to Kefir compared to yoghurt or buttermilk even though Kefir contains more alcohol than Maas.

Furthermore, three sensory groups (clusters I, II and III) with different liking characteristics were identified during the sensory study (Chapter 8). Cluster I was found to represent 36% of the consumer panel and generally disliked all the variants of the Kefir. This general dislike is likely due to the fact that the Cluster I consumers were not used to acidic products. This was not the case for Cluster II consumers (20%) as shown by the high score for Traditional Kefir (7.5), which was moderately acidic compared to the other variants (Candi Kefir, Lacto Kefir and MG Kefir). In addition, it was particularly interesting to notice that cluster II consumers showed sensitivity to alcohol ($p < 0.05$) and that the liking of the products were aligned with the quantity of ethanol present in the variants of Kefir. As for Cluster III consumers (44%), their liking was directed towards the slightly acidic products ($p < 0.05$). Thus, Candi Kefir and Lacto Kefir were scored high, 7.63 and 7.09, respectively. MG Kefir was given the lowest score by cluster II (4.87). This confirms that regular consumers of Kefir (50% of cluster II) find the flavour of MG Kefir unacceptable as stated in the initial hypothesis at the beginning of this dissertation.

According to Ott *et al.* (2000), the perception of acidity conditions the perception of the other attributes. This was verified in this study since acidity (pH) appeared to be the main driver of liking by the total consumer panel. It is very likely that different results would have been obtained if the Kefir variants had been tasted by consumers accustomed to traditional Maas, Kefir or even Sethemi.

This work clearly highlighted the complexity of the "liking of flavour" within a group of consumers. This study showed that Candi Kefir and Lacto Kefir may appeal to large majority of South African consumers whereas Traditional Kefir, being moderately acidic appeals to Maas consumers and obviously to those who use their own Kefir grains.

Further studies would include the characterisation of the microbial population of MG enriched

with the *Lc. diacetylactis* 318 and *C. kefir* 1283, identifying the sensory attributes of the different Kefir variants using a trained panel and studying the acceptability of Kefir by youngsters (8 to 15 years) are also studies than can be further done.

Concluding remarks

In this study, gas chromatography could not successfully be used to quantify lactic acid in Kefir whereas the calibration models obtained using near infrared reflectance (NIR) spectroscopy were good for screening purposes ($2 < \text{RPD} < 3$). In other words, with the calibration models developed, the NIR probe would signal any deviation from the normal acidity. The use of NIR spectroscopy for quality control would require models to have RPD of 5 or higher. This is achievable by scanning different sources of Kefir samples. However, this would be a challenging task since Kefir is not readily available in South Africa (Loretan *et al.*, 2003).

Furthermore, results obtained from the indirect enhancement of the buttery and fruity flavours in Kefir, were successful and of importance to those involved in Kefir production or those interested in expanding their product ranges. However, prior to industrial application, the amount of food additives or the inoculum microorganisms to be added during the fermentation process should be determined by the type of starter used to make Kefir since the metabolic profiles of Kefir are strongly dependant on the microbial composition of the starter culture.

Direct enrichment of MG Kefir grains, led to the production of good mass cultured Kefir grains since the products (Candi Kefir and Lacto Kefir) prepared using those grains were generally liked by the consumers. The main objective of this study was therefore achieved since Candi Kefir and Lacto Kefir generally obtained higher scores than MG Kefir.

Kefir, a lifetime opportunity, not to be missed

The increase in sugar consumption is a worldwide phenomenon. Acceptance of unflavoured and unsweetened Kefir may pose a problem, especially amongst the younger generation, and thus variants may have to be developed. This work is therefore an invitation to the dairy industry to study the potential of Kefir, as a novel product in the South African dairy market.

This is also an invitation to the South African health authorities to evaluate the feasibility of making Kefir grains available to the public and thus contribute in a long term cost saving way in improving the nutritional status of the needy, the sick and the lactose intolerants as well as ensuring more food security at the household level. Truly, Kefir is a freely given lifetime opportunity not to be missed.

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ADDENDUM

Name of the judge:**Email Address:****INSTRUCTIONS:**

PLEASE CIRCLE THE CORRECT ANSWER

Gender:

Male / Female

Age:

18-23 / 24-29 / 30-34 / 35-40 / 41+

Employment status:

Student / Employed / Unemployed / Retired

Education:

Grade 11 (standard 9) or less / Grade 12 (matric) / Diploma or degree

Income group (Rand):

Monthly: below 1000 / below 2500 / 2501 - 5000 / 5001 -10000 / above 10 000

Mother Tongue:

English / Afrikaans / IsiXhosa / IsiZulu / Sesotho / Sepedi / Setswana / Other

How often do you consume Greek yoghurt ?

Every day / 1 x week / 2 x week / 1 x month / 2- 3 times a year / NEVER

How often do you consume plain Bulgarian yoghurt ?

Every day / 1 x week / 2 x week / 1 x month / 2- 3 times a year / NEVER

How often do you consume Inkomasi/Amasi/Maas ?

Every day / 1 x week / 2 x week / 1 x month / 2- 3 times a year / NEVER

How often do you consume Buttermilk ?

Every day / 1 x week / 2 x week / 1 x month / 2- 3 times a year / NEVER

How often do you consume Cheese ?

Every day / 1 x week / 2 x week / 1 x month / 2- 3 times a year / NEVER

Do you add sugar/ fruits to Greek yoghurt ?

Yes / No

Do you add sugar/ fruits to plain Bulgarian yoghurt ?

Yes / No

Do you add sugar/ fruits to Inkomasi/ Amasi / Maas ?

Yes / No

Do you add sugar/ fruits to Buttermilk?

Yes / No

Are you familiar with Kefir ?

Yes / No

If yes, how do you consume it ?**Addendum 1.** Socio demographic information and dietary patterns questionnaire.

TASTING OF KEFIR, A FERMENTED MILK PRODUCT

Instructions

1- RINSE YOUR MOUTH WITH WATER BEFORE TASTING EACH SAMPLE AND BETWEEN EACH SAMPLE.

2- FOR WASH SAMPLE, FIRSTLY RANK THE AROMA AND FLAVOUR FOR DEGREE OF LIKING, AND THEN TRY TO DESCRIBE THE FLAVOUR & AROMA

	CODE	CODE	CODE	CODE
How do you like the AROMA of these products ?	9	Like extremely	9	Like extremely
	8	Like very much	8	Like very much
	7	Like moderately	7	Like moderately
	6	Like slightly	6	Like slightly
	5	Neither like nor dislike	5	Neither like nor dislike
	4	Dislike slightly	4	Dislike slightly
	3	Dislike moderately	3	Dislike moderately
	2	Dislike very much	2	Dislike very much
	1	Dislike extremely	1	Dislike extremely

	CODE	CODE	CODE	CODE
How do you like the FLAVOUR of these products?	9	Like extremely	9	Like extremely
	8	Like very much	8	Like very much
	7	Like moderately	7	Like moderately
	6	Like slightly	6	Like slightly
	5	Neither like nor dislike	5	Neither like nor dislike
	4	Dislike slightly	4	Dislike slightly
	3	Dislike moderately	3	Dislike moderately
	2	Dislike very much	2	Dislike very much
	1	Dislike extremely	1	Dislike extremely

	CODE	CODE	CODE	CODE
DESCRIBE AROMA & FLAVOUR OF EACH SAMPLE				

Addendum 2. Consumer questionnaire on the degree of liking of aroma and taste.