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Ås, May 2012

Heidi Y. Grønnevik

ABSTRACT

Kefir grains contain bacteria and yeasts embedded in a protein and polysaccharide matrix. They constitute the primary starter culture for kefir, a fermented milk drink with a complex sensory profile containing high amounts of lactic acid, ethanol, CO_2, acetaldehyde and diacetyl. In commercial production of kefir in Norway, seven kefir grain cultures of Russian and Romanian origin are used in combination. These kefir grain cultures are subcultured several times every week, and have been treated similarly, yet separately for more than 50 years. Consequently, they constitute a unique material for investigations of treatment influence on microbiota, and microflora investigations were performed using a polyphasic approach of culture-dependent and –independent microbiological methods.

Enumeration on selective growth media showed that lactococci, lactobacilli and yeasts were all found in similar amounts in all kefir grain cultures, log 7.9±0.1, log 7.7±0.2, and log 7.6±0.1 cfu g^{-1} of kefir grain, respectively. Using culture-independent microbiology methods, it was shown that the dominant microflora in all seven kefir grain cultures were the same, consisting of *Lactobacillus (Lb.) kefiranofaciens*, *Kluyveromyces marxianus* and *Kazachstania* spp. 16S rRNA gene sequencing of 245 bacteria isolates demonstrated in addition the presence of a secondary microflora mainly consisting of lactic acid bacteria, and whose composition varied somewhat between the different kefir grain cultures. Bacterial isolates found to belong to the same species of lactic acid bacteria were strain typed by rep-PCR. The results showed no correlation between rep-PCR phylogroups and kefir grain culture, however, a strong correlation was found between the phylogroups and the growth medium used for isolation.

In the commercial production of large volumes of kefir, direct fermentation with kefir grains is impractical as this would demand a correspondingly large quantity of grains. In addition, it would give a product with a disrupted gel and a bulging carton due to high production of CO_2. A stepwise fermentation process is therefore employed, in which the kefir grain fermentate is used for production of a bulk starter. as starter for further inoculations. Kefir grain fermentate and the final kefir commercial product were analysed for microbial content and composition. In the fresh fermented kefir, lactococci and lactobacilli were both present in levels of log 8 cfu mL^{-1}, whereas yeasts were present in lower amounts of log 3.3 cfu mL^{-1}. After 4 weeks of storage, lactococci and lactobacilli showed significant decrease (P<0.01) by 2 and 3 log units, respectively, and remained at this level also after 8 weeks of storage. Conversely, yeast numbers continuously increased during storage, reaching log 5 cfu mL^{-1} after 8 weeks of refrigerated storage. By analyses of total DNA of the fermentates, *Lactococcus lactis* was found both in kefir and kefir grain fermentates, and in

addition *Lb. rhamnosus*, *Lb. kefiri* and *Leuconostoc* spp. could be demonstrated in kefir. None of the fermentates formed detectable amounts of PCR products when using yeast primers.

To reduce the sequencing load when employing denaturing gradient gel electrophoresis (DGGE) for microbial community investigations of dairy products, an additional approach of identification was proposed. By use of high-resolution melt (HMR) analysis and comparison of DGGE band melting profiles to those of known reference strains, rapid species-level identification of lactic acid bacteria can be achieved.

It was of interest to investigate whether kefir grains could be replaced by a pure culture starter of lactic acid bacteria and yeast isolates therefrom. The products of single- and co-culture inoculums of microbial isolates from kefir grains incubated in milk were analysed with emphasis on aromatic volatile compounds, organic acids and carbohydrates. The inoculum compositions were of proportions corresponding to the amounts earlier determined to be present in kefir grains. The results showed that the metabolite profiles of the kefir and kefir grain fermentates contained significantly more aroma- and flavour contributing components than the single- and co-culture fermentates, underlining the crucial role of kefir grains for the manufacure of authentic kefir.

Assessment of free amino acids content in kefir during regfrigerated storage showed reduction of glutamic acid during storage, and a consequent increase in its decarboxylation product γ-aminobutyric acid (GABA). GABA has earlier been found to have blood-pressure-lowering effect in mild hypertensives when consumed in fermented milk in amounts of 10 mg daily over a 12-week period. After two weeks of storage, the amount of kefir necessary for a 10 mg intake of GABA would be 220 g.

SAMMENDRAG (Norwegian abstract)

Kefirkorn inneholder bakterier og gjær omsluttet av en matriks bestående av proteiner og polysakkarider. Kefirkorn er starterkulturen for produksjonen av kefir, en fermentert melkedrikk med kompleks sensorisk profil og høyt innhold av melkesyre, etanol, CO_2, acetaldehyd og diacetyl. I kommersiell kefirproduksjon i Norge benyttes det en kombinasjon av syv kefirkornkulturer med opprinnelse i Russland og Romania. Disse kulturene podes om flere ganger i uken, og har blitt behandlet likt, men samtidig separat i mer enn 50 år. De utgjør derfor et unikt materiale for studier av behandlingsmåtens innvirkning på mikrofloraen. Undersøkelser av den mikrobielle sammensetningen ble gjort ved hjelp av en flersidet tilnærming ved bruk av både vekstavhengige og –uavhengige mikrobiologimetoder.

Vekstforsøk på selektive vekstmedier viste at laktobasiller, laktokokker og gjær var tilstede i omtrent like mengder, henholdsvis log 7.9±0.1, log 7.7±0.2 og log 7.6±0.1 kde g^{-1} kefirkorn. Ved bruk av vekstuavhengige mikrobiologimetoder ble det vist at den dominerende mikrofloraen var lik i alle syv kefirkornkulturer og bestod av *Lactobacillus (Lb.) kefiranofaciens*, *Kluyveromyces marxianus* og *Kazachstania* spp. Sekvensering av 16S rRNA-genet i 245 bakterieisolater påviste tilstedeværelse også av en sekundærflora, i hovedsak bestående av melkesyrebakterier. Sammensetningen av sekundærfloraen varierte noe mellom kefirkornkulturene. Stammetyping ved hjelp av rep-PCR av bakterieisolater funnet å tilhøre samme art av melkesyrebakterie viste sterk korrelasjon mellom fylogruppene og vekstmediet benyttet under isoleringen, mens ingen korrelasjon ble funnet mellom phylogruppene og kefirkornkulturene.

Direkte fermentering med kefirkorn er upraktisk i kommersiell produksjon av store volum kefir på grunn av den store mengden kefirkorn det ville kreve. I tillegg ville det gi et produkt med ødelagt gel og bulende forpakning på grunn av høy CO_2-produksjon. Det benytters derfor en stegvis fermenteringsprosess, hvor kefirkornfermentatet brukes som brukssyre i videre syrninger. Kefirkornfermentat og det endelige kommersielle kefirproduktet ble analysert med hensyn på mikrobielt innhold og sammensetning. I fersk kefir var både laktokokker og laktobasiller tilstede i mengder på log 8 kde mL^{-1}, mens gjær ble funnet i lavere mengder på log 3.3 kde mL^{-1}. Etter fire ukers lagring minket mengden laktokokker og laktobasiller signifikant (P<0.01) med henholdsvis 2 og 3 log-enheter, og holdt seg på dette nivået også etter 8 ukers lagring. Gjærmengden økte kontinuerlig gjennom lagringsperioden, til log 5 kde mL^{-1} etter åtte ukers kald lagring. Analyser av total-DNA fra fermentatene påviste tilstedeværelse av *Lactococcus lactis* både i kefirkornfermentatene og i kefir, i tillegg ble *Lb. rhamnosus*, *Lb. kefiri* og *Leuconostoc* spp. funnet i kefir. Ingen av fermentatene produserte påviselige mengder PCR-produkt ved bruk av gjærprimere.

For å redusere sekvenseringsbelastningen når denatureringsgradient-gelelektroforese (DGGE) benyttes i undersøkelser av mikrobielle samfunn i meieriprodukter, ble det foreslått en ytterligere mulighet for identifisering. Ved å bruke high-resolution melt (HRM) analyse og sammenligning av smelteprofilen til DGGE-båndene med smelteprofilen til kjente referansestammer, kan rask identifisering av melkesyrebakterier på artsnivå oppnås.

Det var av interesse å undersøke om kefirkorn kan erstattes med en starterkultur bestående av renkulturer av melkesyrebakterier og gjær isolert fra kefirkorn. Singel- og co-kulturer av kefirkornisolater ble derfor podet i melk, og fermenteringsproduktene ble analysert med vekt på flyktige aromakomponenter, organiske syrer og karbohydrater. Mengdene i sammensetningen av kulturene var i samsvar med forholdene tidligere påvist i kefirkorn. Resultatene viste at metabolittprofilene til kefir og kefirkornfermentatene inneholdt betydelig mer av aroma- og smakspåvirkende komponenter enn fermentatene fra singel- og co-kulturene, hvilket understreker den avgjørende rollen kefirkorn har i produksjonen av autentisk kefir.

Undersøkelser av innholdet av frie aminosyrer i kefir under kjølt lagring viste at glutaminsyre ble redusert under lagring, mens dens dekarboksyleringsprodukt, γ-aminosmørsyre (GABA), økte tilsvarende. GABA har tidligere blitt funnet å ha blodtrykkssenkende effekt på pasienter med mild hypertensjon når den blir konsumert i fermentert melk i mengder på 10 mg daglig over en periode på 12 uker. Etter to ukers lagring var mengden kefir tilstrekkelig for å innta 10 mg GABA 220 g.

LIST OF PAPERS

Paper I

Grønnevik, H., Østlie, H. M., Rudi, K. & Narvhus, J. A. A polyphasic comparison of the microbiota in seven kefir grain cultures subjected to similar treatment for 50 years. Submitted to *International Dairy Journal*.

Paper II

Porcellato, D. Grønnevik, H., Rudi, K., Narvhus, J. & Skeie, S. (2012). Rapid lactic acid bacteria identification in dairy products by high-resolution melt analysis of DGGE bands. *Letters in Applied Microbiology* **54 (4)**, 344-351.

Paper III

Grønnevik, H., Østlie, H. M. & Narvhus, J. A. Evaluation of the use of single- and co-cultures of lactic acid bacteria and yeasts isolated from kefir grains as a starter culture for kefir. Submitted to *Dairy Science & Technology*.

Paper IV

Grønnevik, H., Falstad, M. & Narvhus, J. A. (2011). Microbiological and chemical properties of Norwegian kefir during storage. *International Dairy Journal* **21 (9)**, 601-606.

Grønnevik, H., Falstad, M. & Narvhus, J. A. (2012). Corrigendum to "Microbiological and chemical properties of Norwegian kefir during storage" [Int Dairy J 21 (2011) 601–606] *International Dairy Journal* **25 (1)**: 1-2.

1. INTRODUCTION

1.1. Background - the history and origin of kefir

The technology of preserving or processing fresh food by fermentation has been used for thousands of years. The history of fermented milk products goes as far back as to around 10 000 BC, when man started the domestication of milk producing animals like cows, sheep and goats [1]. Historically, milk fermentation was spontaneous and induced by microorganisms present in the milk and the environment to which it was subjected, such as the containers used to keep the milk. Often, these containers would be made of animal hide, leather or earthenware, and used over and over again as the finished product was consumed and replaced by fresh milk. A small portion of the fermented product left behind in the uncleaned fermenting container would pass on a large number of microorganisms to the new fresh milk, and the fermenting process was in this way repeated.

The composition of the indigenous microflora responsible for the spontaneous fermentation is assumed to be influenced by the climatic conditions present. Thermophilic lactic acid fermentations with high temperature optimums, around 40-45 °C, have evolved in geographical areas of warm climate, whereas the colder parts, such as Northern Europe, have favourised dominance of mesophilic fermenting bacteria [2]. Today, the knowledge of food microbiology, hygiene and production technology is extensive, and the manufacture of many fermented milk products has been industrialised and now takes place in large scale under controlled conditions with added starter cultures. Nevertheless, small scale traditional spontaneous milk fermentation is globally of major importance particularly in many rural communities, and some examples are the naturally soured raw milks *amasi* (Zimbabwe), *ergo* (Ethiopia), *sethemi* (South Africa) and *kumis* (Columbia) [3-6].

Kefir is a traditional fermented milk drink with a long history now being made both industrially and in household scale. It originates from the Caucasian mountains, an area between the Black Sea and the Caspian Sea (Fig. 1), and was traditionally made by filling leather bags with cow's or goat's milk. The bags would be exposed to sunlight during the day and taken inside at night, and anyone passing should push it to ensure mixing of the contents. The acidulated and somewhat sparkling finished product would be replaced with fresh milk, and the process repeated [7].

Figure 1. Geographical map of the Caucasian mountains (from *Encyclopædia Britannica Online*, accessed December 29, 2011).

Today, kefir is produced commercially in several countries around the world, such as Australia, Spain, Turkey and Norway [8-10], and some examples of commercially available products are shown in Fig. 2. Household production is also common in many countries, for example Taiwan, Argentina, South Africa and Spain [11-14].

Figure 2. Examples of commercially produced kefir fermented with a) kefir grains: Nourish kefir (Carr Foods, UK) and Kefir (TINE SA, Norway), b) pure cultures: Bio Kefir (OLMA, Czech Republic), Ryazhenka kefir (Lifeway Foods Ltd., IL, USA) and Nancy's Organic kefir (Springfield Creamery, OR, USA), and c) starter not specified on the product packaging: Zott kefir (Poland), Jana kefir (Poland), Kefir Berezinskij syrodelnyj zavod (Belarus), Savushkin kefir (Belarus) and Farmi Keefir (Maag, Estonia). (Images from the producers' web sites, accessed December 30, 2011.)

1.2. The technology of kefir fermentation

The common starting point for all authentical kefir making is kefir grains, which grow and propagate in milk, and whose activity can be maintained for years if kept under appropriate conditions [15]. During growth, kefir grains enlarge in size and will eventually divide as pieces break off. These pieces will continue to grow when added to milk, and so the growth and division can continue. Sharing of cultures is thus easy, and today kefir grains can even be aquired over the internet.

For commercial production of large volumes of kefir, direct fermentation with kefir grains is impractical as this would demand a correspondingly large quantity of grains - a 10,000 liters production would require 500 litres of grains. In addition, the vigorous fermentation gives a product that contains a high level of CO_2 and the liberation of gas disturbs the casein acid gel, causing whey separation. Commercial production of kefir thus often involves several fermentation steps, including the making of a mother culture and a bulk starter [16].

The kefir fermentation flowchart in Fig. 3 describes the procedure used in commercial kefir production in Norway by the dairy company TINE SA. It is a stepwise process, similar to that described in the literature based on production routines in the former USSR, and common for kefir production using kefir grains as starter [17, 18]. The milk used is heat treated to 95 °C for a few minutes. For the mother and bulk cultures, the heat treatment is important to inactivate any bacteriophages present and ensure good starter activity, whereas for the final fermentation, the denaturation of whey proteins helps improve the water-binding capacity of the gel and thus the final product viscocity. The first production step involves making a mother culture by inoculating standardised cow's milk (1.2% fat) with 5% kefir grains and incubating for about 18 hours at 18-20 °C. This step takes place in small containers of about 5 litres. At the end of the fermentation, the kefir grains are retrieved by use of a sieve and used for new inoculations. The grain free mother culture is then inoculated at 1% into a larger amount of milk (3.9% fat) and incubated for about 20 hours at 20-22°C to make the bulk starter. Inoculation of milk (3.9% fat) with 1% bulk starter and use of the same incubation conditions as in the previous step leads to the finished kefir of pH ~4.4. The kefir is then cooled to 10-12°C, filled into 1 litre cartons and stored at 4 °C.

Due to in-house factory challenges with the bulk starter tank, the bulk starter step of the fermentation was omitted during the time kefir was sampled for the analyses reported here. During that time, direct inoculation with 0.2% (v/v) of mother culture was used as starter, incubated under the usual fermentation conditions of 20-22 °C for approximately 20 h to pH 4.5.

Koroleva [7] describes a 2-step process used in the Russian dairy industry where the last fermentation step starts at 20-22 °C for 10-12 hours, before a slow cooling process over 10-12 hours to 8-10 °C acts as a ripening period, to ensure development of the characteristic taste and aroma of kefir.

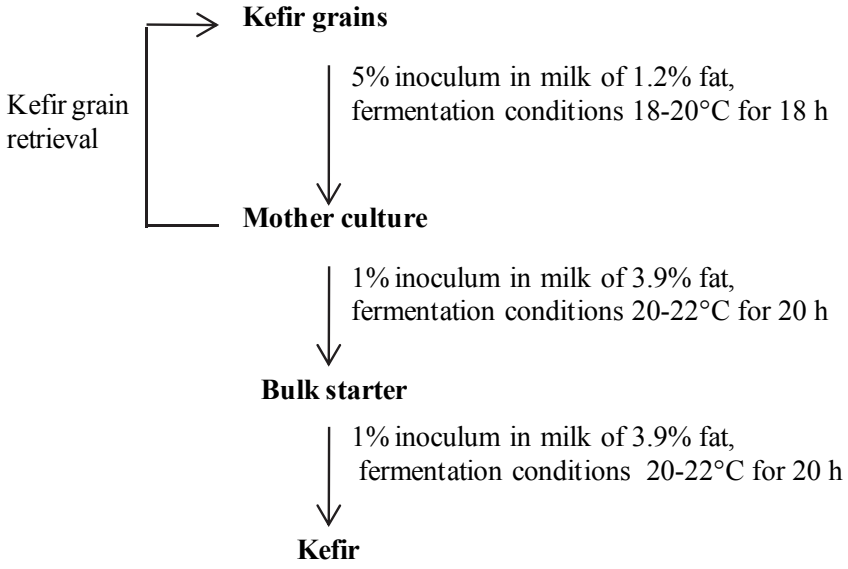

Figure 3. Schematic process diagram of the step-wise industrial kefir production using kefir grains as starter culture.

The kefir grains are recovered from the fermented milk and can in theory be re-used *ad infinitum*. The retrieval is done using sterile sieves in the commercial production, and with as clean sieves as possible in household production. Rinsing of the grains with water prior to reuse is practised by some producers. In a production plant, monitoring of the kefir grain culture activity is not an easy task, as the microbial composition is complex and to a large extent unknown both in content and quantities. A good and immediate indication of grain activity is the location of the kefir grains at the end of fermentation. The grains should float on top of the fermented milk as shown in Fig. 5b, which is a sign of production of CO_2 and therefore indicates good yeast activity. If not, the grains should be replaced by new grains in subsequent fermentations. Other quality tests mainly include analysis of pH and yeast amounts, as well as sensory analysis of the finished kefir drink. The kefir grain biomass increase makes it possible to amass a backup storage of grains. It is, however, time-consuming to propagate frozen grains until sufficient activation of the microflora, particularly the yeast part. The experience of the Norwegian kefir producing dairy TINE Meieriet Oslo is that when kefir grain cultures from the frozen stock are to be employed, satisfactory activity is achieved only after approximately 3 weeks of daily subculturings in fresh milk.

The main difference between household and commercial kefir manufacture is the number of fermentation steps; the product in consumer households is equivalent to a mother culture and

intermediate step in larger scale productions. The technology of home-made kefir has been developed through the experience aquired over a long period of time [7]. Several internet sites are published by people particularly interested in kefir and its properties, dedicated to inform about household kefir production, complete with videos on how to make kefir and with kefir grain purchase possibilities (web page examples accessed December 30, 2011: www.pocketsofthefuture.com/KefirGrains.htm, http://users.sa.chariot.net.au/~dna/Makekefir.html).

1.3. Kefir characteristics

Kefir is a viscous and smooth, slightly carbonated fermented milk drink with a refreshing somewhat sour taste with yeasty notes. Drinking it gives a prickly sensation on the tongue due to CO_2 produced by yeast fermentation. Kefir contains small amounts of alcohol, up to 1% ethanol, although the content seldom exceeds 0.1% in modern commercial kefir production [7].

The composition criteria for kefir both with regards to chemical and microbial content are given in the Codex standard for fermented milks [19] in the FAO/WHO food standards (Table 1).

Table 1. Kefir composision criteria (the FAO/WHO Codex standard for fermented milks [19]).

Milk protein (% w/w) [a]	≥ 2.8
Milk fat (% m/m)	≤ 10
Titrable acidity, expressed as % lactic acid (% m/m)	≥ 0.6
Sum of microorganisms, cfu g^{-1} [b]	$\geq 10^7$
Yeasts, cfu g^{-1}	$\geq 10^4$

[a] Total Kjeldahl nitrogen determined multiplied by 6.38

[b] cfu = colony forming units

Whereas criteria are given for the sum of microorganisms and for yeast content, a more detailed demand for composition is difficult due to geographical and manufacturing differences. Table 2 shows an overview of numbers of some bacterial groups and yeasts reported in the literature for kefirs of different origins, manufactured both industrially and in households, and how these numbers can vary by up to 2 and 3 log units within each microbial group. The milk source used will also influence the properties of the product. The main milk types used in kefir making in Caucasus are cow's and goat's milk [17]. A wide range of other kinds of milks have also been employed, such as ewe, camel and buffalo milk, as well as the vegetarian versions soy, rice, peanut and coconut milk [20-23]. Studies of milk type influence on kefir microflora population development have even suggested that milk type can have greater influence on the sensory profile of the end product than the starter cultures [24].

Table 2. Bacteria and yeast numbers found in kefir. If noting else is stated, the kefir was made by direct inoculation with 1-5% kefir grains.

		log cfu g^{-1} [a]					
Country	Kefir grain source	Lactobacilli	Lactococci	*Leuconostoc* spp.	Yeasts	Acetic acid bacteria	Reference
Canada	Commercial grains				5.5		[25]
Taiwan	3 different household grains	9 - 10	10 - 11.5		5 - 7		[12]
Poland	Polish grains in Polish milk and Scottish milk	8.85, 8.07	9.13, 8.34	< 1	3.64, 2.77		[16]
Bulgaria	Culture collection grains (two-step fermentation; B=mother culture, C=kefir)	B: 6.9, C: 6.3	B: 9.9, C: 9.8		B: 5.3,C: 4.0		[15]
South-Africa	7 different household grains	7.5 - 8.5 (incl *Leuc.* spp)	6 - 9		8.3 - 8.5	3.1 - 5.1	[20] [b]
Turkey	Private grains	8	8.64		6.16		[26] [c]
Spain	Private household grains	8	8.2		5.4 - 5.8	6 - 6.2	[11] [d]
Spain	Freeze-dried starter culture, ''Kefir Culture type C''	7.4	8	6.8	0.5		[27] [d]
Slovenia	Grains from local dairy	6.3	8.8		6.2		[28] [e]

[a] cfu = colony forming units
[b] Data after 18 h of fermentation
[c] Data after 22 h of fermentation
[d] Data after 24 h of fermentation
[e] Fermentation at 19 °C

In Norway, kefir has traditionally been a thirst-quenching drink, particularly during the summer season. This has also been the case in Poland, where the production of kefir is five times higher in June and July compared to the winter months [29]. In the areas of its origin and where it is an important and usual part of the diet, kefir has been credited with various health-promoting properties. In Russia, kefir has traditionally been widely used in the diets of patients hospitalized for various disorders, such as intestinal diseases, atherosclerosis and allergy [7]. Recent research have confirmed several health-beneficial properties of kefir and bacterial isolates therefrom, ranging from reduction in lactose intolerance symptoms to antitumour, antiallergic and anticolitis effects [30-33]. The current knowledge on the functional properties of kefir was recently reviewed by Guzel-Seydim *et al.* [34].

1.4.The starter culture - kefir grains

1.4.1. Origin

The exact origin of kefir grains is unknown. One legend says that the first grains were given to the Orthodox people living in the Caucasian mountains by the Prophet Mohammed, with strict instructions to keep secret the grains and how to use them [17]. Tales about how the first grains were obtained by Russian dairies in the start of the 20th century tell of kidnapping involving a beautiful young woman and a prince, and kefir grains being used as ransom [7], and add to the myths and mystery surrounding the kefir grains and their use.

A more likely explanation is the formation of a biofilm on the inside of the kefir fermentation container. As this continued to grow, pieces would fall off and constitute the beginning of the starter culture that today is known as kefir grains. Motaghi *et al.* [35] showed how this theory is plausible with an experiment where a goat-hide bag was filled with pasteurized milk and intestinal flora from sheep. It was kept at 24-26 °C for 48 h, at which point 75% of the coagulate was replaced with fresh milk. After repetition of the process for 3 months, a spongy polysaccharide layer had formed on the inside of the hide, which was then propagated in milk and could be used as kefir starter.

1.4.2. Microbial composition

For most types of commercial fermented milk, the starter cultures are specific and defined, with a partially or completely known composition consisting of one, a few or several species or strains of microorganisms. Kefir grains are examples of a starter culture that is undefined in terms of both quantitative and qualitative composition. In appearance, they resemble cauliflower florets; white to yellow in colour, resilient in texture, irregular in size from a few millimetres up to 3-4 centimetres, and with a folded and uneven surface (Fig. 5). They consist of a matrix of polysaccharides and coagulated milk proteins, and the typical dry weight chemical composition is 47% (w/w)

polysaccharide and 34% (w/w) protein [21]. Embedded within the matrix is an undefined mix of microorganisms, found to be relatively stable and active if preserved and incubated under appropriate conditions, and existing in a complex symbiotic relationship dominated by a particularly stable population of yeasts and lactobacilli [15, 36]. The microflora is also believed to be self-regulating, meaning that different kefir grain cultures cultivated under the same conditions will have an increasingly similar microflora [37].

Whether all kefirs originate from a single original starter culture has been much pondered. This remains unclear as reports of microbial composition of kefir grains indicate differences both in species of microorganisms present as well as their numbers, depending on place of origin, grain cultivation method and storage conditions [2, 38-40]. The collection of reported results of numbers of yeasts and bacteria, mainly lactic acid bacteria, found in kefir grains of different origin presented in Table 3, demonstrate how the microbial composition and balance of different cultures varies greatly, with reported values spanning over 2-3 log differences. Reported results are dependent on the analytical methods used, and as molecular techniques have become increasingly available over the recent years, it has been possible to extend the knowledge on the microbial compositions.

Kefir grains continue to intrigue researchers world-wide, and both conventional and molecular microbiology tools are now used in the work of unravelling the complete microflora and fermentation mechanisms of kefir grain cultures. The complete description of a kefir grain culture microbiota is a starting point in understanding the symbiotic relationships, the forming of bioactive components and in particular the mechanisms involved in the formation of the grains [41].

Table 4 shows an overview of species of bacteria and yeasts whose presence has been demonstrated in kefir and kefir grains. The table is based on the review by Farnworth [38], and has been extended with data on species not previously noted that have been reported in later publications from 2006 and to date. The Codex standard for fermented milks in the FAO/WHO food standards [19] states that kefir is made from a specific starter culture: “Starter culture prepared from kefir grains, *Lactobacillus kefiri*, species of the genera *Leuconostoc, Lactococcus* and *Acetobacter* growing in a strong specific relationship. Kefir grains contain both lactose fermenting yeasts *(Kluyveromyces marxianu*s) and non-lactose-fermenting yeasts *(Saccharomyces unisporus, Saccharomyces cerevisiae* and *Saccharomyces exiguu*s)”. Research subsequent of the release of these regulations are questioning the importance of acetic acid bacteria, as their presence cannot always be proven [39, 42].

Altogether, more than 30 different species of bacteria and over 20 different species of yeasts have been identified from kefir grains and kefir world-wide, and the species present differ in samples of

different origins. The primary kefir grain microflora consists of lactic acid bacteria (LAB) and yeasts. LAB are Gram positive, non-sporulating, catalase-negative, acid-tolerant, facultative anaerobic organisms, and typical LAB species found in kefir and kefir grains belong to the genera *Lactobacillus (Lb.)*, *Lactococcus (Lc.)* and *Leuconostoc (Leuc.)* (Table 4). Recent reports using a combination of conventional and molecular microbiology methods establish how the bacterial flora of kefir grains is dominated by lactobacilli, whereas the fermented kefir is dominated by lactococci [10, 41-43]. Two of the most often reported species found in kefir grains are *Lb. kefiri* and *Lb. kefiranofaciens* [10, 41, 42, 44-46]. The latter is not specified in the Codex standard, although this has been found to be an important contributor to the production of the exopolysaccharide (EPS) kefiran, which is a major constituent of the kefir grain matrix [47, 48].

Table 3. Bacteria and yeast numbers demonstrated in kefir grains.

Kefir grain origin	log cfu g^{-1} [a]					Reference
	Lactobacilli	Lactococci	*Leuconostoc* spp.	Yeasts	Acetic acid bacteria	
Ireland	6	9	8	6.55	5	[49]
Argentina (households)	7.1 - 8.2	6 - 7.1	-	7.3-8.4	-	[21]
Argentina (households)	7.7 - 8.3 [b]		-	6.6 - 7.4	4.9 - 5.6	[14]
Poland	8.92	8.91	-	6.52	-	[16]
Turkey	9.05	8.87	-	6.55	-	[26]
Slovenia [c]	8.3	10.6	-	8.2	-	[28]
Turkey	6.7 - 8	6.5 - 6.8				[10]
South Africa (households)	6.0 - 7.6	4.8 - 7.8	0 - 7.7	5.2 - 8.6	5.6 - 8.2	[13]

[a] cfu = colony forming units

[b] Total lactobacilli+lactococci

[c] Fermentation temperature 19°C

Table 4. Species of bacteria and yeasts found in kefir grains and kefir, as reviewed by Farnworth in 2005 [38] and updated with species not previously noted found in later publications (2006-2012) [10, 41-46, 50, 51].

Bacteria		**Yeasts**	
Lactobacilli	**Lactococci**	*Kluyveromyces marxianus*	*Torulaspora delbrueckii*
Lactobacillus kefiri	*Lactococcus lactis* subsp. *lactis*	*Saccharomyces* sp.	*Brettanomyces anomalus*
Lactobacillus kefiranofaciens	*Lactococcus lactis* subsp. *cremoris*	*Saccharomyces cerevisiae*	*Issatchenkia occidentalis*
Lactobacillus kefirgranum	*Lactococcus raffinolactis* [10]	*Saccharomyces unisporus* *	*Pichia fermentans*
Lactobacillus parakefiri	**Streptococci**	*Saccharomyces exiguus* *	*Kluyveromyces lactis* [44]
Lactobacillus brevis	*Streptococcus thermophilus*	*Saccharomyces turicensis* *	*Kazachstania aerobia* [44]
Lactobacillus plantarum	**Enterococci**	*Saccharomyces dairensis*	*Lachancea meyersii* [44]
Lactobacillus helveticus	*Enterococcus durans*	*Saccharomyces delbrueckii*	*Saccharomyces lipolytica* [50]
Lactobacillus acidophilus	**Acetic acid bacteria**	*Candida friedrichii*	*Saccharomyces humaticus* [51] *
Lactobacillus delbrueckii	*Acetobacter* sp.	*Candida pseudotropicalis*	
Lactobacillus rhamnosus	*Acetobacter pasteurianus*	*Candida tenuis*	
Lactobacillus casei	*Acetobacter aceti*	*Candida inconspicua*	
Lactobacillus paracasei	*Acetobacter lovaniensis* [44]	*Candida maris*	
Lactobacillus fructivorans	*Acetobacter syzygii* [10, 46]	*Candida lambica*	
Lactobacillus hilgardii	*Gluconobacter japonicus* [46]	*Candida tannotelerans*	
Lactobacillus fermentum	**Other bacteria**	*Candida valida*	
Lactobacillus viridescens	*Bacillus* sp.	*Candida kefyr*	
Lactobacillus parabuchneri [44]	*Bacillus subtilis*	*Candida holmii*	
Lactobacillus satsumensis [46]	*Micrococcus* sp.		
Lactobacillus uvarum [46]	*Escherichia coli*		
Leuconostocs	*Bifidobacterium bifidum* [43]		
Leuconostoc sp.	*Bifidobacterium sp.* [10, 42]		
Leuconostoc mesenteroides	*Pseudomonas sp.* [10, 41, 45]		
	Paenibacillus sp. [10]		

* Current taxonomy [52]:
Kazachstania unispora, Kazachstania exigua, Kazachstania humatica, Kazachstania turicensis

1.4.3. The exopolysaccharide (EPS) kefiran and kefir grain biomass increase

Kefiran is a water-soluble glucogalactan containing approximately equal proportions of glucose and galactose residues (Fig. 4) [53]. La Riviére *et al.* [54] first isolated kefiran from kefir grains in 1967, designated the producer to be *Lactobacillus brevis* and reported that the capsule formation was lost with the first transfer after the isolation of the strains. Kooiman [53] argued how the variety of linkage types may account for the rather poor accessibility of kefiran to enzymatic attack, a property that might be important in the ecology of the kefir grain and the stability of the matrix. In the later years, kefiran has been of interest to many researchers. Production kinetics have been explored, [55-59], and kefiran's potential as a food additive with functional properties has been discussed [60, 61]. Recently, Wang *et al.* [62] sequenced the complete genome of a strain of *Lb. kefiranofaciens* from Tibetan kefir grains, ZW3, which has been found to have the ability to produce high-yield EPS. They could identify a 14.4-kb EPS gene cluster containing 17 EPS-related genes, 5 of which were uniquely present in the ZW3 and regarded as the key enzymes to determine the formation of kefiran.

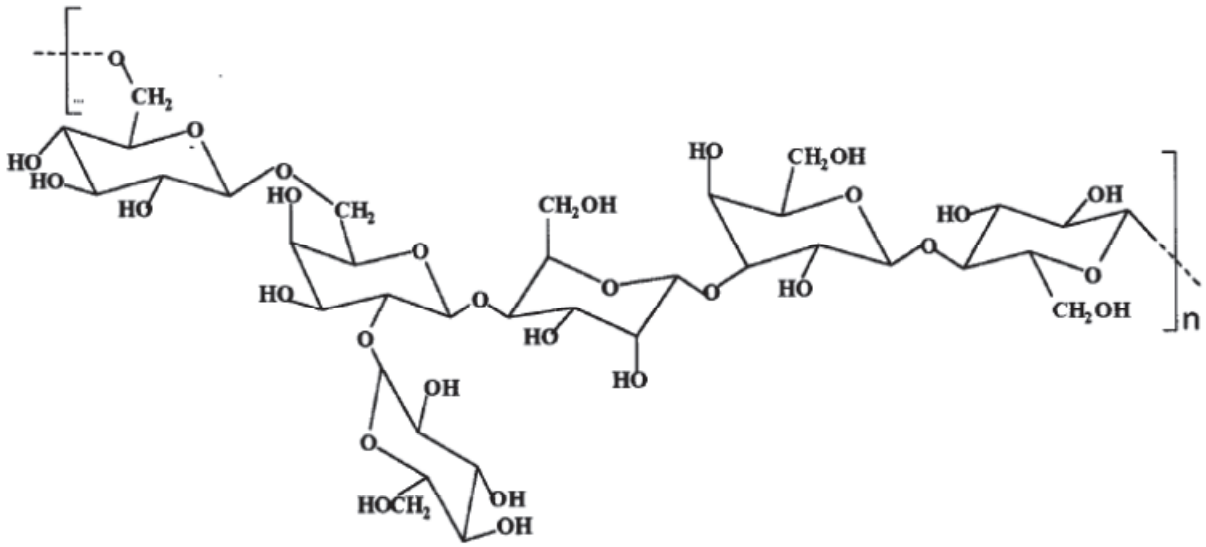

Figure 4. Chemical structure of kefiran, a major constituent of the kefir grain matrix [63].

During the milk fermentation process, the biomass of the grains increases as the microbiota increases. Kefiran is produced and milk protein is precipitated due to the low pH and sticks to the grain surface. Conditions involving agitation at 25 °C and daily replacement of the fermented milk, has been found to give a 5-7% biomass increase per day, making it possible to double the kefir grain weight within about 10 days [29, 54, 64]. Despite the relative simplicity of increasing the grain amounts, direct inoculation with kefir grains in industrial production would demand a large amount of grains and be impractical. Instead, stepwise fermentation as described in section 1.2 is employed.

1.4.4. Balance and stability of kefir grain microflora

With every fermentation step in the kefir making process (kefir grains → mother culture → bulk starter → kefir), the composition of the microflora slightly shifts towards dominance of lactococci in the kefir [38]. The origin of the kefir grains used, as well as the proportion of the initial kefir grain inoculum and the number of fermentation steps influence the microbial composition [15, 65]. Marshall and Cole [36] found that the balance of the microflora in the fermented milk was lost on successive subculturing, particularly shown by a retention of the yeast part of the microflora. Yeast numbers decreased by 1 log per subculture reaching <10 cfu mL^{-1} after 5 subcultures, showing how kefir grains are essential to achieve the right microbial balance in kefir and for obtaining stable product characteristics. Microbial stability also implies a resistance towards contamination that could arise as a consequence of insufficient production hygiene. La Riviére *et al.* [54] assigned this attribute in kefir grains to the selection taking place each time the fermented milk is poured off, carrying with it all suspended microbes not embedded in grains, including contaminants. More recent suggestions indicate that the microbial stability is due to properties like high organic acid concentration and the presence of other antimicrobial substances, such as bacteriosins and bacteriophages that have been demonstrated in strains of *Lb. plantarum* isolated from kefir grains [66-68]. Whereas the microbial stability within the kefir grains is strong, the fermentation conditions have an impact on the microbial balance of the fermentate. Ninane *et al.* [69] found that the heterogenous surroundings of the kefir grain (whey, air, curd) influenced the grain microbial growth of lactobacilli, lactococci and yeasts, particularly when the fermentation was still (no agitation), and that of the microbial groups investigated, the lactococci were most affected.

Whereas the microflora of a kefir grain is in symbiotic equilibrium, the species and quantitative structure of the various groups of microorganisms change significantly along the pathway kefir grains→ bulk starter→ kefir [15]. The final kefir microflora is very different from the starter kefir grains, and consequently kefir cannot be used as a starter for new kefir fermentations [38]. Another approach to omit the use of kefir grains has been to isolate pure strains of the involved microrganisms and combine these to constitute a starter. Many of these isolates will not grow (such as lactose-negative yeasts) or grow poorly with reduced biochemical and metabolic activity in milk, and cannot produce new grains [66]. It has also been shown that kefir produced with kefir grains gave more desirable organoleptic properties compared to kefir produced with a commercial starter or a starter culture of yeast and bacteria in cell concentrations equal to amounts found per g of kefir grain [27, 70].

Under unfavourable conditions, a change in the microbial balance can occur, giving problems with the kefir grain growth or their activity in milk. An effective way for recovery of growth dynamics,

kefir grain size and microbiota balance has been found to be continued daily transfer of the kefir grains into fresh milk. Sometimes a true trial of patience, up to 45 days for frozen grains. This is yet more proof of the extraordinary resilience of the kefir grain environment [71].

1.4.5. Norwegian kefir grain cultures

In Norway, kefir has been industrially produced since the 1930's by TINE SA, Norway's largest producer, distributor and exporter of dairy products, and the production today is about 85,000 litres per week. From the 1950's to present time, the starter culture has been the same, consisting of seven different kefir grain cultures. These cultures have three different origins - five from Russia (denoted RII, RIIx, RIII, RIV and RVI), one from Romania (denoted Rm) and one from the commercial starter culture supplier Chr.Hansen (Hørsholm, Denmark) (denoted CH). Unfortunately, there is a lack in documentation on the details of exact grain origin, and of their microbial composition at the time of acquisition as well as during this long time of production. In the kefir producing dairies, the kefir grain cultures are inoculated several times every week, and they are always treated similarly, yet separately. The argument for the continued use of the seven kefir grain cultures in combination, despite the resource demanding handling this requires, is that omitting some cultures simply gives a less organoleptically complex kefir.

Figure 5. Images of Norwegian kefir grains, displaying the characteristic wrinkled surface (a), floating of grains during milk fermentation (b), and grains after the fermented kefir milk has been sieved off (c and d) (Photo: Heidi Grønnevik).

1.5. Metabolic pathways important in kefir fermentation

Kefir's desirable organoleptic properties result from both lactic acid fermentation by lactic acid bacteria (LAB) and alcohol fermentation by yeasts, and the major fermentation end products are lactic acid, acetic acid, acetaldehyde, acetoin, diacetyl, ethanol and CO_2 [72]. The development of flavor compounds can derive from fermentation of lactose and citrate, from degradation of milk proteins and fat, and from amino acids and free fatty acids metabolism [73, 74]. The complexity of the kefir grain microbiota composition and its physical arrangement in the kefir grain matrix complicates the understanding of the pathways employed in the production of these compounds [18, 75].

1.5.1. Metabolism of carbohydrates and citrate in milk by lactic acid bacteria

LAB degrade carbohydrates present in milk to lactic acid to generate energy and precursor metabolites needed for biomass synthesis, and the metabolic pathways involved are well established [76, 77]. LAB are unable to perform oxidative phosphorylation through respiration as they lack an active electron transport chain, and so energy is produced by substrate-level production of adenosine triphosphate (ATP) [78].

In milk, the main carbohydrate present is lactose, and this is transported into the cell either via a permease system or via the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS). In the former, lactose is subsequently hydrolysed to glucose and galactose moieties by intracellular β-galactosidase, whereas in the latter, glucose and D-galactose 6-phosphate are formed by phospho-β-galactosidase hydrolysation. For catabolism of glucose, two main fermentation pathways are utilized. Homofermentative (homolactic) LAB, such as *Lactococcus* spp., convert one molecule of glucose in the Embden-Meyerhof-Parnas (EMP) pathway to yield two molecules of pyruvate and two molecules of ATP (Fig. 6A). As pyruvate is further reduced to lactate, NAD^+ is regenerated, maintaining the intracellular redox balance and the continued run of the pathway. In heterofermentative (heterolactic) LAB, such as *Leuconostoc* spp. and some species of *Lactobacillus*, glucose is converted using the pentose phosphate pathway (PPP) (Fig. 6B), yielding two intermediates; glyceraldehyde-3-phosphate, which is converted to lactate as in homofermentation, and acetyl phosphate. The latter is converted to acetate as well as reduced to ethanol via acetyl-CoA and acetaldehyde intermediates, regenerating NAD^+. PPP also leads to the production of CO_2, due to decarboxylation as hexoses are converted to pentoses. In theory, PPP produces equimolar amounts of lactate, ethanol, CO_2 and ATP per mol of glucose. From heterofermentation, most of the carbon converted is used to produce secretion products in preference to cell biomass increase. LAB are very adaptable to condition changes, and can modify their metabolism accordingly. Consequently, under certain conditions other products than lactic

acid may be formed through EMP, and in addition, some LAB regarded as homofermentative use PPP when metabolising certain substrates [76].

Galactose transported into the cell by the permease system, or released by lactose degradation, is processed by the enzymes of the Leloir pathway, converting it over several steps before entering EMP at glucose-6-phosphate level (Fig. 6C). Galactose entering the cell through the PEP-PTS is processed via the tagatose-6-phosphate pathway by conversion over several steps before entering EMP at glyceraldehyde-3-phosphate level (Fig. 6D) [79]. Some LAB, such as many strains of *Streptococcus thermophilus*, lack systems for galactose handling and thus excrete it, leading to galactose accumulation in the growth medium [80]. The excretion of galactose is done by use of a metabolic pump, in which lactose is imported and galactose excreted in a one-to-one exchange, functioning as a driving force for the rapid uptake of lactose, allowing fast growth [81].

The main end product of glucose and galactose catabolism, lactate, leads to the lowered pH during LAB fermentation of milk, causing casein micelle aggregation and gel formation, along with the sour taste of the fermented milk. Several of the other end metabolites, such as CO_2 and acetic acid, also have great impact on the organoleptic qualitites of the fermented milk.

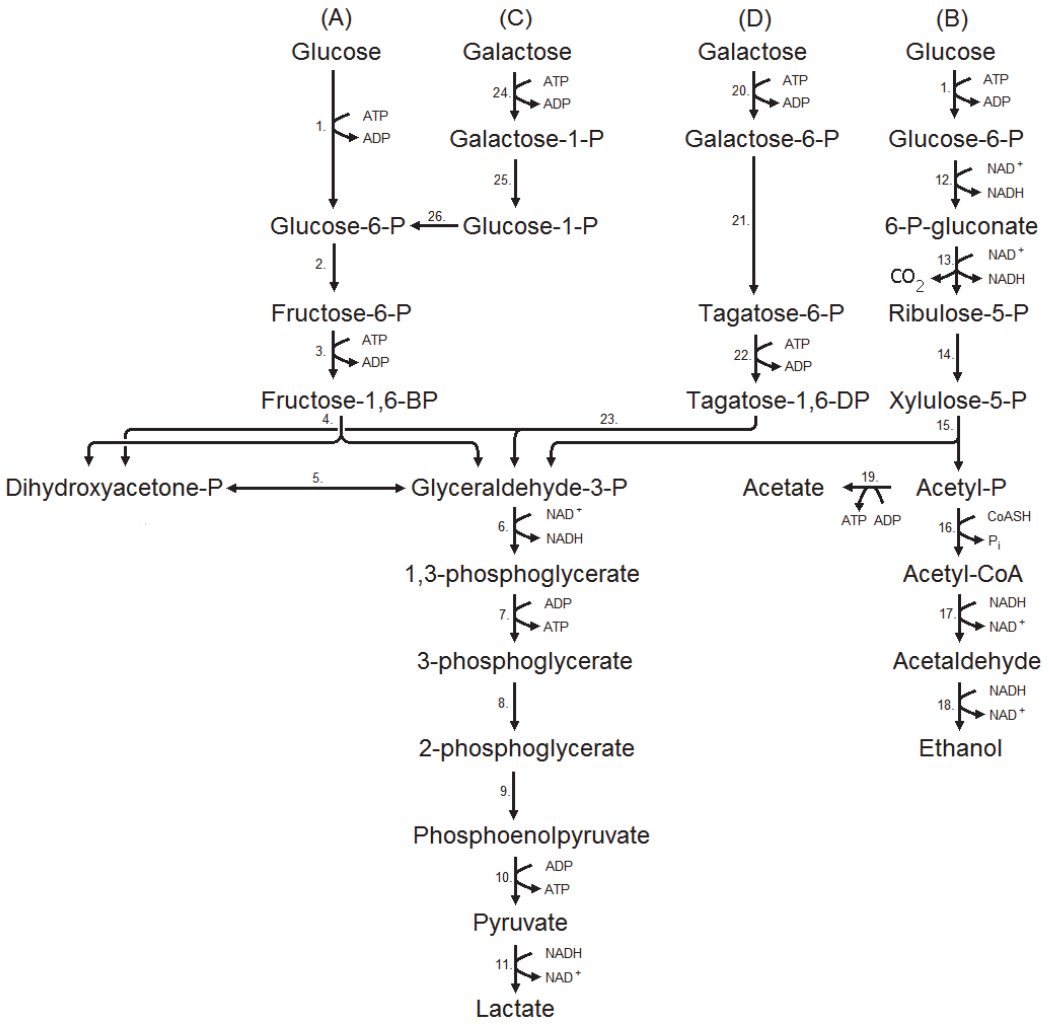

Figure 6. Main fermentation pathways of glucose and galactose in lactic acid bacteria, showing the homofermentative Embden-Meyerhof-Parnas (EMP) pathway (A), the heterofermentative pentose phosphate pathway (PPP) (B), the Leloir pathway (C) and the tagatose-6-phosphate pathway (D). Catalysing enzymes: 1. glucokinase; 2. glucose-phosphate isomerase; 3. phosphofructokinase; 4. fructose- diphosphate aldolase; 5. triosephosphate isomerase; 6. glyceraldehyde-phosphate dehydrogenase; 7. phosphoglycerate kinase; 8. phosphoglycerate mutase; 9. enolase; 10. pyruvate kinase; 11. lactate dehydrogenase; 12. glucose-6-P dehydrogenase; 13. 6-P-gluconate dehydrogenase; 14. ribulose-5-P-3 epimerase; 15. D-xylulose-5-P phosphoketolase; 16. phosphotransacetylase; 17. acetaldehyde dehydrogenase; 18. alcohol dehydrogenase; 19. acetate kinase; 20. 6-P-β-galactosidase; 21. galactose-6-P isomerase; 22. tagatose-6-P kinase; 23. tagatose-1,6-diphosphate aldolase; 24. galactokinase; 25. galactose-1-P-uridylyltransferase; 26. phosphoglucomutase.

In addition to sugars, several LAB species have the capability of metabolising citrate, a natural component in milk present in amounts of about 2 g kg^{-1}. *Leuconostoc* spp. and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* are the most common citrate degrading LAB used in milk fermentation, but also some lactobacilli are able to utilise this compound [82]. Citrate is transported into the cell by membrane-associated permeases, and then converted into acetate and oxaloacetate, before the latter is decarboxylated, generating pyruvate and CO_2. The pyruvate produced from citrate is primarily reduced to D-lactate, however, citrate fermentation by LAB also leads to the production

of 4-carbon (C4) compounds, such as diacetyl, acetoin and butanediol (Fig. 7). The aromatic properties of diacetyl impart the typical aroma of many dairy products, including kefir. In addition to lactate and C4 compounds, other products such as acetate and formate are also formed from pyruvate (Fig. 7), and the balance between the end-products of citrate fermentation depends on the redox state of the cells [77]. For instance, for reduction of pyruvate to lactate, it is essential that co-fermentation with carbohydrates takes place providing the cells with the necessary reducing power, rendered possible by the heterofermentative *Leuconostoc* spp. by producing acetate instead of ethanol during sugar metabolism [82].

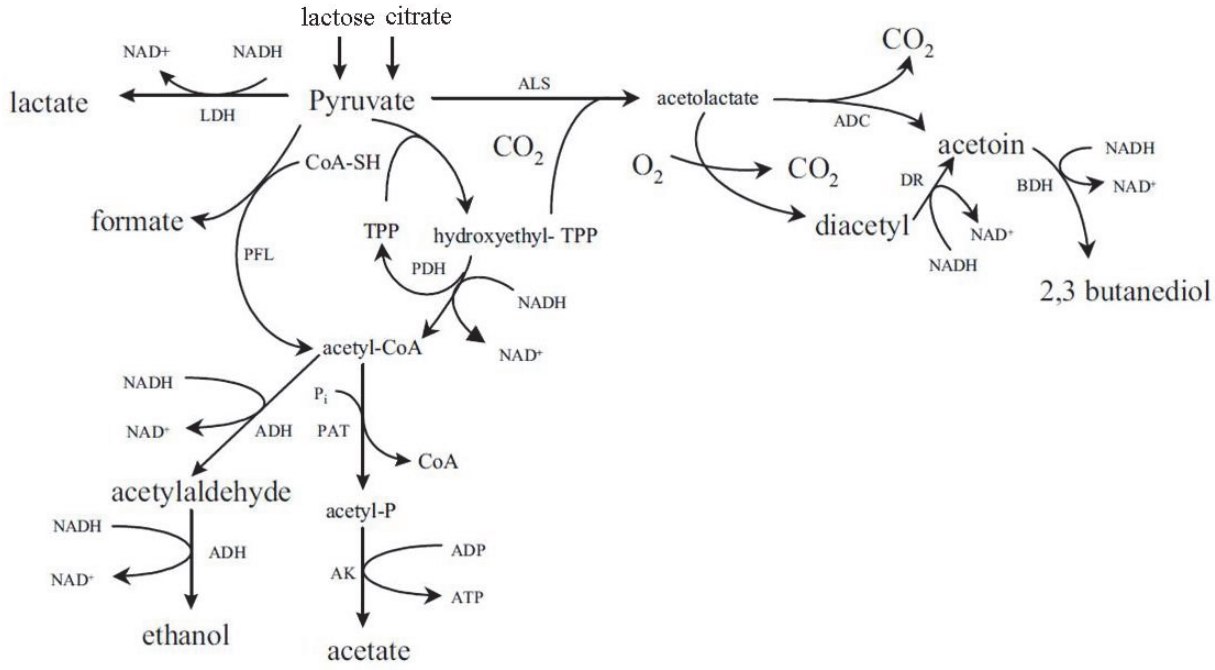

Figure 7. Alternative end-products of pyruvate catabolism – products found extracellularly are given in large letters. LDH, lactate dehydrogenase; PFL, pyruvate formate lyase; ADH, alcohol dehydrogenase; PAT, phosphotransacetyl transferase; AK, acetate kinase; ALS, acetolactate synthase; ADC, acetolactate decarboxylase; DR, diacetyl reductase; BDH, butanediol dehydrogenase; PDH, pyruvate dehydrogenase [78].

1.5.2. Carbohydrate metabolism in milk by yeasts

Yeasts have a large metabolic capacity, and can utilise a wide range of substrates under different environmental conditions [83]. However, most yeasts prefer sugars as their primary source of carbon and energy [84]. The sugar composition of the media and oxygen availability are the two main environmental conditions that have a strong impact on yeast metabolic physiology [85]. The majority of the known yeast species are facultatively fermentative, meaning that they can utilise both oxidative and substrate-level phosphorylation as a source of ATP for biosynthesis, depending on the growth conditions [86]. When milk is the growth medium, the ability to make use of lactose

is important for growth. Microorganisms lacking this property may still have good growth conditions if present together with species able of lactose utilisation. One of the yeast species often found in kefir, *Kluyveromyces (K.) marxianus*, is among the only 2% of yeasts that possess the lactose-hydrolysing enzyme β-galactosidase [87], and has positive, albeit slow galactose fermentation and assimilation [88]. When yeasts are present in co-culture with LAB in milk, another possible carbon and energy source is lactate. *K. marxianus* has the ability to utilise this compound [88].

Most of the energy from carbohydrate degradation by yeasts is harvested through aerobic respiration, whereas under anaerobic conditions, the degradation takes place by fermentation. In alcohol fermentation, two molecules of ATP are produced per molecule of glucose converted into ethanol, and NADH is re-oxidised to NAD^+ [85]. The reducing power generated is used for biosynthesis of fatty acids, amino acids and sugar alcohols, as well as ribose sugars for the synthesis of nucleotides, the precursors of the nucleic acids RNA and DNA [84]. The main end metabolites of the yeast fermentation of milk are ethanol and CO_2 (Fig. 8), with the latter responsible for the prickly sensation on the tongue when drinking kefir, and the reason why kefir has been referred to as the champagne of cultured dairy products [89].

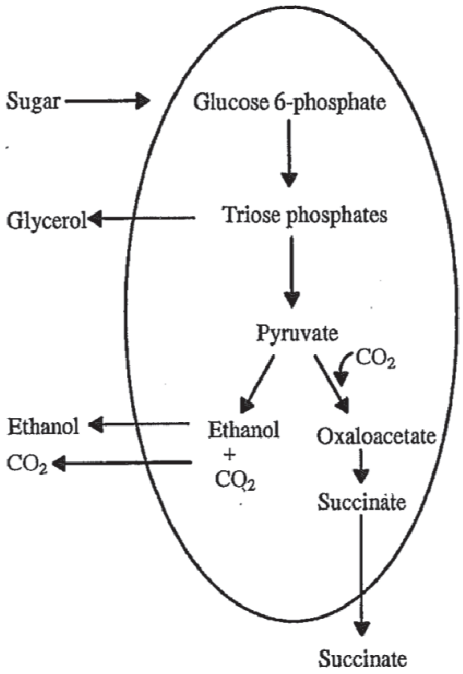

Figure 8. Simplified scheme of carbohydrate catabolism through anaerobic fermentation in yeasts [84].

EMP in yeasts takes place in the cytoplasmic matrix. Depending on the yeast species and the environmental conditions, pyruvate can have 3 different metabolic fates – carboxylation to oxaloacetate to re-plenish the TCA cycle intermediates that are used for biosynthesis; transportation into the mitochondria and subsequent oxidation to acetyl-CoA, which enters the TCA cycle; or decarboxylation by cytoplasmic pyruvate decarboxylase to acetaldehyde (Fig. 9) [85, 86]. The acetaldehyde in turn can be oxidized to acetate or reduced to ethanol. One common way to satisfy the redox balances during growth under anaerobic conditions is the split of glucose metabolism towards glycerol (Fig. 8). Glycerol is produced by reduction of the glycolytic intermediate dihydroxyacetone phosphate to glycerol 3-phosphate followed by a dephosphorylation of glycerol 3-phosphate to glycerol [85].

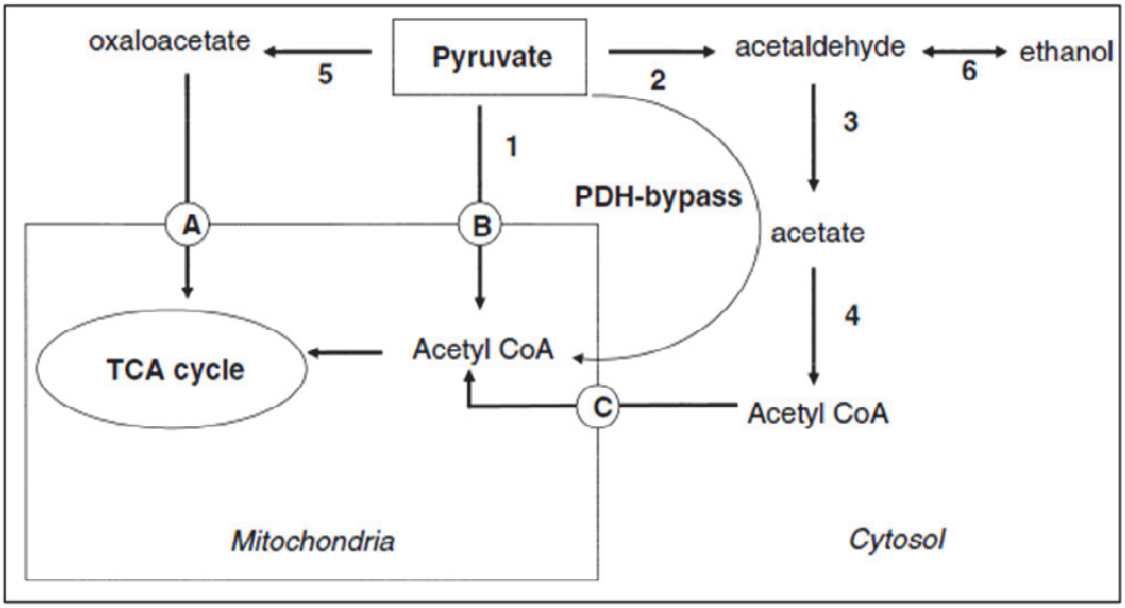

Figure 9. Pyruvate conversion in yeasts. Pyruvate formed in the EMP is converted to acetyl-cofactor A (CoA) and/or oxaloacetate, both intermediates of the tricarboxylic acid cycle, or decarboxylated to acetaldehyde. Enzymes involved: 1) pyruvate dehydrogenase complex, 2) pyruvate decarboxylase, 3) acetaldehyde dehydrogenase, 4) acetyl-CoA synthetase, 5) pyruvate carboxylase and 6) alcohol dehydrogenase. A) mitochondrial oxaloacetate carrier; B) mitochondrial pyruvate carrier and C) carnitine acetyltransferase [85].

1.5.3. Amino acid metabolism

A wide range of volatile compounds that may impact the flavour formation in fermented products can be produced by LAB through catabolism of amino acids (Fig. 10) [77]. The amino acid converting abilities differ between strains, and are also linked to the ability to synthesize these compounds. In particular, the conversion of methionine, the aromatic (tyrosine, phenylalanine) and the branched-chain (leucine, isoleucine, valine) amino acids are crucial [90]. For yeasts, the nitrogen utilisation abilities and degradation pathways vary according to species. Many L-amino acids are known to serve as nitrogen sources for yeasts, and generally, all degradative pathways

lead to ammonia or glutamate or both, which again can be used for amino acid synthesis (Fig. 11) [91]. In LAB, co-operation between strains can result in complementation of biochemical pathways [90], and it is likely that this kind of synergism can take place also between species of bacteria and yeasts in the dense environment of kefir grains.

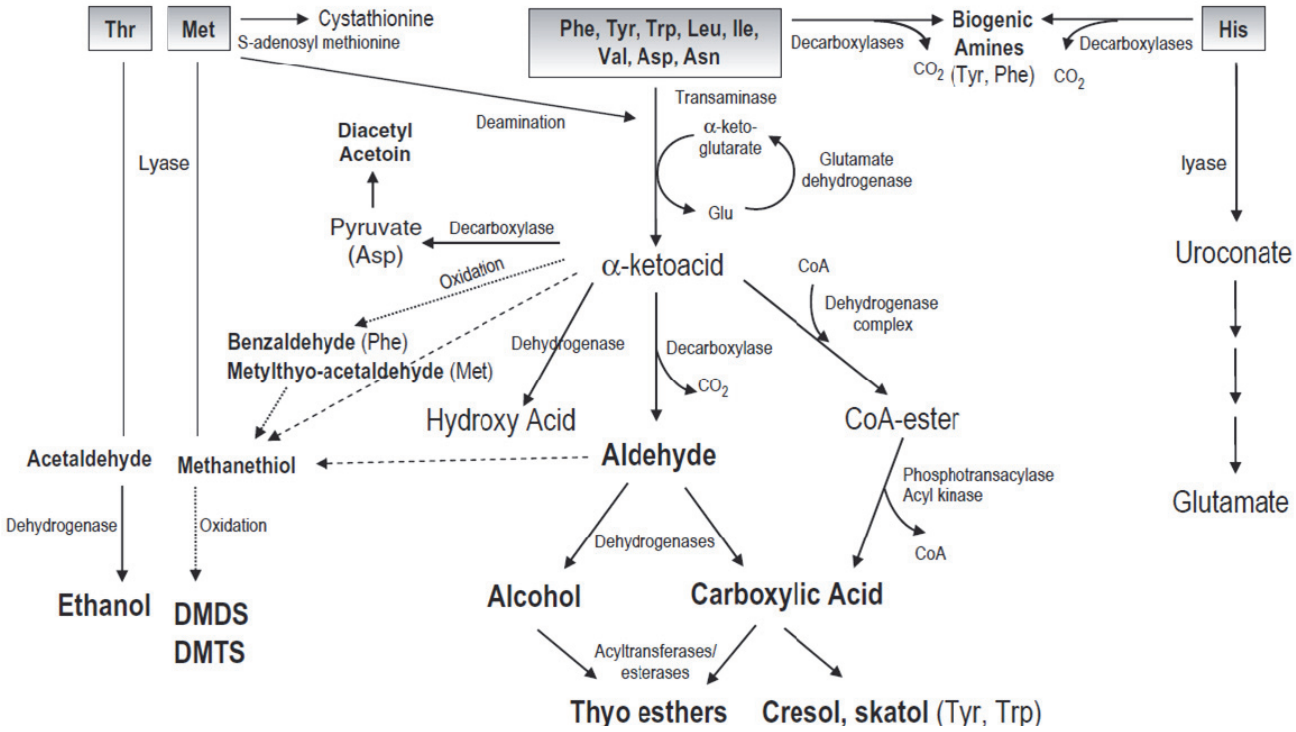

Figure 10. Catabolic pathways of the principal amino acids involved in the production of aroma compounds in LAB. Continuous lines show enzymatic reactions catalyzed by the enzymes indicated. Dotted and broken lines show spontaneous chemical reactions and poorly defined pathways, respectively. The most important compounds formed are shown in bold [77].

Accumulation of free amino acids has been observed in kefir, as well as in milk fermented with co-cultures of kefir LAB isolates [92, 93]. The concentrations of free amino acids analysed may be considered a result of a combination of proteolytic activity, assimilation of peptides and release of amino acids from the cells, and significant proteolysis of α-lactalbumin and κ-, α-,and β-caseins has been observed in milk incubated with kefir grains for 48 h [94]. An interesting amino acid with regards to alleged positive health effects of kefir is γ-aminobutyric acid (GABA), a decarboxylation product from glutamic acid [95]. This amino acid has earlier been found to have blood-pressure-lowering effect in mild hypertensives when amounts of 10 mg in a fermented milk were consumed daily over a 12-week period [96].

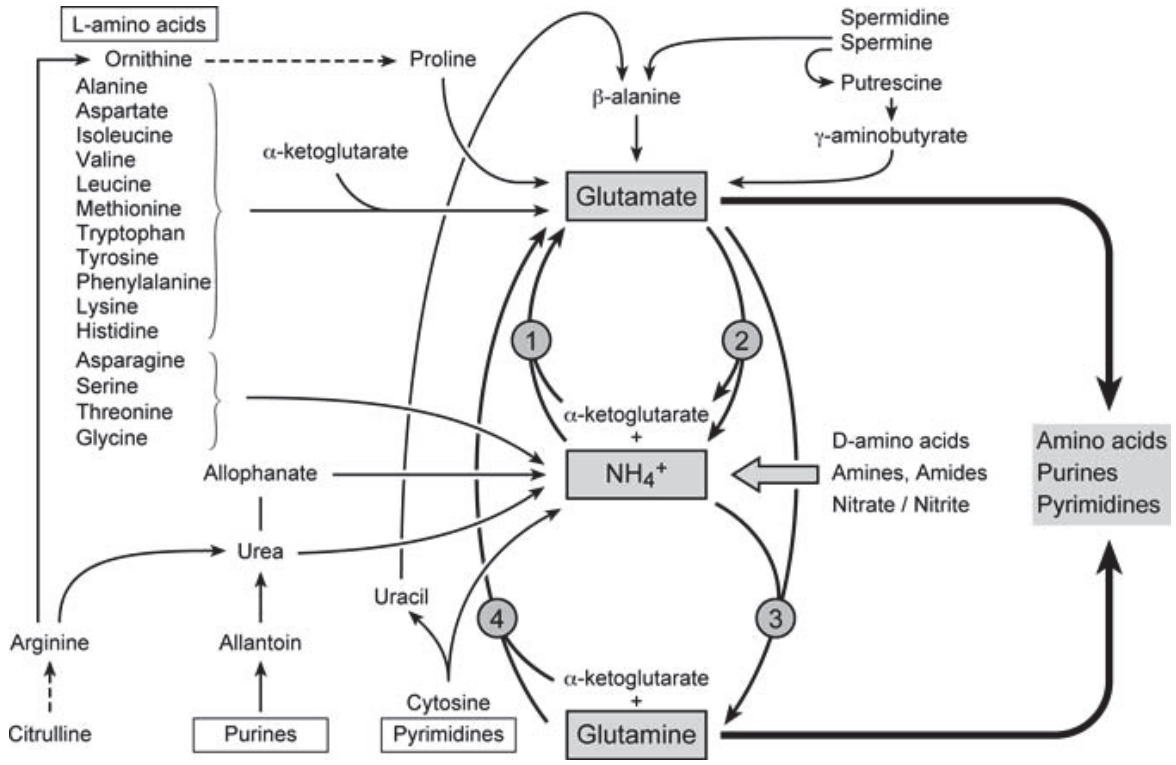

Figure 11. Schematic representation of the main reactions involved in nitrogen utilization in yeasts grown on various nitrogenous compounds [91].

Microbial decarboxylation of amino acids can also lead to formation of biogenic amines, which can cause intoxication in humans when consumed in excessive amounts. Investigations of the biogenic amine content of Turkish kefir determined that tyramine was the prevailing one, however, the concentrations of both individual as well as total biogenic amines were far below the allowed limits [97].

1.5.4. Lipid metabolism

Free fatty acids (FFA) as well as their esters contribute to the flavour development in fermented milks. Conversion of pyruvate obtained from EMP via oxidative decarboxylation to acetyl-CoA can result in released FFA, or further conversion to esters [98]. Critical precursors for ester formation are alcohols, and these are present in high amounts in fermentations where yeasts are present, such as kefir. The lipolytic activity leading to formation of free fatty acids and the final levels of these, their carbon chain length and level of saturation is likely to vary between strains and is affected by the environmental conditions, as well as a subject of yeast-bacteria interactions [99]. Most LAB have weak lipolytic activity due to intracellular lipases and esterases, which are released when cells lyse and are of particular importance in the maturation and flavour formation in cheese [100]. Lipolytic activity has been demonstrated in *K. marxianus* isolated from Ugandan self-fermented milk [101], and this yeast is also often found in kefir [38]. Ethyl acetate is one of the main volatile

compounds produced by kefir yeasts, and *K. marxianus* has in addition been found to be able to produce several 2-phenylethyl esters [102].

1.5.5. Possible interactions of the kefir grain microbiota inhabitants

In a microbial community like kefir grains, where both yeasts and LAB are present, several kinds of interrelationships can occur that influence the growth, metabolism and the characteristics of the end-product. The interrelationships can be LAB-LAB, LAB-yeast or yeast-yeast, and of several different natures – e.g. mutualism/synergism, commensalism or predation. The interactions may appear simultaneously or sequentially, and could involve competition for the same substrate or metabolite production promoting synergistic growth [103].

Yeasts produce compounds essential for LAB growth, such as vitamins, amino acids and purines. In addition, some yeasts utilise lactic acid and other organic acids, consequently increasing the pH and allowing continued growth of LAB [93, 103]. It has already been mentioned (section 1.5.2) how *Kaz. humatica*, unable to utilise lactose or lactic acid, can still have good growth conditions in co-culture with LAB or the lactose-hydrolysing *K. marxianus* providing galactose. Considering the complexity of the kefir microbiota, all the interactions previously mentioned may take place, maintaining an adequate balance among the kefir grain microorganisms [66]. Cell numbers of *Lb. kefiranofaciens* have been found to increase when co-cultured with *Saccharomyces (S.) cerevisiae*, as opposed to when grown in single-culture in milk [56, 104], and the same has been found for *Lb. kefiri* co-cultured with *Candida kefyr* [105, 106]. It has been suggested that direct physical contact between the LAB and yeasts cells is important for this improvement in growth numbers [107, 108], and investigations of the distribution of microorganisms in kefir grains by scanning electron microscopy showed bacilli cells growing in close association with lemon-shaped yeasts cells, and strong surface interaction [44, 50]. In this study, it was shown how milk fermented with single and co-cultures of kefir grain isolates of LAB and yeasts gave different and less complex profiles of aroma and flavour compounds compared to kefir grain fermentates (similar inoculation rates and incubation conditions), indicating a simpler metabolism of less interrelation (**paper III**). Of the two yeasts present in the kefir grain cultures investigated here (**paper I**), *K. marxianus* posesses the lactose-hydrolysing enzyme β-galactosidase and thus utilises lactose for energy production [87]. *Kaz. humatica* lacks this enzyme, but metabolises the galactose moiety of lactose excreted by *K. marxianus* as well as by LAB, demonstrated as extensive accumulation of galactose was shown in co-cultures containing only LAB (**paper III**).

1.6. Microbiological methods employed; descriptions and considerations

In this study, a polyphasic approach was used to identify and compare the microbiota of seven different kefir grain cultures, as well as their fermentates and the end product kefir. Both culture-dependent conventional microbiology techniques as well as culture-independent molecular methods were employed, and some initial considerations were done to identify their possible limitations and biases. Whereas the culture-dependent methods might be biased by selective culture media making some of the strains unculturable, or by failure to pick colonies from all strains from the agar plates, culture-independent methods such as PCR-DGGE eliminates the necessity for strain isolation, thereby negating the potential biases inherent to microbial enrichment [109]. Still, culture-independent methods also have inherent biases, introduced by e.g. selective extraction of nucleic acids, selective amplification of the target gene(s), and comigration of bands of different sequences in a DGGE analysis [110].

A schematic outline of the analytical work in this thesis, the methods employed and the papers in which the results are reported, is shown in Fig. 12. For the examination of microbial development during storage of kefir, as well as isolation of bacteria and yeasts strains, conventional methods of plate spreads and phenotypic characterisation were used (**papers I and IV**). For isolate identification, DNA extraction followed by PCR amplification with universal primers and Sanger sequencing was used (**papers I and II**).

For investigations on total sample DNA from kefir grains, mother culture, bulk starter and kefir, DNA extraction was done using a combination of enzymatic cell lysis combined with mechanical disruption by bead beating and column purification (**papers I and II**). PCR amplification was done using universal bacterial and yeast primers, and community microflora analysis was done using DGGE (denaturing gradient gel electrophoresis) (**papers I and II**), HRM (high resolution melt) analysis (**paper II**) and direct sequencing (**paper I**). For metabolite investigations in fermentates, head space gas chromatography (HSGC) was used to analyse the amounts of aromatic volatiles (**papers III and IV**), whereas high performance liquid chromatography (HPLC) was used to analyse the amounts of free amino acids (**paper IV**), carbohydrates and organic acids (**papers III and IV**).

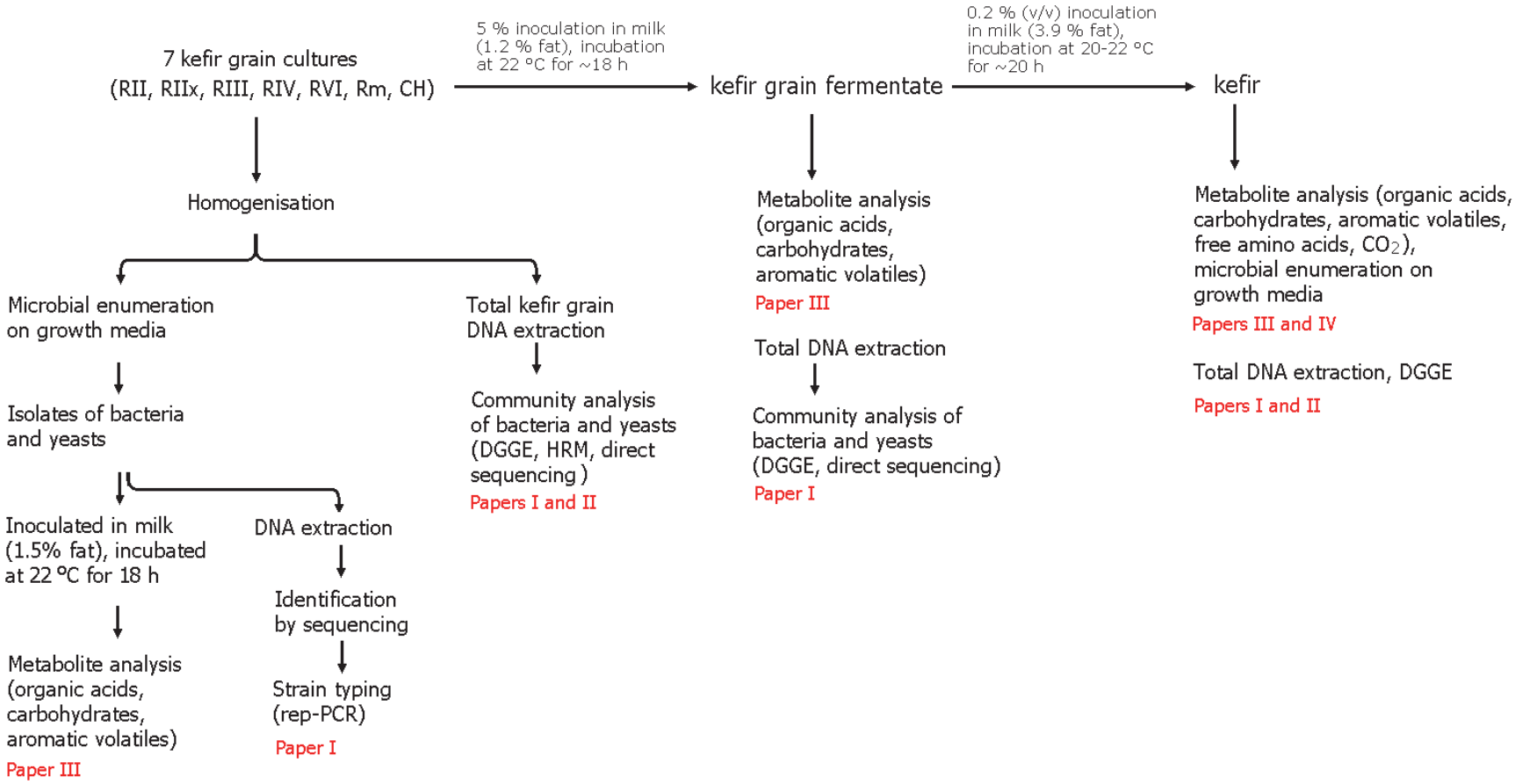

Figure 12. Schematic outline of the analytical work in the thesis, the methods employed and the papers in which the results are reported.

1.6.1. Culture-dependent conventional methods

Growth media cultivation and plate count results indicate how many of the cells that can replicate under the conditions provided for growth, and microbial cells might exist in several states, in which they are viable, yet will not form colonies on growth media [111]. For stressed cells, plate counts may indicate viability in less than 50% of the true viable population [112]. Several species of LAB are also able to grow on more than just one specific medium, as clearly seen by the sequencing results in this study (**paper I**) and also demonstrated in earlier work on kefir grains [39]. For LAB, inherent similarities within the group have made the development of selective media only partially successful. In most cases the media used for differential counts of LAB must be regarded as elective rather than selective. A further problem is that, in the absence of truly selective media for isolation, the chances of isolating a strain that is present in a considerable minority (say 1%) are extremely small.

As all plating procedures are selective to a greater or lesser extent and therefore may exclude parts of the microbial community, several different enumeration media were used to cover a wider spectrum of metabolic activity and find as much of the microbial diversity in the kefir and kefir grain samples as possible. The media chosen were based to a large extent on previously reported investigations on kefir and kefir grains, as well as own research group experience with LAB cultivation. A range of additives were included in the different nutrient media to prevent yeast growth on bacterial plates and *vice versa*. This approach may still fail to provide sufficient growth conditions for everything that might be present in the incompletely understood microbial community of kefir grains. Also, dominating species or strains might outcompete others present in lower amount, resulting in an analysed microbial balance of different proportions than the original sample environment. In addition, some bacteria can form aggregates, for instance in relation to EPS production. In this way, one colony on a growth plate may not be not representative of only one bacteria as starting point. Due to the complex matrix of kefir grains, it is to be expected that organisms requiring symbiotic interactions are present. These conditions can be difficult to reconstruct in a growth medium, leaving the particular organisms unable to form colonies from isolated cells [113].

1.6.2. Culture-independent molecular methods

An advantage of the culture-independent approach is the ability to detect novel microorganisms, which might not be cultivable using known media, and the ability to recover known microorganisms which are either stressed or have entered a viable but non-cultivable state [114]. The known considerations and limitations begin with the step of extracting the total nucleic acids of the sample. A complex microbial environment as expected in kefir grains is likely to contain cells

that are both easy and more difficult to lyse, and the intension is to secure that the DNA extracted is representative of the microbial diversity and proportions present in the original sample. In addition to species differences, metabolic state and position in the growth curve and cell cycle are probably of importance for lysis efficiency, and the amount of nucleic acid recovered by different isolation methods varies substantially [115]. Previously reported work comparing DNA extraction methods (lysozyme, sonication and CTAB methods) on kefir showed how this step was an important factor affecting the microbial diversity analysis using PCR-single strand conformation polymorphism [116]. The DNA extraction protocol chosen in this study for kefir and kefir grain samples thus contained both enzymatic as well as mechanical lysis, and in addition a CTAB-treatment step to remove EPS was added (**paper I**).

The step of amplification of the DNA target of interest by PCR also involves some possible sources of bias, such as cellular debris inhibiting PCR or unavailable target or primer DNA due to blocking or binding. Milk proteins and Ca^{2+} ions in milk have been reported as inhibitory, and proteases present in the product analysed may lead to polymerase inhibition [115]. Other possible challenges include comparative efficacy of amplification between strains, amplification of dead cell DNA, and in the case of quantification, gene copy numbers and possible differences beween species. Also, unrecognised primer specificity may occur, e.g. related to the GC-content, or the lengths of the primers and amplicons [115]. When working with strain typing such as rep-PCR, one should be aware of the possible poor interlaboratory reproducibility, and the need to standardise PCR and gel running conditions.

Until recently, the sequencing techniques available have been dominated by Sanger sequencing [117], so-called first-generation sequencing, as was used here (**papers I and II**). Recently, and during the time of this PhD project, second-generation sequencing systems have developed and become more available. First introduced in 2005, the systems are based on different platforms and sequencing chemistries, all allowing for high throughput of increasingly longer read lengths of reduced error rate, and all constantly improved. Since they are still quite costly and generate large volumes of data that need skilled handling, the availability is somewhat limited, and these methods were not employed in this study.

For microbial community analysis, DGGE is a well established method that has also earlier been used for kefir grain investigations and has contributed to the demonstration of species not found by cultivation [41, 45, 46, 118]. The principle of DGGE is a separation of amplified PCR fragments in a denaturing gradient gel based on different melting profile of the amplicons [119]. The melting characteristics of each amplicon based on their base composition cause them to migrate differently

through the denaturing gradient in the polyacrylamide gel, resulting in a unique fingerprint of bands for each given sample. For band identification purposes, known pure strains can be used as markers, and their band positions on the gel compared to the bands in the sample fingerprint. However, PCR heteroduplexes may generate more than one band per bacteria species on the gel, and co-migration of amplicons with different sequence, but same melting behaviour can result in one band representing more than one species [119, 120]. Reliable identification of DGGE bands was previously only ensured by excision of the bands from the gel, reamplification and sequencing. During the work with this thesis, a simpler and more economically advantegeous DGGE band identification method was proposed, using high-resolution melt (HRM) analysis to compare the melting profiles of excised bands to those of known strains for identification, and so reducing the sequencing load (**paper II**).

In the case of DGGE and community analyses, detection limits have been indicated, ranging between log 4 and log 8 cfu mL^{-1}, depending on species and the number and concentration of the other members of the microbial community leading to possible competition among templates [121]. Species with a large population size in the mixture might generate larger amounts of template DNA, and therefore have a higher probability of detection [122]. In this study, culture-independent analysis was performed using both DGGE and direct sequencing. In the latter method, mixed sequence spectra are generated based on a sequencing primer in a conserved region of the gene. These mixed sequences represent signatures of the dominant bacteria in the sample, and are used for subsequent downstream multivariate statistical analyses. [123, 124]. In the kefir grain analyses, both methods could only detect a dominating microflora of one LAB and two yeasts, whereas cultivation methods also revealed the presence of a secondary LAB microflora (**paper I**).

In addition to the above-mentioned issues, there could always be spontaneous intramolecular genomic arrangements such as point mutations, insertions and deletions occuring, complicating the comparison of isolates of e.g. different sampling times [115]. It has been said that regarding the flaws of molecular methods providing an incomplete view, the saying ‘in the land of the blind, the one-eyed man is king” might apply [125]. Getting some insight is better than being completely blind, and by combining molecular methods with conventional microbiology, more pieces are added to the puzzle.

Sequences and target positions of the primers employed

In the work of this thesis, so-called universal primers targeting bacteria and eukaryotes were employed, with the intention to unravel the microbial composition of kefir and kefir grains. The bacterial primers mainly targeted conserved regions of the 16S rRNA gene flanking different

variable regions (V1 or V3) (Fig. 13), whereas the eukaryotic primers mainly targeted the D1/D2 domain of the 26S rRNA gene or the internal transcribed spacer region ITS1-5.8S rRNA-ITS2 (Fig. 14). In addition to the primer positions on the targets shown in Fig. 13 and 14, the sequences are listed in Tables 5 and 6 for the bacterial and eukaryote primers, respectively. The primers used for rep-PCR are listed in Table 7.

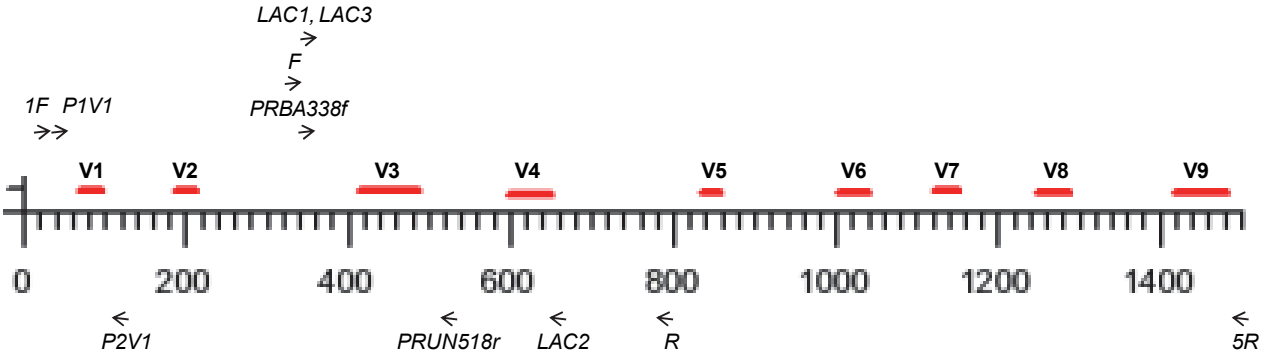

Figure 13. The positions of the universal eubacterial primers used for bacteria amplification of the 16S rRNA gene. Positions are based on *Escerichia coli* 16S, and the location of the hypervariable regions are positioned as described in Gray *et al.* [126].

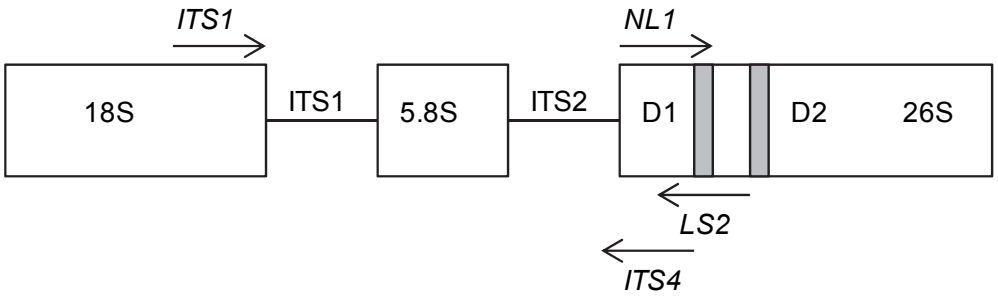

Figure 14. The positions of the universal eukaryot primers used in the project for yeast amplification (figure based on Deak [83]).

Table 5. Sequences of the prokaryote primers used in the project.

Primers	Sequence	Position (bases)[a]	Used in paper
1F	5'GAG TTT GAT CCT GGC TCA G [127]	8-27	I
5R	5'GGT TAC CTT GTT ACG ACT T [127]	1510-1492	
F	5'TCC TAC GGG AGG CAG CAG T [128]	331-349	I
R	5'GGA CTA CCA GGG TAT CTA ATC CTG TT [128]	797-772	
PRBA338f	5'ACT CCT ACG GGA GGC AGC AG [129]	338-357	I
PRUN518r	5'ATT ACC GCG GCT GCT GG [129]	518-534	
P1V1	5'GCG GCG TGC CTA ATA CAT GC [130]	41-60	I
P2V1	5'TTC CCC ACG CGT TAC TCA CC [130]	130-111	
LAC1	5'AGCAGTAGGGAATCTTCCA [131]	352-370	II
LAC2	5'ATTTCACCGCTACACATG [131]	679-662	
LAC3	5'AGCAGTAGGGAATCTTCGG [132]	352-370	
GC clamp [b]	5'CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G		I

[a] The numbering of positions is based on *Escherichia coli* 16S rRNA
[b] The GC clamp was attached to the 5' end of the PRBA338F and P1V1 primers

Table 6. Sequences of the eukaryote primers used in the project.

Primer	Sequence	Amplicon size, bp	Used in paper
ITS1	5'TCC GTA GGT GAA CCT GCG G [133]	Varies with species,	I
ITS4	5'TCC TCC GCT TAT TGA TAT GC [133]	~300-900 [134]	
NL1	5'GCC ATA TCA ATA AGC GGA GGA AAA G [135]	~250	I
LS2	5'ATT CCC AAA CAA CTC GAC TC [135]		
GC clamp [a]	5'CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG		I

[a] The GC clamp was attached to the 5' end of the NL1 primer

Table 7. Primers used for rep-PCR

Primer	Sequence	Used in paper
GTG_5	5'GTG GTG GTG GTG GTG [136]	I
BOXA1R	5'TCC TCC GCT TAT TGA TAT GC [136]	I

2. OBJECTIVES OF THE STUDY

For more than 50 years, seven kefir grain cultures of partly different origin have been treated similarly, yet separately and subcultured nearly daily in commercial kefir production in Norway. The microbiotas of the cultures have been unknown, as have the microbiological and chemical profiles of the products of the step-wise fermentation process, both in the fresh end-product kefir and during storage. To explore these areas and expand the knowledge on kefir and kefir grains, the project was divided into 3 subgoals:

i) To determine and compare the microbiotas of the seven kefir grain cultures of partly different origin in maximal detail by use of a polyphasic approach including culture-dependent and –independent microbiology methods (**papers I and II**)

ii) To investigate whether co-culture inoculums of kefir grain isolates, in proportions corresponding to the amounts present in kefir grains, can be used as starter culture in a single-step fermentation process, thereby obtaining a kefir of similar aroma and flavour profile as with the existing manufacture process (**paper III**)

iii) To monitor the development in chemical and microbial properties in kefir during fermentation and up to 8 weeks of refrigerated storage (**paper IV**)

3. MAIN RESULTS AND DISCUSSION

The microbiota of kefir grains is poorly defined both qualitatively and quantitatively, and reported analyses results vary with kefir grain origin, grain cultivation method and storage conditions [2, 38-40]. In this study, the first aim was to identify the microbiota of seven different kefir grain cultures used for commercial production for more than 60 years in Norway, with separate, yet identical treatment and multiple subculturings every week. A polyphasic approach was employed, using both culture-dependent and -independent methods to survey the microbial community compositions in maximal detail (**papers I and II**). The product of kefir grain fermentation in milk is here denoted kefir grain fermentate. In a metabolite study, the fermentates of the seven kefir grain cultures were analysed and compared, with emphasis on aromatic volatile compounds, organic acids and carbohydrates (**paper III**). These properties were also analysed for kefir, the product of kefir grain fermentate culturing in milk, in addition to the assessment of free amino acids and microbiological properties. The kefir was analysed after fermentation and during 8 weeks of cold storage (**paper IV**). In addition, single- and co-cultures of pure strains of LAB and yeasts isolated from kefir grains (**paper I**) were evaluated as possible starter cultures for kefir, based on comparison of content of aromatic volatile compounds, organic acids and carbohydrates in the fermented milk compared to that of kefir grain fermentates and kefir (**paper III**).

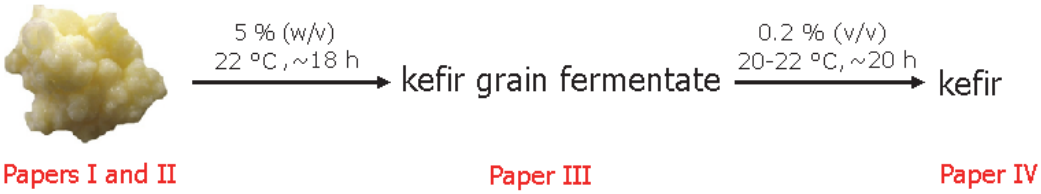

Figure 15. Overview of the materials studied in the different papers of the thesis.

Enumeration of lactic acid bacteria and yeasts in kefir grains

Enumeration on selective growth media showed that lactococci, lactobacilli and yeasts were all found in very similar amounts in the kefir grains, log 7.9±0.1, log 7.7±0.2, and log 7.6±0.1 cfu g^{-1} of kefir grain, respectively. Also, the seven different kefir grain cultures showed very similar results, and the only significant difference found in growth numbers was significantly lower (P<0.05) yeast numbers in RIV and CH compared to RVI (**paper I**). In the literature, reported numbers of the different microbial groups vary quite considerably, due to differences in e.g. kefir grain origin and grain cultivation method [2, 37-40, 92, 137]. In kefir grains from Ireland, Argentina, Poland, Turkey, Slovenia and South Africa numbers of lactobacilli per g range from log 6 to log 9, lactococci range from log 5 to log 10, and reported yeasts numbers range from log 5 to log 8 cfu g^{-1} kefir grain [10, 13, 14, 16, 21, 26, 28, 49].

Enumeration of lactic acid bacteria and yeasts in kefir

For the commercially produced kefir investigated in this study, a two-step fermentation had been employed, in which fermentate of a 5% (w/v) inoculation of kefir grains was used as bulk starter at a 0.2% (v/v) inoculation rate to produce kefir (Fig. 15). In the fresh fermented kefir, lactococci and lactobacilli were both present in levels of log 8 cfu mL^{-1}, in agreement with findings in Spanish, Turkish, South African, Scottish and Polish kefir [11, 16, 20, 26, 27]. Yeasts were present in lower amounts of log 3.3 cfu mL^{-1} (**paper IV**). Yeast amounts in kefir vary, with reported values ranging from log 3 to log 6 cfu mL^{-1} [11, 12, 15, 25, 26, 28, 38], and log 3 cfu mL^{-1} is the lower limit employed by the dairy company manufacturing the investigated kefir. After 4 weeks of storage, lactococci and lactobacilli showed significant decrease ($P<0.01$) by 2 and 3 log units, respectively, and remained at this level also after 8 weeks of storage. Conversely, yeast numbers continuously increased during storage; a significant increase to log 4 cfu mL^{-1} occurred after 3 weeks and a further significant increase to log 5 cfu mL^{-1} occurred after 8 weeks of storage (**paper IV**).

Kefir grain microbiota identification

The kefir grain microflora is believed to be self-regulating [37], and genus-level differences have been shown in cultures of different geographical location [9, 38, 46]. According to the dairy company's historical documentation, the kefir grain cultures investigated in this study are of two different geographical origin, Russia (RII, RIIx, RIII, RIV, RVI, CH) and Romania (Rm). Unfortunately, there is a lack in documentation on the details of exact grain origin, and of their microbial composition at the time of acquisition and during this long time of production. Nevertheless, the seven grain cultures are subcultured several times every week, and have been treated similarly, yet separately for the last 50 years. A polyphasic approach including culture-dependent and -independent methods was employed to survey the microbial community composition of the kefir grain cultures. Culture-independent DNA-based methods using 16S rRNA V3 region primers showed that a dominant microflora consisting of *Lb. kefiranofaciens*, *K. marxianus* and *Kaz.* spp. was present in all the kefir grain cultures (**paper I**). Lactobacilli have been found to be the dominant bacterial species in kefir grains from wide-spread parts of the world, and more specifically, one of the two species *Lb. kefiri* or *Lb. kefiranofaciens* is most often dominant [8, 10, 41, 42, 46]. The latter produces the exopolysaccharide kefiran, which is a major constituent of the kefir grain matrix [53].

In the material included in paper II, cultures RIV and Rm were chosen as they represent two different origins, Russia and Romanina, respectively. The use of more specific primers targeting the genera *Lactococcous, Enterococcus* and *Streptococcus* (LAC3-LAC2), showed presence of *Lc.*

lactis in both RIV and Rm (**paper II**), and this species was also detected in all grains using culture-dependent isolation (Table 8). When using universal 16S V3 rRNA primers and DGGE separation of the amplicons or direct sequencing (**paper I**), *Lc. lactis* was not detected, even though enumeration results showed lactococci and lactobacilli to be present in almost the same amounts, log 7.9±0.1, and log 7.7±0.2 cfu g^{-1} of kefir grain, respectively. One possible source of bias can be the growth media specificity, although here this was found to be good - only lactococci were found on M17, whereas 41 out of 43 MRS isolates were lactobacilli and the two remaining isolates were lactococci. During growth, some bacteria can form aggregates, leading to underreporting of actual numbers during plate counting. This can be related to EPS production, which is found in the kefiran-producing *Lb. kefiranofaciens*. In the molecular analyses, cell lysis and the DNA extraction and purification method could cause possible bias due to differences in bacterial lysis. However, it was observed during the isolations of pure strain DNA that lactococci were easier to lyse than lactobacilli. Furthermore, the primer specificity was equally good for both genera, and the GC-content of the amplicons of *Lc. lactis* and *Lb. kefiranofaciens* using the universal 16S rRNA primers in the direct sequencing [128] was quite similar, 50.4% and 50.0%, respectively. The number of rRNA operons varies between species, and a template with a high number of copies will yield more amplicons than a template with a low number of copies in an equimolar mix, presuming equal primer match [138]. *Lc. lactis* has 6 copies, and whereas no information could be found for *Lb. kefiranofaciens*, most of the lactobacilli listed in the *rrn* database [138] have 4-7 copies. The possible copy number difference would thus be little or in favour of the lactococci, not explaining their absence in the culture-independent analysis using universal bacterial primers. A strain of *Lc. lactis* subsp. *lactis,* INF L2*,* has been found to have high autolytic activity after 12 h of growth, giving accumulation of free DNA which is susceptible to degradation by DNases [139]. If this occurs in kefir grains, it could leave the lactococci outcompeted by the lactobacilli as template in amplifications using universal primers, or amplified in amounts below the detection limits of the methods employed here. However, as the more genera-specific primers did show presence of lactococci, the latter possibility seems unlikely.

In the culture-dependent approach, a total of 245 bacteria isolates and 69 yeast isolates were randomly selected from plates of different growth media, chosen to cover all expected genera based on a wide literature survey. This allowed isolation of species that were present in lower amounts than the dominating microflora, here denoted the secondary microflora. 16S rRNA gene sequencing of the bacterial isolates showed that this secondary microflora mainly consisted of lactic acid bacteria, and that its composition varied somewhat between the different kefir grain cultures (Table 8). The lactic acid bacteria demonstrated, *Lb. kefiri*, *Lb. parakefiri*, *Leuc. mesenteroides*, *Lc. lactis*

subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, are all part of the most frequently reported lactic acid bacteria in kefir grains world-wide [10, 38, 44]. In contrast to the dominant obligate homofermentative *Lb. kefiranofaciens*, several of the species found are producers of aroma and flavour compounds such as diacetyl and acetate, shown to be present in high amounts in the fermentate of kefir grains (**paper III**).

Whereas acetic acid bacteria have earlier been considered to play an important role in maintaining the symbiosis among the kefir grain microflora [37], none could be demonstrated in this study in any of the kefir grains or fermentates, neither by culture-dependent nor -independent methods. Their importance has become increasingly questioned, and it has been suggested that they are nonessential contaminants of kefir [9, 39, 40, 42].

Table 8. Species of bacteria and yeasts demonstrated to be present in the different kefir grains, kefir grain fermentates and kefir using a combination of culture-dependent and -independent metods.

	Kefir grain cultures							**Kefir grain fermentates**							**Kefir**		
	RII	RIIx	RIII	RIV	RVI	Rm	CH	RII	RIIx	RIII	RIV	RVI	Rm	CH	Fresh	3 weeks	8 weeks
Lactobacillus (Lb.) kefiri	+	+	+	+	+	+	+								+	+	
Lb. kefiranofaciens	+	+	+	+	+	+	+										
Lb. parakefiri			+			+											
Lb. rhamnosus															+	+	
Staphylococcus (S.) pasteuri / S. warneri					+												
Lactococcus (Lc.) lactis subsp. *lactis*	+	+	+	+	+	+	+										
Lc. lactis subsp. *cremoris*		+		+	+												
Lc. lactis [1]								+	+	+	+	+	+	+	+	+	+
Leuconostoc (Leuc.) spp.															+	+	
Leuc. mesenteroides subsp. *mesenteroides*	+	+	+		+	+	+										
Kazachstania (Kaz.) spp.	+	+	+	+	+	+	+										
Kaz. humatica	+	+	+	+	+	+	+										
Kluyveromyces marxianus	+	+	+	+	+	+	+										

[1] Primers targeting 16s rRNA V3 region used, do not distinguish between subspecies

Rep-PCR was used to investigate the diversity of the isolates found to belong to the same bacterial species, though they were in some cases isolated from different kefir grains and different growth media. Using a 90% similarity cut-off in the strain typing [140, 141], it was shown that the strains of *Lb. kefiranofaciens* (23 strains), *Lb. kefiri* (28 strains), and *Lc. lactis* (34 strains) formed 11, 8, and 10 clusters, respectively, whereas the 10 strains of *Leu. mesenteroides* grouped into two clusters. In further analysis of the *Lb. kefiri* isolates, no correlation was found between rep-PCR phylogroups and kefir grain culture. However, a strong correlation (P=0.005) was found between the phylogroups and the growth medium used for isolation. All *Lb. kefiri* phylotypes isolated on APM, containing 3% (v/v) ethanol, formed phylogroups separate from the remaining phylotypes

isolated on other media. A further test showed that this ethanol tolerance was not a unique acquired property, as the *Lb. kefiri* strains isolated from media other than APM could grow on APM, albeit to lower numbers and smaller colony sizes than under similar growth conditions on MRS, indicating that niche selection is important for the grain composition. Although the total ethanol content of the kefir drink seldom exceeds 0.1%, it is likely that local areas with considerably higher amounts will occur during the first step of the fermentation, causing a spatial heterogeneity of the habitat, and the ability to tolerate this is vital for microorganisms present.

Palys *et al.* [142] pointed out that closely related groups of bacteria from the same geographical area falling into distinct clusters based on sequence similarity must represent different ecological subpopulations inhabiting own ecological niches. Within the complex and folded kefir grain matrix, niches of different local conditions are likely to occur, e.g. related to localisation of different microbial groups. Several studies of kefir grains using scanning electron microscopy have been performed [26, 143-145]. Although the results are unambiguous with regards to structural positioning of the different microbial groups present, results imply that the microbial diversity is not uniform. Recently, high-throughput sequence-based analysis demonstrated a greater level of diversity associated with the interior kefir grain compared with the exterior [42]. Niche selection is probably important for maintaining the species composition of kefir grains, as indicated by the strain typing results in this study.

Kefir grain fermentate and kefir microbiota

PCR-DGGE using 16S V3 rRNA primers showed the same band pattern for all seven kefir grain fermentates and fresh, 3 week old and 8 week old kefir, and identified by sequencing as *Lc. lactis*. Further identification was not possible as the subspecies *lactis* and *cremoris* are identical in the 16S V3 rRNA region [146]. When using primers specific for the genera *Lactobacillus, Leuconostoc* and *Pediococcus*, strong bands representing *Leuconostoc* spp. and two somewhat weaker bands identified as *Lb. kefiri* and *Lb. rhamnosus* were found in both fresh and 3 week old kefir (**paper II**). *Leuconostoc* spp. and *Lb. kefiri* are often reported as kefir and kefir grain constituents and have been demonstrated in for example Taiwanese, Argentinean, Turkish, Brazilian, Canadian and Portuguese samples [10, 14, 40, 41, 46]. Use of high-throughput parallel sequencing with universal 16S rRNA V4 primers has earlier demonstrated dominance of lactobacilli in the microflora of Irish kefir grains, whereas the kefir fermentate was found to be mainly composed of lactococci [42].

No yeasts could be demonstrated present in the kefir grain fermentates or in the kefir using culture-independent methods, although they were found to be present in amounts of log 3.3 cfu mL^{-1} in fresh kefir increasing to log 5 cfu mL^{-1} after 8 weeks of refrigerated storage. In the PCR reaction,

the yeast part of the total kefir grain fermentate or kefir DNA was attempted amplified with universal eukaryote primers covering the D1/D2 domain of the 26S rRNA gene or the internal transcribed spacer region ITS1-5.8S rRNA-ITS2, however, none of the kefir grain fermentates or the kefir samples formed detectable amounts of PCR products during PCR-DGGE or direct sequencing PCR.

Less diversity was shown in the microbiotas of kefir and kefir grains using PCR-DGGE and direct sequencing than when using an initial enrichment stage on nutritive media and subsequent identification of isolates. It is well known that detection limits apply when employing methods for total community analysis, where so-called universal primers are used to amplify fragments from a total DNA extract. Detection limits are influenced by several factors, such as the food matrix composition and the diversity and concentration of the different members of the microbial community. These conditions affect the efficiency of DNA extraction and the template competition during PCR [121]. Consequently, amplification to detectable amounts of only the dominating species in the sample or in the DNA extract is likely.

Method improvement proposal: high-resolution melt (HRM) analysis for identification of DGGE bands and total community monitoring

During the work with total community DNA from dairy samples, the use of high-resolution melt (HRM) analysis was investigated and suggested as an additional approach for identification of DGGE bands (**paper II**). The method is based on comparison of HRM melting profiles with known reference strains, and was found to reduce the sequencing load when working with cheese and kefir samples and the lab collection of reference strains. The analysis relies on a preliminary construction of a collection of reference species that have to be included in the analysis. Consequently, to maximize the potential of the HRM method, some foreknowledge about the expected microbial composition of the sample analysed is required. Analysis of a new sample type should therefore start with identifying expected species, through literature searches and isolation of pure cultures from the samples, to use as references. For initial validation of the method, as well as for those bands not identified by HRM reference strain comparisons, sequencing is still required. The largest time-saving potential with this method will be when working with samples of somewhat similar character and making use of a constructed reference collection covering the most common species. As in this case for fermented milks and cheeses, it was possible to build an adequate library within a short time, and this is continuously expanded as unknown bands are identified. In the present work (**paper II**), 10 of 13 bands were identified using HRM melting curve comparison and gene scanning analysis, while only three needed sequencing for an unambiguous identification.

Metabolite analyses – carbohydrates and lactic acid production

The main carbohydrate in milk is lactose, abundantly present in amounts of 4.7 – 4.9% in the milk employed in this study and in the commercial kefir production in Norway. One of the two yeasts found present in the kefir grains in this study, *K. marxianus*, is among the only 2% of yeasts that possess the lactose-hydrolysing enzyme β-galactosidase and thus can utilise lactose for energy production [87]. The other yeast present in the kefir grains, *Kaz. humatica*, lacks this enzyme, but is able to metabolise galactose and so will be able to grow in milk when co-cultured with *K. marxianus* or LAB [147]. In the investigations of kefir during storage, no significant change was found in lactose content during weeks 0-4, whereas a significant decrease occurred between weeks 4 and 8 (**paper IV**). The significantly higher yeast numbers in the last half of the storage period compared to the first, and the presence of *K. marxianus* indicate that lactose was utilised by yeast metabolism in weeks 4-8 of storage.

Whereas the glucose moiety of lactose is easily metabolised by both LAB and yeasts, the galactose moiety was found to accumulate both in kefir during weeks 0-4 of storage as well as in the LAB and yeast isolate co-culture combination (hereafter denoted CC) without the *Kaz. humatica* strain (CC5), to around 500 and 1200 mg kg^{-1}, respectively (**papers III and IV**). The *Leuc. mesenteroides* strain showed high galactose accumulation in single culture in milk, 1100 mg kg^{-1}. Galactose accumulation has earlier been found during LAB fermentation in yoghurt [18], and has been suggested to contribute in LAB-yeast interaction in kefir grains, as it could favour the growth of lactose-negative yeasts [37]. This seems to be the case in the kefir grains investigated here, as *Kaz. humatica* utilised the galactose excreted by LAB and the lactose-positive *K. marxianus* when co-cultured, leading to galactose amounts under 100 mg kg^{-1} at the end of the fermentation time. In kefir during storage, the galactose accumulated during weeks 0-4 decreased significantly as yeast numbers increased during weeks 4-8 of storage (**paper IV**).

In kefir and the kefir grain fermentates, the amounts of lactic acid were significantly higher ($P<0.05$) than in all pure strain CCs, ranging between 7.9 and 9.0 g kg^{-1} (**paper III**). Both alone and in combinations, *Lc. lactis* subsp. *lactis* 44 was the main lactate producer of the pure strains, generating more than 6 g kg^{-1}, whereas single-strains of the homofermentative *Lb. kefiranofaciens* 119 and the heterofermentative *Leuc. mesenteroides* 141 produced smaller amounts, 0.82±0.04 g kg^{-1} and 0.55±0.02 g kg^{-1}, respectively. In the CCs without *Lc. lactis* subsp. *lactis* 44, lactic acid amounts were below 2 g kg^{-1}. CC8 was the only CC to contain the lactate-utilising yeast K. marxianus 182 [88], and its fermentate consequently showed a much lower level of lactic acid (3 g kg^{-1}).

Products of citrate degradation and alcohol fermentation

Citrate is a natural component in milk at levels of about 2 g kg^{-1}. In the kefir grain fermentates and single culture inoculation of *Lc. lactis* subsp. *lactis* 44, citrate was reduced by more than 95%, and a 90% reduction was seen during the second fermentation step producing kefir (**papers III and IV)**. In both kefir grain fermentate and kefir, a concurrent increase was found in the concentrations of acetate and the volatile compounds diacetyl and acetoin. The acetate amounts ranged between 680-760 mg kg^{-1} (Table 9) and were well in agreement with reported amounts produced from citrate metabolism by *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* [148]. In the kefir grain isolate study (**paper III**) it became clear that the strain of *Lc. lactis* used belonged to the biovariant *diacetylactis* due to the citrate degradation and the produced amounts of diacetyl, acetoin and acetate during milk fermentation [149]. A subsequent decrease found in amounts of diacetyl and acetoin in kefir after the first week of storage was probably due to further reduction to 2,3-butanediol [82], although this could not be confirmed as this component was not measurable on the HSGC used. In the kefir grain isolate study (**paper III**), *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* 44 was responsible for significantly (P<0.05) higher production of acetoin in single-culture and in CC3 than any in other CC, in amounts close to those seen in kefir and kefir grain fermentates. In all other CCs containing *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* 44, *Leuc. mesenteroides* was also present. *Leuconostoc* can convert acetoin to 2,3 butanediol [150], and this was likely the reason for the lower acetoin levels seen in these CCs.

The ethanol content in modern commercial kefir production seldom exceeds 1000 mg kg^{-1} [7]. In the fresh commercial kefir, the ethanol content was only 29±2 mg kg^{-1}, however, it increased during storage as the yeast numbers increased, reaching 893±308 mg kg^{-1} after 8 weeks. In the kefir grain fermentates, ethanol amounts were varying, from the lowest content shown in CH at 640±127 mg kg^{-1} to the highest in RII at 1603±14 mg kg^{-1}. During kefir grain fermentation, microorganisms are lost from the grains to the milk, called shedding, and this was shown be higher for lactic acid bacteria than for yeasts (**papers I and IV**). In the second fermentation step of kefir production, there are thus fewer yeasts cells present and a correspondingly lower ethanol production will take place. This was also seen for acetaldehyde, a metabolite formed during growth of both yeasts and LAB, that can be reduced to ethanol [76, 86]. In the kefir grain fermentate, acetaldehyde amounts were considerably higher, 13.1±7.6 mg kg^{-1}, than in kefir and CC3 (Table 9). The amounts shown in the kefir grain fermentates was in agreement with the levels shown in Turkish kefir inoculated directly with 5% kefir grains, that reached 11-25 mg kg^{-1} after fermentation and during up to 3 weeks of storage [72, 151]. In the kefir investigated in this study, the acetaldehyde concentration was stable until 4 weeks of storage, then increased significantly to 5.7±0.71 mg kg^{-1} (Table 9)

between 4 and 8 weeks of storage, corresponding to the observed increase in yeast numbers and ethanol concentration during the same period.

Table 9. Amounts of aroma and flavour impacting compounds in kefir grain fermentates, fresh and stored kefir and co-culture combination 3 (CC3) containing *Lb. kefiranofaciens, Kaz.humatica* and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* (paper III). Results are mean values of the seven kefir grain fermentates combined, of five kefir productions and of two analysis replicates of one fermented milk sample for CC3.

	Kefir grain fermentates	**Kefir**			**CC3** [1]
		Fresh	4 weeks	8 weeks	
Lactic acid, g kg^{-1}	8.4 ± 0.4	7.9 ± 0.3	8.2 ± 0.2	8.1 ± 0.1	6.6 ± 0.3
Acetic acid, mg kg^{-1}	761 ± 31	680 ± 9	742 ± 14	741 ± 6	733 ± 2
Acetaldehyde, mg kg^{-1}	13.12 ± 7.56	0.37 ± 0.11	1.65 ± 0.83	5.66 ± 0.71	1.84 ± 0.01
Diacetyl, mg kg^{-1}	1.48 ± 0.58	1.02 ± 0.55	0.14 ± 0.21	0.64 ± 0.59	1.93 ± 0.11
Ethanol, mg kg^{-1}	1183 ± 427	29 ± 2	157 ± 64	893 ± 308	89 ± 2
Ethyl acetate, mg kg^{-1}	2.11 ± 1.41	0.11	0.12 ± 0.11	1.03 ± 0.34	ND
2-methyl butanal, mg kg^{-1}	0.054 ± 0.034	ND*	0.010 ± 0.00	0.010 ± 0.004	ND

*ND = not detected

[1] The combination with chemical properties closest to kefir and kefir grain fermentate (paper III)

When comparing the single- and co-cultures, it was evident that presence of yeasts led to the highest production of ethanol (**paper III**). In yeasts single-cultures, *K. marxianus* 182 produced significantly higher ethanol amounts than *Kaz. humatica* 228, 1870 mg kg^{-1} compared to 80±33 mg kg^{-1}. Of the LAB, the heterofermentative *Leuc. mesenteroides* 141 produced ethanol at about the same level as *Kaz. humatica* 228, 54±0.6 mg kg^{-1}. The highest ethanol amount of 1.7 g kg^{-1} was found in CC8, which contained all these ethanol producing strains.

Ethyl acetate is one of the main volatile compounds produced by kefir yeasts [102]. This component was present in significantly (P<0.05) higher amounts in Rm than in any of the other kefir grain cultures, and only in *K. marxianus* among the pure yeast strains. Consequently, ethyl acetate was present in the highest amount in CC8 of all the CCs, the only CC to contain *K. marxianus*. When all seven kefir grain fermentates were compared with regards to measured metabolites, the difference in ethyl acetate between Rm and the remaining cultures was the only significant difference found. Rm is the only of the kefir grain cultures with a definite different origin, acquired from Romania, whereas the remaining cultures all are believed to be of Russian origin. From these results, the necessity of including all seven fermentates in the present manufacture process could be questioned.

Free amino acids

LAB require a complex mix of nutrients for growth, among them different amino acids. Milk contains only trace amounts of amino acids, so LAB need proteolytic systems to obtain sufficient amounts [18]. In kefir, LAB might be provided with essential growth factors such as amino acids and small peptides resulting from yeast metabolism [87, 152-154]. The concentrations of free amino acids measured in the kefir at each time of analysis may be considered a result of a combination of proteolytic activity, assimilation of peptides and release of amino acids from the cells. Significant proteolysis of α-lactalbumin and κ-, α-,and β-caseins has been observed in milk incubated with kefir grains for 48 h [94].

In this study (**paper IV**) amino acid levels were measured after fermentation of milk with kefir bulk starter was completed, and compared to amino acid levels in the original milk as well as the development in the kefir during storage for up to 8 weeks. It was shown how isoleucine (Ile), alanine (Ala), glycine (Gly), arginine (Arg) and glutamine (Gln) present in the milk decreased during fermentation, whereas the total free amino acids increased and continued increasing throughout the 8 week storage period. Accumulation of free amino acids has been observed also by other authors in kefir, as well as in milk fermented with co-cultures of kefir LAB isolates [92, 93]. One interesting amino acid is γ-aminobutyric acid (GABA), a product of decarboxylation of glutamic acid [95]. This amino acid has earlier been found to have blood-pressure-lowering effect in mild hypertensives when amounts of 10 mg in a fermented milk were consumed daily over a 12-week period [96]. Approximately 1 kg of newly fermented kefir corresponds to 10 mg of GABA, but as GABA levels increased during storage, the amount of kefir necessary for a 10 mg intake of GABA would decrease to a more easily consumable amount, 220 g, after 2 weeks (**paper IV, corrigendum**).

Isoleucine can be metabolised to 2-methyl butanal, an aldehyde with a malty taste and flavour threshold of 0.13 mg kg^{-1} in skim milk [155]. In this study, *K. marxianus* was found to produce high amounts of 2-methyl butanal in milk in single-culture, 0.38 mg kg^{-1}, whereas none was detected in the single-culture LAB fermented milks (**paper III**). In the fermentates made with kefir grains, however, the total amounts of 2-methyl butanal were <0.07 mg kg^{-1} and thus under the flavour threshold. In the next fermentation step, 2-methyl butanal was not detectable (**paper IV**).

Pure cultures as kefir starter culture

Single-strains and different co-culture combinations (CC) of isolated pure strains of LAB and yeasts were used to ferment milk, to investigate whether kefir grains can be replaced as starter culture for

kefir. The single-strain inoculations made it possible to evaluate the contribution of each isolate alone in milk. It has been stated that pure cultures of kefir bacteria and yeasts either do not grow in milk or have a low biochemical activity [66]. This was only partially true for the strains analysed in this study. Whereas *Lb. kefiri* 88 did not produce measurable metabolites and *Leuc. mesenteroides* 141 and *Kaz. humatica* 228 grew poorly in single-culture in milk with little production of lactic acid or reduction of pH, *Lc. lactis* subsp. *lactis* biovar. *diacetylactis 44* and *K. marxianus* 182 produced considerable amounts of metabolites indicating substantial metabolic activity.

Of all the co-culture combinations, CC3 containing *Lb. kefiranofaciens* 119*, Kaz. humatica* 228 and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* 44 grouped closest to the kefir and kefir grain fermentates when all the results of all measured compounds were analysed by principal component analysis (PCA) (Fig. 16). CC8, the only CC containing all pure cultures including *K. marxianus* 182, was positioned separate from all other samples, due to high amounts of formic acid and ethanol, as well as extensive lactose degradation. CC8 also showed significantly (P<0.05) higher reduction of lactose and corresponding higher production of acetic and formic acid compared to kefir and kefir grain fermentates, as well as significantly (P<0.05) lower lactate levels due to utilisation by *K. marxianus* 182. CC7, without *K. marxianus 182*, showed lower yeast metabolism activity, with significantly (P<0.05) lower production of acetaldehyde, ethanol and 2-methyl butanal, and less utilisation of lactose and galactose compared to CC8.

The isolated kefir grain microorganisms co-cultured with inoculums with cell numbers equivalent to those present in kefir grains and incubated under similar conditions, gave different profiles of aroma and flavour compounds compared to kefir grain fermentates or commercial 2-step fermentation kefir (**paper III**). The kefir and kefir grain fermentates were more complex and contained significantly more aroma- and flavour contributing components than the single- and co-culture fermentates. This underlines the impact and importance of the dynamic interactions taking place in the microbiota of the kefir grain matrix on the fermentation end product kefir, and the crucial role of kefir grains for the manufacure of authentic kefir.

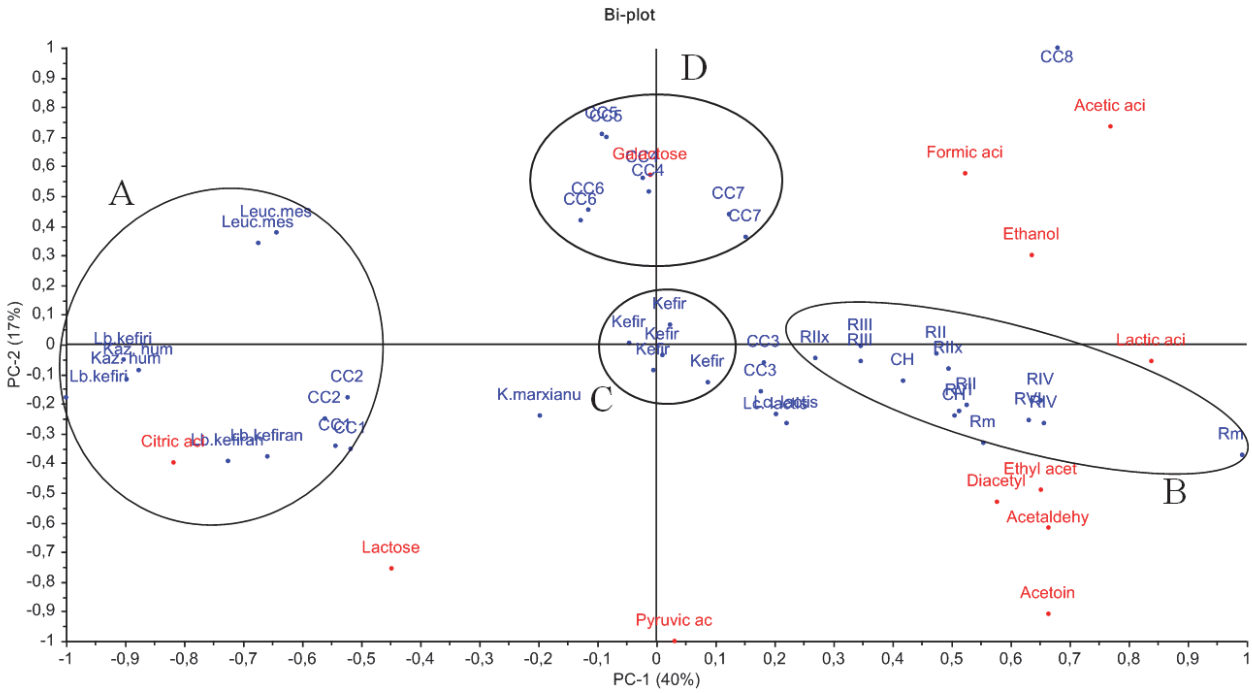

Figure 16. The scores and loadings bi-plot for volatile compounds, organic acids and carbohydrates analysed in milk fermented with kefir grains and single and co-cultures of *Lactobacillus (Lb.) kefiranofaciens* (in all CCs), *Kazachstania humatica* (in all CCs except 5), *Lb. kefiri* (in CC2 and CC5-8), *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (in CC3-5, CC7 and CC8), *Leuconostoc mesenteroides* (in CC4-8) and *Kluyveromyces marxianus* (CC8), and in the untreated milk and the kefir end product.

The complexity of the kefir grain microbiota composition and its physical arrangement in the kefir grain matrix complicates the understanding of the pathways employed in the collective metabolism [18, 75]. Different interrelationships, both of LAB-yeast, LAB-LAB and yeast-yeast character, are likely to occur. These interactions may have significant influence on the activities of the different strains, e.g. by complementary metabolisms, where a compound produced by one organism may be metabolised further by another [153]. It is known that yeasts produce compounds essential for LAB growth, such as vitamins, amino acids and purines [103]. In addition, some yeasts can utilise lactic acid and other organic acids, thus increasing the pH and allowing continued growth of LAB [93, 156]. It is probable that the microorganisms are less exposed to stress conditions such as low pH or suboptimal temperatures when inside the kefir grain matrix [66], and there could also be presence of uncultivable organisms requiring symbiotic interactions that are difficult to reconstruct [66, 113], all adding to the difficulty of replacing grains with pure cultures.

4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This study has described the microbial communities of seven kefir grain cultures used in commercial kefir production in Norway, both by quantification of the microbial groups present and by identification of the microbiotas using culture-dependent and –independent methods. These characteristics were also analysed for the fermentates of the 2-step kefir manufacture process, both when fresh and during 8 weeks of refrigerated storage. The results showed that seven kefir grain cultures of Russian and Romanian origin that have been treated separately, yet identically for more than 50 years, all had the same dominant microflora, whereas the composition of the secondary microflora varied somewhat between the different kefir grain cultures. Metabolite analyses were performed for the kefir grain fermentates and kefir, as well as for milk fermented with single- and co-cultures of kefir grain isolates of LAB and yeasts. The results underlined the crucial role of kefir grains for the manufacure of authentic kefir.

Suggestions for future work to expand the knowledge of kefir and kefir grains include:

- The kefir grain backup storage contains samples of RIV and Rm from 1999 to date, kept at -30 °C. qPCR of total DNA amplified using both universal eubacterial and eukaryote primers could be used to make a relative comparison of bacteria:yeast ratio, to substantiate the assertion of stability and self-regulation of the kefir grain microbiota.
- Working with extracted RNA in preference to DNA would reveal transcriptionally active populations.
- The HRM and cluster analyses of sample melting profiles without performing DGGE may offer interesting industrial applications, as the method could be used to investigate changes in microbial balance. One application could be the comparisons of dairy starter culture profiles or monitoring the microbial composition of products over time. Additionally, as the HRM is based on real-time PCR equipment, a further improvement of the present method could involve combining identification with quantification protocols.
- Further characterisation of the isolated bacterial and yeast strains with regards to potentially probiotic characteristics, with reference to the alleged positive health related aspects of kefir. Epithelial binding properties and adhesion to Caco-2 cells may be investigated, as S-layer proteins have been demonstrated in both *Lb. kefiri* and *Lb. parakefiri* isolated from kefir grains [106]. As the outermost layer in different species of lactobacilli, the S-layer is in direct contact with bacterial environment and thus may be involved in many of their surface properties, and different studies have found lactobacilli S-layer proteins to mediate bacterial aggregation as well as adhesion to epithelial cells and to intestinal components like mucus or extracellular matrix proteins [157].

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6. PAPERS I-IV

Paper I

A polyphasic comparison of the microbiota in seven kefir grain cultures subjected to similar treatment for 50 years

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Abstract

Kefir grains represent a symbiotic relationship between bacteria and yeasts, and are the starter culture for the fermented milk kefir. Culture-independent analyses of the microbiota of seven kefir grain cultures of Russian and Romanian origin that have been treated separately, yet identically for more than 50 years, were conducted. The results showed that the dominant microflora in all seven kefir grain cultures were the same, consisting of *Lactobacillus kefiranofaciens*, *Kluyveromyces marxianus* and *Kazachstania* spp. 16S rRNA gene sequencing of 245 bacteria isolates demonstrated the presence also of a secondary microflora mainly concisting of lactic acid bacteria, and whose composition varied somewhat between the different kefir grain cultures. Rep-PCR strain typing of bacterial isolates found to belong to the same species of lactic acid bacteria showed a strong correlation between the phylogroups and the growth medium used for isolation. These results indicate that niche selection is important for maintaining the species composition of kefir grains.

1. Introduction

Kefir grains are the starter culture for making kefir, a fermented milk drink with long history originating in the Caucasian mountains. Traditionally, kefir was made by filling leather bags with cow's or goat's milk, which would spontaneously ferment. The fermented product would then be replaced with fresh milk, and the procedure repeated (Koroleva, 1988a). A biofilm evolving on the inside of the leather container, continuously growing thicker to the point where pieces (grains) would fall off, is thought to be the origin of kefir grains. There is no scientific literature confirming this, however, one group of researchers have suceeded in making kefir grains using a goat-hide bag filled with pasteurized milk and intestinal flora from sheep (Motaghi et al., 1997). Today, kefir grains constitute the starting point for all modern production of genuine kefir, occuring both in households and industrially in many parts of the world as wide-spread as Norway, Taiwan, Brazil and South-Africa (Grønnevik, Falstad, & Narvhus, 2011; Lin, Chen, & Liu, 1999; Magalhaes, Pereira, Campos, Dragone, & Schwan, 2011; Witthuhn, Schoeman, & Britz, 2004). In appearance, kefir grains resemble cauliflower florets - white to yellow in colour, up to 30 mm in size, with a folded or uneven surface and an elastic texture. Structurally they consist of a protein and polysaccharide matrix, and embedded within is a complex and symbiotic mixture of bacteria and yeasts (Duitschaever, Kemp, & Smith, 1988; Farnworth & Mainville, 2003; Koroleva, 1991).

Kefir grain cultures subjected to microbiota analyses are often denoted geographically by the authors, for example "taiwanese", "turkish" or "portuguese" kefir grains (Güzel-Seydim, Twyffels, Seydim, & Greene, 2005; Kuo & Lin, 1999; Pintado, Da Silva, Fernandes, Malcata, & Hogg, 1996), however, their origin and procurement path previous to the collection source at the time of analysis most often lacks documentation. It is

unclear whether all kefir grains initially originate from the same, or a few, starter cultures. Genus-level investigations of kefir grain microbiotas have shown some differences in cultures of different geographical location (Angulo, Lopez, & Lema, 1993; Farnworth, 2005; Miguel, Cardoso, Lago, & Schwan, 2010). The balance of the microbial composition is affected by the treatment of the cultures, and in early records the kefir grain microflora is described to be self-regulating (Koroleva, 1988b). This implies that kefir grains of the same origin could develop somewhat different microbiotas if treated under different conditions, and that kefir grain cultures of different origin cultivated under the same conditions are likely to have an increasingly similar microflora. However, this is an area within the field of kefir grain research that has been less studied, probably much due to the lack of access to appropriate sample material.

In Norway, kefir has been produced industrially since the 1930's, and since the 1960's, the starter culture has been the same, consisting of seven different kefir grain cultures originating from Russia and Romania. Unfortunately, there is a lack in documentation on the details of exact grain origin, and of their microbial composition at the time of acquisition and during this long time of production. Nevertheless, since the seven grain cultures are subcultured several times every week, and have been treated similarly, yet separately for the last 50 years, they constitute a unique material for investigations of treatment influence on microbiota. Based on prior assertions of the self-regulation and treatment-dependent influence on kefir grain microbiota, the aim of this work was to compare the microbiota of the seven kefir grain cultures today, with genus-level identification of present species of bacteria and yeasts, as well as strain-level analyses of some of the most abundant bacterial species. A polyphasic approach was employed, using

both culture-dependent and -independent methods to survey the microbial community compositions in maximal detail.

Materials and methods

2.1. Kefir grains

Seven different cultures of kefir grains denoted RII, RII_x, RIII, RIV, RVI, Rm and CH, were provided by the Norwegian dairy company TINE Meieriet Oslo (Oslo, Norway). Each kefir grain culture was daily subcultured (5 %) in UHT milk (1.5 % fat) in a sterile 1 L glass jar before incubation at 20° C for 18 h for 6 days. At the end of each incubation the kefir grains were retrieved using a sterile sieve and transferred to a new sterile 1 L glass jar with UHT milk. In the industrial kefir production, the fermentate of all seven kefir grain cultures are mixed and used as a mother culture to make the bulk starter, which in turn is used to produce kefir. Samples of fermentate from each kefir grain culture, of the bulk starter, as well as of freshly made kefir and kefir after 3 and 8 weeks of storage were also provided by the dairy.

2.2. Culture-dependent microbial analysis

2.2.1. Microbial enumeration

For each kefir grain culture, 10 g of kefir grains were mixed with 90 mL of sterile Ringers solution (Merck, Darmstadt, Germany) for 2 min at level 4 in an Omni mixer (Omni International, Waterbury, CT, USA), and suitable dilutions were plated in duplicate on various selective growth media. The dilution media used were sterile Ringers solution (Merck) for bacteria and sterile 0.1 % peptone water (LP0040, Oxoid, Cambridge, UK) for yeasts (Mian, Fleet, & Hocking, 1997). The enumeration results were expressed as log colony forming units per gram (cfu g^{-1}) of kefir grain.

109 Dilutions were plated on M17 broth (Merck) added 12.75 g L^{-1} agar (Merck) for
110 presumptive *Lactococcus (Lc.)* spp., on MRS agar (Merck) for presumptive *Lactobacillus*
111 *(Lb.)* spp., and on yeast extract glucose chloramphenicol agar (YGCA) (Merck) for yeasts.
112 To avoid growth of yeasts on the bacterial plates and vice versa, 200 mg L^{-1}
113 cycloheximide (Calbiochem, La Jolla, CA, USA) was added to M17- and MRS agar, and
114 100 mg L^{-1} oxytetracycline hydrochloride (Calbiochem) was added to YGCA agar (Chen,
115 Wang, & Chen, 2008; Irigoyen et al., 2005). For presumptive *Leuconostoc (Leu.)* spp.
116 LD-agar (Grønnevik et al., 2011) added 200 mg L^{-1} vancomycin hydrochloride (Sigma-
117 Aldrich) was used. For propionic acid bacteria sodium lactate broth (Brede et al., 2004)
118 added 15 g L^{-1} agar (Merck) (SLA) was used, and for acetic acid bacteria *Acetobacter*
119 *peroxydans* medium (APM) (DSMZ, 2004) added 25 mg L^{-1} pimaricin (Sigma-Aldrich)
120 for inhibition of yeasts and 3 mg L^{-1} Penicillin G (Sigma-Aldrich) for inhibition of lactic
121 acid bacteria (DSMZ, 2004; Irigoyen, Arana, Castiella, Torre, & Ibáñez, 2005; Rea et al.,
122 1996; Witthuhn, Schoeman, & Britz, 2005) was used. VRBA (Oxoid) was used to test for
123 coliforms.
124
125 M17 plates were incubated aerobically at 30 ^{o}C for 2 d (mesophilic) or at 45 ^{o}C for 2 d
126 (thermophilic), LD plates at 20 ^{o}C for 4 d, YGCA plates at 25 ^{o}C for 5-7 d, APM plates at
127 25 °C for 2-5 d, VRBA plates at 37 ^{o}C for 1 d, MRS plates in a CO_2 incubator (W.C.
128 Heraeus GmbH, Hanau, Germany) at 10 % CO_2 and 30 ^{o}C for 4 d, and SLA plates in
129 anaerobic jars (AnaeroGen^{TM} 3.5 L) (Oxoid) at 30 °C for 5-10 d.
130
131 *2.2.2. Isolation of strains and DNA extraction from pure cultures*
132 For each of the 7 kefir grain cultures, 10 colonies were randomly picked from each of the
133 growth media M17, MRS and YGCA. From SLA, APM and LD media, 5 colonies were

134 picked from each medium for each kefir grain culture. All isolated colonies were purified
135 by at least three passages on appropriate growth media. A total of 69 yeast isolates and
136 245 bacteria isolates were obtained and all were stored in appropriate broth media
137 containing 15 % (v/v) glycerol at -80 °C.
138
139 For collection of cells for DNA extraction, 9 mL tubes of 48-72 h cultures of isolates in
140 appropriate growth broths were centrifuged in a tabletop centrifuge (Kubota 2010, Tokyo,
141 Japan) at 2000 x *g* for 10 min. The pellet was resuspended in 200 µL spheroplast buffer
142 (10 % sucrose, 2 mg mL^{-1} lysozyme, 0.4 mg mL^{-1} RNase A, 25 mM Tris pH 8.4, 25 mM
143 EDTA pH 8.0) followed by incubation at 37°C for 10 min (30 min for presumed
144 lactobacilli). For further cell disruption, 75 µL 5% SDS (sodium dodecyl sulfate) and 75
145 µL 5 M NaCl was added before incubation at 65°C for 5 min (30 min for presumed
146 lactobacilli). Then, 150 µL of a protein precipitating solution (60 % 5M potassium acetate,
147 11.5 % glacial acetic acid, 28.5 % dH_2O) was added before mixing and incubation on ice
148 for 5 min, followed by centrifugation in a tabletop centrifuge (5415D, Eppendorf AG,
149 Hamburg, Germany) at 13 000 x *g* at 4 °C for 15 min. DNA was then precipitated by
150 adding an identical volume of 2-propanol to the supernatant, followed by incubation for 5
151 min and centrifugation (5415D, Eppendorf) at 13 000 x *g* for 15 min, both at room
152 temperature. The pellet was washed with 70 % ethanol, dried, resuspended in 50 µL 1 x
153 Tris-EDTA and stored at -20 °C.
154
155 *2.2.3. PCR amplification and sequencing*
156 PCR was performed in a total reaction volume of 50 µL containing 5 µL of 10x PCR
157 buffer, 10 mM dNTP mix, 1 U DyNAzyme™ II DNA Polymerase (Finnzymes Oy, Espoo,
158 Finland), 50 pmol of each primer (Invitrogen Ltd, Paisley, UK) and 3 µL of template

159 DNA. The universal primer set used for bacteria was 1F (5'-GAG TTT GAT CCT GGC
160 TCA G-3') and 5R (5'-GGT TAC CTT GTT ACG ACT T-3'), amplifying the 16S rRNA
161 gene. For yeasts, ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC
162 TCC GCT TAT TGA TAT GC-3') was used, amplifying the internal transcribed spacer
163 region ITS1-5.8S rRNA-ITS2 (Fell, Boekhout, Fonseca, Scorzetti, & Statzell-Tallman,
164 2000; Jespersen, Nielsen, Hønholt, & Jakobsen, 2005). Amplification was done in a
165 thermal cycler (PTC-200, MJ Research, Waltham, MA, USA), and the PCR conditions for
166 bacteria were initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for
167 30 s, annealing at 55°C for 30 s and elongation at 72°C for 3 min, and final extension at
168 72°C for 10 min. For yeasts, initial denaturation at 94°C for 3 min was followed by 30
169 cycles of denaturation at 94°C for 2 min, annealing at 60°C for 60 s and elongation at
170 72°C for 2 min 30 s, and final extension at 72°C for 7 min. Purification of the PCR
171 products was done using QIAquick PCR Purification Kit (Qiagen GmbH, Hilden,
172 Germany) according to the manufacturer's instructions.
173
174 The chain termination and labeling reactions for sequencing of bacteria and yeast isolates
175 were carried out using the same primer sets as for the first amplification step and the ABI
176 Prism® BigDye® Terminator v3.1 Cycle Sequencing chemistry (Applied Biosystems,
177 Foster City, CA, USA) according to the manufacturer's instructions. Sequence data were
178 obtained using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems), and
179 compared to the sequences reported in the GenBank using the BLAST algorithm.
180
181 *2.2.4. Rep-PCR strain fingerprinting*
182 PCR was performed in a total reaction volume of 25 µL containing 50 pmol $(GTG)_5$
183 primer (5'-GTGGTGGTGGTGGTG-3') (Invitrogen) (Versalovic, Schneider, De Bruijn,

& Lupski, 1994), 100 ng of template DNA, 5 mM dNTP mix, 2 U μL^{-1} DyNAzyme™ II DNA Polymerase and 2.5 µL of 10x PCR buffer (both Finnzymes). Amplification was done as described in Versalovic et al. (1994), with an exception in extension time, which was changed to 3 minutes per cycle. Five µL of PCR amplicons were loaded on a 1.5 % (w/v) agarose gel (15 x 25 cm) and run for 4.5 h in 1xTAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) at 50V. The gels were stained with 0.5 mg L^{-1} ethidium bromide (Bio-Rad, Hercules, CA, USA) in milliQ water for 30 min with gentle shaking, visualized under UV light (Gel Doc 1000, Bio-Rad) and analyzed using the GelCompar II software v.6.1 (Applied Maths NV, Sint-Martens-Latem, Belgium). Cluster analysis was done using the Dice similarity coefficient and UPGMA (unweighted pair-group method) dendrograms were derived from the band pattern profiles.

2.3 Culture-independent microbial analysis

2.3.1. Extraction and purification of total kefir grain DNA

Total DNA extraction from kefir grains was performed as described by Alegría et al. (2010), with some modification. Briefly, 1 kefir grain of about 3 g was diluted 1:10 in sodium citrate solution (20 g L^{-1}, Merck) and homogenized in an Omni mixer (Omni International) at level 4 for 1 min. Two mL of the mix was pelleted by centrifugation at 3000 x *g* for 15 min (5415D, Eppendorf), and the pellet added 200 µL of enzymatic solution (20 mM Tris HCl pH 8, 2 mM EDTA, 1.2 % Triton X-100, 20 U mutanolysine [Sigma-Aldrich]) and lysed at 37 °C for 1 h. DNA was then extracted and purified using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen) following the manufacturer's recommendations, apart from the addition of a mechanical disruption step by bead-beating with 0.5 g of glass beads (Ø ≤ 106 µm, Sigma-Aldrich) at 6 ms^{-1} for 20 s following the

enzymatic lysis. For the kefir grain fermentates, the bulk starter and the kefir samples, DNA was extracted directly from two mL of sample.

2.3.2. PCR-DGGE

PCR was performed in a total reaction volume of 50 µL containing 5 µL of 10X DreamTaq Green buffer (Fermentas, Vilnius, Lithuania), 10 mM dNTP mix, 75 mM $MgCl_2$, 0.5 µL formamide, 0.5 µg bovine serum albumin, 1.25 U DreamTaq™ DNA Polymerase (Fermentas), 5 pmol of each primer (Invitrogen) and 1 µL of template DNA. For bacteria, two sets of universal bacteria primers were used separately to validate the results; PRBA338fGC/PRUN518r (Øvreås, Forney, Daae, & Torsvik, 1997), amplifying a ~200 bp part of the V3 region of the 16S rRNA gene, and P1V1GC/P2V1 (Klijn, Weerkamp, & de Vos, 1991), amplifying a ~90 bp part of the V1 region of the 16S rRNA gene. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 60 s for V3 or 54 °C for V1, and elongation at 72 °C for 60 s, and final extension at 72 °C for 10 min.

For yeasts, the eukaryotic universal primers NL1GC and LS2 were used (Cocolin, Bisson, & Mills, 2000; Nielsen, Hønholt, Tano-Debrah, & Jespersen, 2005), amplifying a ~250 bp long fragment of the D1/D2-region of the 26S rRNA gene. The PCR conditions were initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 60 s, annealing at 52 °C for 45 s and elongation at 72 °C for 60 s, and final extension at 72 °C for 7 min.

PCR amplicons were separated using a DGGE apparatus (INGENYphorU system, Ingeny International B.V. Goes, The Netherlands) by loading PCR samples directly to a 9 % (v/v)

polyacrylamide gel with a 35-50 % (bacteria) and 40-55 % (yeasts) urea-formamide denaturing gradient (100 % denaturant corresponding to 7M urea and 40 % [v/v] formamide) in 1 x TAE buffer. Electrophoresis was performed for 16 h at 60 °C and 75V. After staining with ethidium bromide for 30 min, band patterns were visualized using the Gel Doc™ XR System (Bio-Rad).

DGGE bands were excised with a sterile scalpel and eluted in 50 µl 1 x Tris-EDTA buffer overnight at 4 °C to allow diffusion of the DNA. One µL of the DNA was reamplified using the same primers as in the DGGE-PCR, and then sequenced using 16S rRNA V3 primers for bacteria and 26S rRNA D1/D2 primers for the yeasts, and the ABI Prism® BigDye® Terminator v3.1 Cycle Sequencing chemistry (Applied Biosystems) as described in section 2.2.3.

2.3.3. PCR amplification for direct sequencing of total kefir grain DNA

The analysis was performed as described by Sekelja et al. (2012). For bacteria, the primers used were the universal primer pair F (5'-TCC TAC GGG AGG CAG CAG T-3') and R (5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3') (Nadkarni, Martin, Jacques, & Hunter, 2002), whereas for yeasts the primer pair used was ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Fell et al., 2000; Jespersen et al., 2005).

2.4. Statistical analysis

The significance of differences in analysed variables between the different kefir grain cultures were tested by Student's t test for the equality of means (assuming equal variances) using Unscrambler® X (Camo Software AS, Oslo, Norway), and the correlation

calculations between isolate phylogroups, kefir grain source and growth media were performed using Pearson's chi-squared test.

3. Results

3.1 Enumeration of yeasts and bacteria in kefir grains

The enumeration results on the selective growth media demonstrated quite similar results for all seven kefir grain cultures, especially for the lactic acid bacteria (fig. 1). When comparing all kefir grain cultures, the average of lactococci, lactobacilli and yeasts were all found in very similar amounts, log 7.9±0.1, log 7.7±0.2, and log 7.6±0.1 cfu g^{-1} of kefir grain, respectively. The average amounts of presumed *Leuconostoc* spp. and presumed acetic acid bacteria were log 7.6±0.2 and log 4.4±0.3 cfu g^{-1} of kefir grain, respectively. There was no growth on M17 plates at 45 °C or on VRBA plates for any of the kefir grain cultures, indicating absence of thermophilic lactic streptococci and coliforms. Results from the SLA plates showed overgrowth at the highest dilution of 10^{-7}. The only significant difference found in growth numbers between the seven kefir grain cultures was significantly lower yeast numbers in RIV and CH compared to RVI ($P < 0.05$).

3.2 Identification of kefir grain cultivation isolates of bacteria and yeasts

The 245 bacteria and 69 yeast isolates picked from agar plates were grouped according to the results of several phenotypic and biochemical tests; optimal growth temperature (30°C or 37°C for MRS and LD isolates, 22°C or 30°C for M17 isolates), colony morphology and broth tube growth characteristics, Gram reaction, cell morphology, CO_2 production and catalase test (results not shown). A total of 162 bacterial and 69 yeast isolates were sequenced, and seven bacterial species and two yeast species were identified: *Lactococcus (Lc). lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lactobacillus (Lb.) kefiranofaciens*,

283 *Lb. kefiri*, *Lb. parakefiri*, *Leuconostoc (Leu.) mesenteroides*, *Staphylococcus (S.)*
284 *pasteuri/S. warneri*, *Kluyveromyces (K.) marxianus*, and *Kazachstania (Kaz.) humatica.*
285 The distribution of identified isolates per species for each kefir grain culture from the
286 media M17, MRS, LD and YGCA is presented in figure 2. The media APM and SLA did
287 not give growth of their target bacterial groups; however, several of the lactic acid bacteria
288 grew well on these media. The growth medium specificity of M17, MRS and YGCA was
289 good, only lactococci were found on M17, whereas 41 out of 43 MRS isolates were
290 lactobacilli and the two remaining isolates were lactococci (table 1). *Lb. kefiri* tolerated
291 environments containing vancomycin (LD) and high amounts of ethanol (APM). Half of
292 the LD isolates were identified as *Leu. mesenteroides,* and the rest as *Lb. kefiri.*
293
294 Rep-PCR was used to investigate the diversity of the isolates that were found to belong to
295 the same bacterial species, though they were in some cases isolated from different kefir
296 grains and different growth media. Initial testing of two single oligonucleotide primers,
297 BOXA1R and $(GTG)_5$, showed that the latter gave the highest band pattern complexity
298 (results not shown), and this was chosen for further fingerprinting analysis. Guidelines for
299 strain-level discrimination in rep-PCR in commercial tests available suggest that similarity
300 below 95 % is considered as different. However, 90 % similarity has also been used as a
301 threshold in strain typing of bacteria and moulds (Palencia, Klich, Glenn, & Bacon, 2009;
302 Pasanen et al., 2011), and was set as the cut-off value in these comparisons (fig. 3). The
303 strains of *Lb. kefiranofaciens, Lb. kefiri,* and *Lc. lactis* formed 11, 8, and 10 clusters,
304 respectively, whereas the 10 strains of *Leu. mesenteroides* grouped into two clusters (fig.
305 3). In further analysis of the *Lb. kefiri* isolates, no correlation was found between rep-PCR
306 phylogroups and kefir grain culture, however, a strong correlation ($P = 0.005$) was found
307 between the phylogroups and the growth medium used for isolation. An additional test of

ethanol toleration of *Lb. kefiri* strains isolated from other media than APM showed that they grew to lower numbers and smaller colony sizes on APM than on MRS under similar growth conditions (results not shown).

3.3 Identification of bacteria and yeasts from total sample DNA

DGGE bacterial profiles using 16S rRNA V3 region primers of the seven different kefir grain cultures, their fermentates, the bulk starter, and the fresh, 3 week old and 8 week old kefir showed the same number, positioning and intensity of bands for all kefir grain cultures (fig. 4a). Sequencing of band 1 identified it as *Lb. kefiranofaciens*. Similarly, all kefir grain fermentates, the bulk starter and the kefir samples gave the same band pattern, and sequencing of band 2 identified it as *Lc. lactis*. Amplification with 16S rRNA V1 region primers demonstrated the same; identical band fingerprints for all seven kefir grain cultures, as well as for the fermentates, bulk starter and kefir samples (data not shown). Direct sequencing of total DNA using universal bacterial primers confirmed the DGGE results, as all samples had one consensus sequence, identified as *Lb. kefiranofaciens* for the kefir grain cultures, and as *Lc. lactis* for all fermentates and kefir samples. The distribution of identified bacterial strains for each kefir grain culture, by use of both culture-dependent and –independent analysis, showed how *Lc. lactis* subsp. *lactis, Lb. kefiranofaciens* and *Lb. kefiri* were found in all cultures, whereas the presence of the remaining identified species varied: *S. pasteuri/S. warneri* was found only in RVI, *Lb. parakefiri* only in RIII and Rm, and *Lc. lactis subsp. cremoris* only in RIIx, RIV and RVI, whereas *Leu. mesenteroides* was found in all cultures except for RIV (table 2).

PCR-DGGE using universal eukaryotic primers also revealed identical band patterns for all seven different kefir grain cultures (fig. 4b). Sequencing of band 3 identified it as *K.*

marxianus. The sequence of the 169 bp long amplicon in band 4 was identical to both *Kaz. exigua* and *Kaz. turicensis*, whereas it had 3 mismatches with *Saccharomyces cerevisiae* and 18 mismatches with *Kaz. humatica*. Direct sequencing of total kefir grain DNA using yeast primers gave one consensus sequence, identified as *Kaz. humatica,* whereas none of the fermentates, the bulk starter or the kefir samples formed detectable amounts of PCR products during PCR-DGGE or direct sequencing PCR.

4. Discussion

As kefir grains can be viewed as microbial community units of selection, constituting lineages that occupy stable niches (Doolittle & Zhaxybayeva, 2010), they are highly interesting as subjects for ecological studies. Microbiota comparisons of the seven kefir grain cultures were carried out to explore the influence of external treatment and internal selection, and a polyphasic approach using both culture-dependent and culture-independent methods was employed. Two levels of kefir grains microorgansims were identified; a dominating microflora consisting of a few species that were the same for all seven kefir grain cultures, and a secondary microflora consisting of a greater number of species, and whose composition varied somewhat between the investigated cultures. Just as in the kefir grain cultures investigated here, lactobacilli have been found to be the dominant bacterial species in kefir grains from wide-spread parts of the world. More specifically, one of the two species *Lb. kefiri* or *Lb. kefiranofaciens* are most often dominant (Chen, Wang, & Chen, 2008; Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011; Heo & Lee, 2006; Kesmen & Kacmaz, 2011; Miguel et al., 2010). The latter produces the exopolysaccharide kefiran, which is a major constituent of the kefir grain matrix (Kooiman, 1968).

Yeast examinations showed the presence of both *K. marxianus* and *Kaz.* spp. in all kefir grain cultures. The yeasts in kefir have been less studied than the bacterial community, however the most reported yeast species are *K. marxianus* and *S. cerevisiae* (Farnworth, 2005; Heo & Lee, 2006; Wang, Chen, Liu, & Chen, 2008). *K. marxianus* has an advantage in a milk environment, as it is one of the few yeast species that possesses *β*-galactosidase, and has the ability to ferment and assimilate glucose and, at a slower rate, galactose (Dickinson & Kruckenberg, 2006; Graciano Fonseca, Heinzle, Wittmann, & Gombert, 2008; Kurtzman & Fell, 1998). A study of microbiological and chemical properties of Norwegian kefir (Grønnevik et al., 2011) showed how both lactose and galactose were significantly reduced during storage at the same time as yeast numbers increased and lactic acid bacteria numbers decreased, thus indicating the now confirmed presence of galactose and lactose utilising yeasts.

The culture-dependent approach also allowed for isolation of species that were present in lower amounts, a secondary microflora, whose composition varied somewhat between the kefir grain cultures. The lactic acid bacteria demonstrated, *Lb. kefiri*, *Lb. parakefiri*, *Leu. mesenteroides*, *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, are all part of the most frequently reported lactic acid bacteria in kefir grains world-wide (Farnworth, 2005; Kesmen & Kacmaz, 2011; Magalhaes et al., 2011). In contrast to the dominant obligately homofermentative *Lb. kefiranofaciens*, several of the species found are producers of aroma and flavour compounds such as diacetyl and acetate. The dairy producer's argument for the continued use of the seven kefir grain cultures in combination, despite the resource demanding handling this requires, is that omitting some cultures simply gives a less organoleptically complex kefir. The differences found in the secondary microflora composition could be contributing to this.

For rigorous comparative strain fingerprint analysis using rep-PCR, between 8 and 15 bands per lane are desirable (Versalovic et al., 1994). Here, between 2 and 9 bands per lane was found for all strains analysed. All *Lb. kefiri* phylotypes isolated on APM, which contains 3 % (v/v) ethanol, formed phylogroups separate from the remaining phylotypes isolated on other media, and a strong correlation was found between the phylogroups and isolation media. A further test showed that this ethanol toleration was not a unique acquired property, as all of the cluster 1 phylotypes could also grow on APM, albeit to lower numbers and smaller colony sizes than on MRS under similar growth conditions, indicating that niche selection is important for the strain composition. Although the total ethanol content of the kefir drink seldom exceeds 0.1%, it is likely that local areas with considerably higher amounts will occur during the first step of the fermentation, causing a spatial heterogeneity of the habitat, and the ability to tolerate this is vital for microorganisms present.

The amounts of both lactococci and lactobacilli found in the kefir grains corresponded to the amounts earlier found in newly fermented kefir made from the same kefir grains as analysed here; around log 8 cfu mL^{-1} each (Grønnevik et al., 2011). For yeasts, log 7.6±0.1 cfu g^{-1} was found in the grains, whereas only log 3.3 cfu mL^{-1} was recovered in the kefir. The transfer of microorganisms from kefir grains to the kefir fermented milk, often called shedding, was thus higher for lactic acid bacteria than for yeasts, and though the yeasts in the kefir continuously increased in number during storage, they never reached the amounts found in the grains (Grønnevik et al., 2011). In the literature, reported numbers of the different microbial groups vary quite considerably, due to differences in e.g. kefir grain origin and grain cultivation method (Farnworth, 2005; Koroleva, 1988b; Lin et al., 1999; Pintado et al., 1996; Tamime & Marshall, 1997; Witthuhn et al., 2005; Wouters, Ayad,

Hugenholtz, & Smit, 2002). In kefir grains from Ireland, Argentina, Poland, Turkey, Slovenia and South Africa numbers of lactobacilli per g range from log 6 to log 9, lactococci range from log 5 to log 10, and reported yeasts numbers range from log 5 to log 8 cfu g^{-1} kefir grain (Abraham & De Antoni, 1999; Garrote, Abraham, & De Antoni, 2001; Güzel-Seydim et al., 2005; Kesmen & Kacmaz, 2011; Rea et al., 1996; Witthuhn et al., 2004; Wszolek, Tamime, Muir, & Barclay, 2001; Zajsek & Gorsek, 2010). Whereas acetic acid bacteria have earlier been considered to play an important role in maintaining the symbiosis among the kefir grain microflora (Koroleva, 1988b), none could be demonstrated in this work neither by culture-dependent nor -independent methods. Their importance has become increasingly questioned, and it has been suggested that they are nonessential contaminants of kefir (Angulo et al., 1993; Dobson et al., 2011; Pintado et al., 1996; Witthuhn et al., 2005).

Conclusion

The dominant microflora of the seven kefir grain cultures subjected to separate, yet similar treatment for 50 years was found to be the same, consisting of the lactic acid bacteria *Lb. kefiranofaciens* and two yeasts, *K. marxianus* and *Kazachstania* spp. The composition of the secondary microflora varied somewhat between the different kefir grain cultures and was found to consist of several species of lactic acid bacteria, some of which produce aroma and flavour components.

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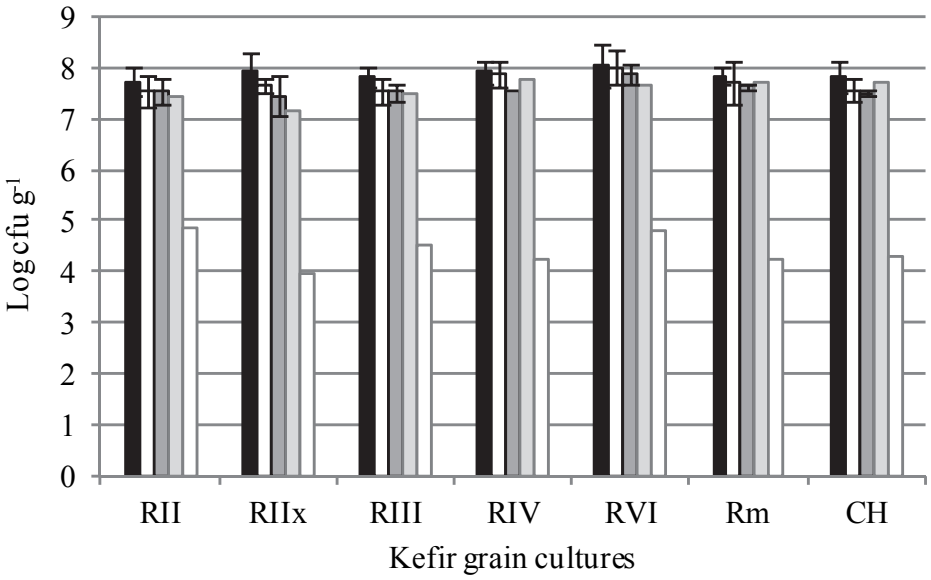

Figure 1. Microbial contents of seven kefir grain cultures: lactococci (M17 agar) (■), lactobacilli (MRS agar) (□), yeasts (YGCA) (■), presumed *Leuconostoc* spp. (LD agar) (□), and presumed acetic acid bacteria (APM) (□). LD and APM results are average per gram of kefir grain of single samples plated in duplicate, whereas M17, MRS and YGCA results are average of three samples plated in duplicate; error bars show standard deviation.

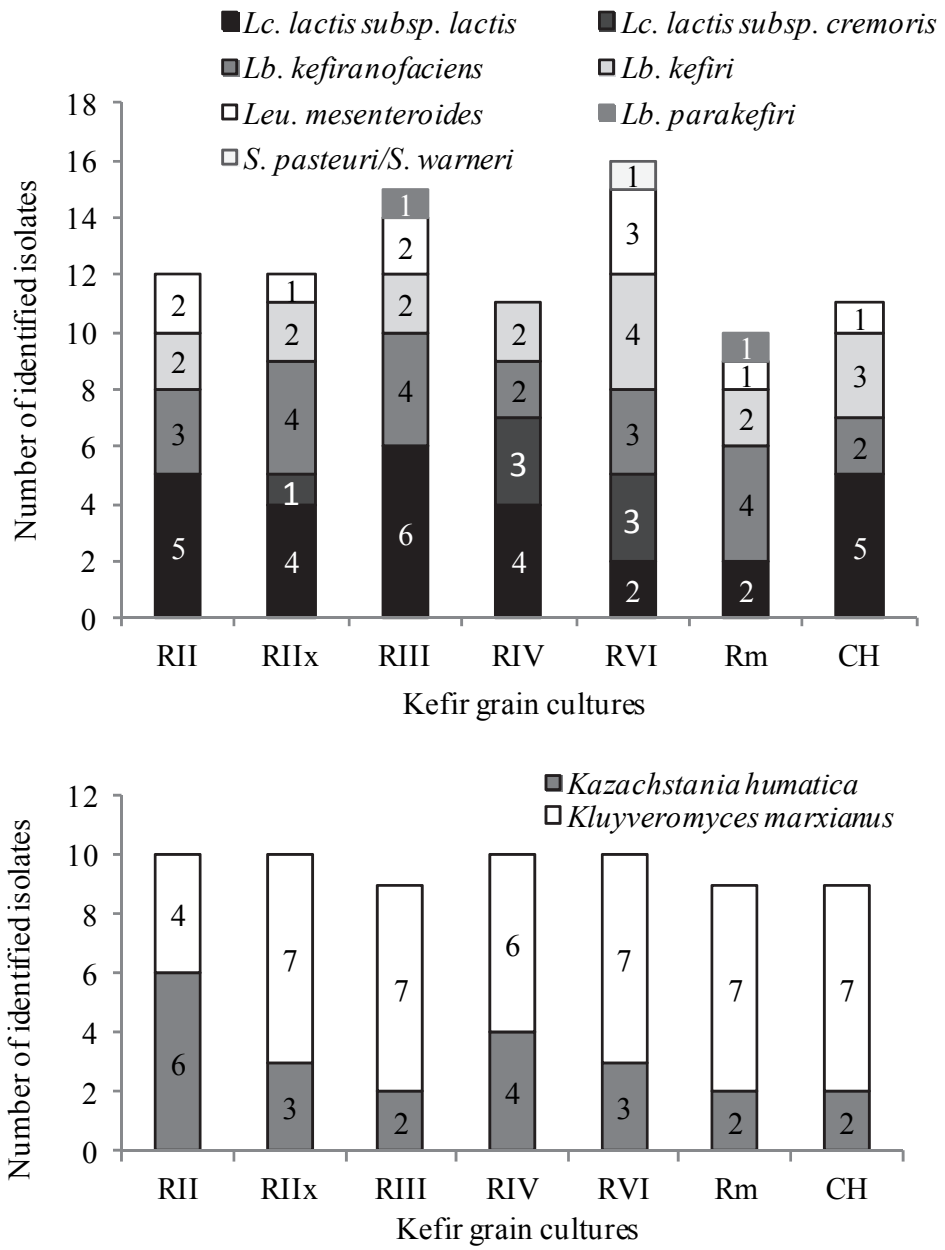

Figure 2. Sequencing identification results of a) bacterial and b) yeast isolates from seven kefir grain cultures. The lactococci were isolated from M17 plates, the lactobacilli from MRS plates, *Leuconostoc* spp. from LD plates and *Staphylococcus* spp. from SLA plates. For all isolates, the identical nucleotides percentage in the sequences compared to the NCBI match were all 98-100% and the E-value (probability of chance hits) was 0.0.

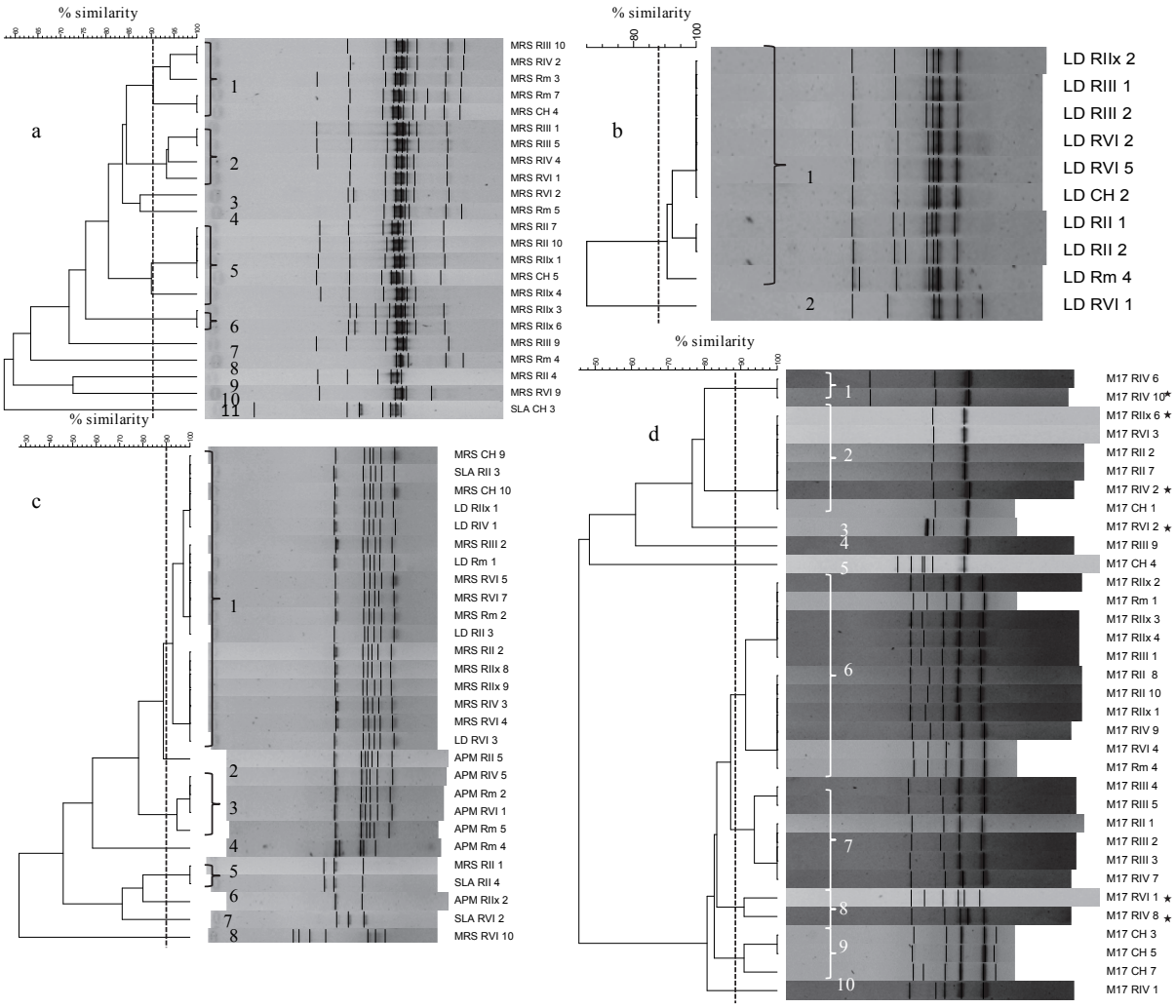

Figure 3. $(GTG)_5$-PCR amplicon gel patterns and cluster analysis dendrogram derived by UPGMA linkage of Dice similarity coefficients of a) 23 isolates of *Lactobacillus (Lb.) kefiranofaciens*, b) 10 isolates of *Leuconostoc mesenteroides* subsp. *mesenteroides*, c) 28 isolates of *Lb. kefiri*, and d) 34 isolates of *Lactococcus (Lc.)lactis* (the stars indicate the isolates identified as *Lc. lactis* subsp. *cremoris*). The vertical lines on the dendrograms indicate the 90% similarity cut-off.

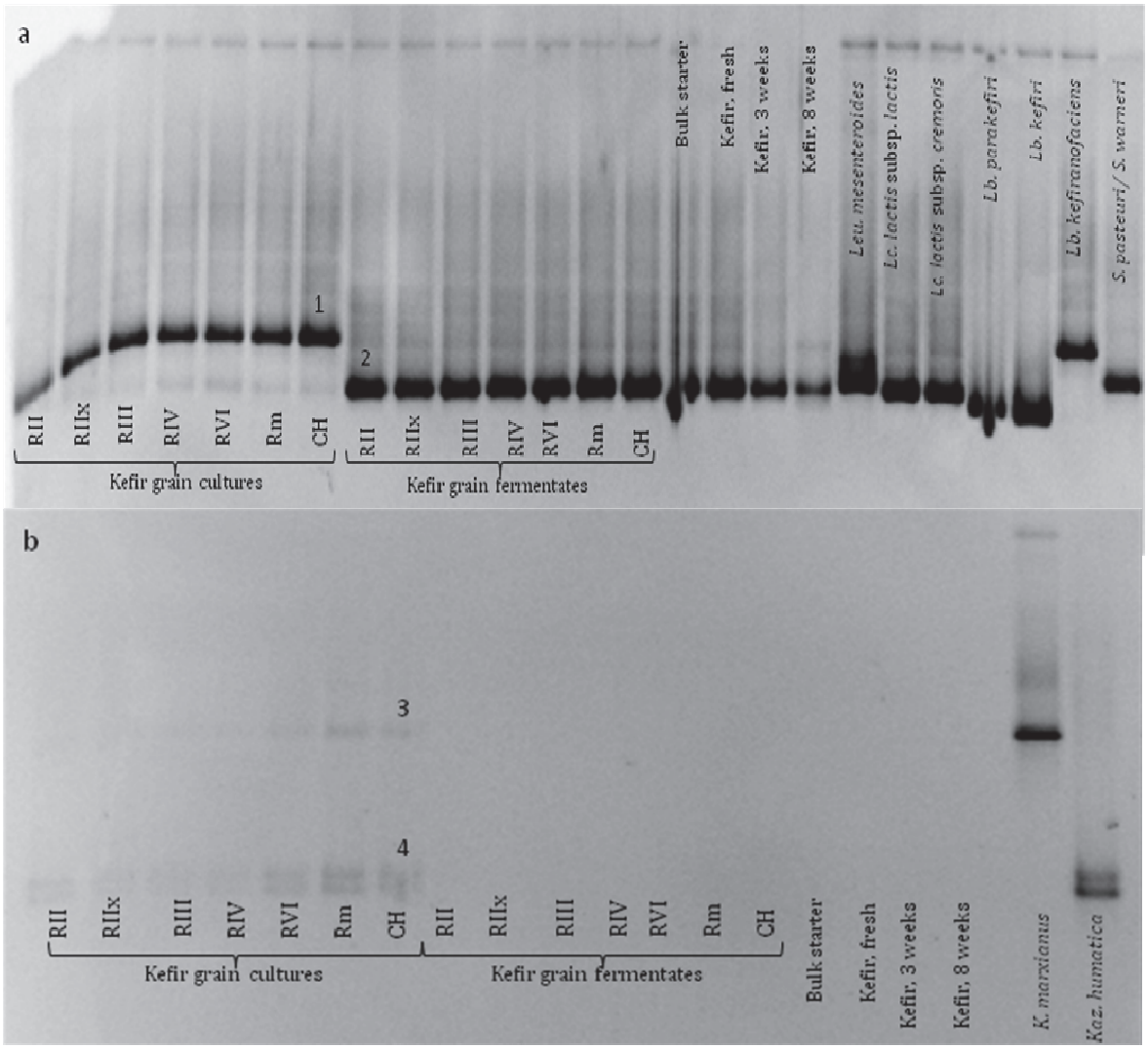

Figure 4. DGGE band pattern profiles of the seven kefir grain cultures, their fermentates, the bulk starter, the fresh kefir and the kefir after 3 and 8 weeks of storage, and the isolated pure cultures using a) universal 16S V3 rRNA primers for bacteria (denaturing gradient 35-50 %) and b) universal 26S rRNA D1/D2-region primers for yeasts (denaturing gradient 40-55 %).

Table 1. Distribution of identified species of bacteria and yeasts from kefir grains on the different cultivation media used.

Species	Growth media					
	M17[a]	MRS[b]	SLA[c]	LD[d]	APM[e]	YGCA[f]
Lactococcus (Lc.) lactis subsp. *lactis*	28	2	22	-	-	-
Lc. lactis subsp. *cremoris*	7	-	-	-	-	-
Lactobacillus (Lb.) kefiranofaciens	-	22	1	-	-	-
Lb. kefiri	-	17	9	10	31	-
Lb. parakefiri	-	2	-	-	-	-
Staphylococcus (S.) pasteuri/S. warneri	-	-	1	-	-	-
Leuconostoc (Leu.) mesenteroides subsp. *mesenteroides*	-	-	-	10	-	-
Kazachstania humatica	-	-	-	-	-	22
Kluyveromyces marxianus	-	-	-	-	-	45

[a]M17 for lactococci, [b]MRS for lactobacilli, [c]SLA for propionic acid bacteria,

[d]LD for *Leu.* spp., [e]APM for acetic acid bacteria, [f]YGCA for yeasts

Table 2. Species present in the different kefir grain cultures, demonstrated by culture-dependent and culture-independent analyses. The identical nucleotides percentage in the sequences compared to the NCBI match were all 98-100% and the E-value (probability of chance hits) was 0.0.

	Kefir grain cultures						
	RII	RIIx	RIII	RIV	RVI	Rm	CH
	CD[1] / CI[2]	CD / CI	CD / CI	CD / CI	CD / CI	CD / CI	CD / CI
Lactococcus (Lc.) lactis subsp. *lactis*	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -
Lc. lactis subsp. *cremoris*	- / -	+ / -	- / -	+ / -	+ / -	- / -	- / -
Lactobacillus (Lb.) kefiranofaciens	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
Lb. kefiri	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -
Lb. parakefiri	- / -	- / -	+ / -	- / -	- / -	+ / -	- / -
Staphylococcus (S.)pasteuri/ S. warneri	- / -	- / -	- / -	- / -	+ / -	- / -	- / -
Leuconostoc mesenteroides subsp. *mesenteroides*	+ / -	+ / -	+ / -	- / -	+ / -	+ / -	+ / -
Kazachstania spp.	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
Kluyveromyces marxianus	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +

[1] Culture-dependent

[2] Culture-independent (DGGE, direct sequencing)

Paper II

Letters in Applied Microbiology

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ORIGINAL ARTICLE

Rapid lactic acid bacteria identification in dairy products by high-resolution melt analysis of DGGE bands

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Keywords
cheese, DGGE, DGGE band identification, high-resolution melt analysis, kefir, lactic acid bacteria.

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Abstract

Aim: To investigate the application of high-resolution melt (HRM) analysis for rapid species-level identification of lactic acid bacteria (LAB) communities in dairy products, as well as for bacterial community profiling and monitoring.
Methods and Results: First, comparisons of HRM profiles of known reference strains of LAB and their denaturing gradient gel electrophoresis (DGGE) bands showed very good agreement, allowing species recognition and identification from DGGE bands by HRM. Second, samples of cheese, kefir grains and kefir were characterized by PCR-DGGE, and melting profiles of DGGE bands were compared with known reference strains. Of the 13 DGGE bands, ten were identified by HRM by comparison with the reference strains and only three required sequencing for identification. Use of HRM profiling for comparison and monitoring of total LAB communities from dairy products or starter cultures was also evaluated, and good agreement was found when comparing clustering of DGGE band profiles with clustering of HRM melting profiles.
Conclusion: Identification of DGGE bands is possible by comparison of HRM melting profiles with known reference strains.
Significance and Impact of the Study: HRM profiling is suggested as an additional approach for identification of DGGE bands.

Introduction

High-resolution melt (HRM) analysis is a sensitive post-PCR method that can be used to study sequence variation down to a single nucleotide polymorphism. Target sequences are amplified by PCR in a real-time qPCR instrument, in the presence of a saturated fluorescent double-stranded DNA (dsDNA) intercalating dye. The PCR amplicons are then melted by slowly increasing the temperature and, as dsDNA converts to single-stranded DNA (ssDNA), fluorescence decreases sharply. DNA from samples or pure strains can thus be characterized according to their melting profile and specific melting temperature (T_m), T_m being defined as the temperature where 50% of the DNA duplexes are melted (Reed *et al.* 2007; Patel 2009). The melt analysis has previously been applied for identification and classification of bacterial communities by 16S rRNA restriction fragment melting curve analyses (Rudi *et al.* 2005, 2007). Since then, improvement of instrumentation has increased the resolution and specificity of HRM, making it even more attractive as a molecular microbiology tool (Vossen *et al.* 2009). The HRM is a simple, rapid and less expensive method, with high-throughput possibilities. As it is a closed-tube, nondestructive method, downstream usage of the PCR amplicons is possible, such as sequencing or loading onto a denaturing gradient gel electrophoresis (DGGE) gel. Earlier work in our laboratory has shown reliable identification of pure strains of lactic acid bacteria (LAB) by cluster analysis of HRM melting profiles (D. Porcellato, unpublished results).

Recently, high-throughput parallel sequence techniques, such as pyrosequencing, have become increasingly available. However, the large amount of data generated limits its usefulness in industrial applications. Hence, there is still a need for simpler and more cost-effective techniques for the screening of bacterial communities. One of the most used methods to study the diversity of microbial systems and for monitoring their dynamic development is

DGGE. This method has been applied for more than two decades to study the microbial community of many food products, with dairy products and the LAB that often dominate their microflora being among the most studied (Ercolini *et al.* 2001; Ercolini 2004; Chen *et al.* 2008; Randazzo *et al.* 2009). The principle of DGGE is a separation of amplified PCR fragments in a denaturing gradient gel based on the amplicons' denaturing properties. Identification of DGGE bands has earlier been achieved by sequencing each band. Identification may also be carried out by comparison of migration distance between reference species and DGGE bands.

We present the use of HRM for DGGE band identification and its application to fermented dairy products. In addition, melting curve comparisons of total dairy product community DNA was investigated, exploring its potential as a LAB community screening and monitoring method suitable for industrial applications.

Materials and Methods

Reference strains and DNA isolation

The following bacteria strains, previously isolated from dairy products, were used as reference strains: *Lactobacillus (Lact.) paracasei* INF448, *Lactobacillus plantarum* INF15D, *Lactobacillus curvatus* H13, *Lactobacillus helveticus* INF1001, *Lactobacillus fermentum* INF10031, *Lactobacillus brevis* NG0012, *Lactobacillus kefiri* MRS RIIx8, *Lactobacillus kefiranofaciens* MRS RVI9, *Leuconostoc (Leuc.) mesenteroides* INF10171, *Pediococcus pentosaceus* INF10023, *Lactococcus (Lc.) lactis* subsp. *lactis* INF1005, *Lc. lactis* subsp. *cremoris* Ar1 and *Enterococcus faecalis* NG0005. In addition, a strain of *Lactobacillus rhamnosus* INFG001 was included in the study. *Lactobacillus* and *Leuconostoc* strains were grown in de Man, Rogosa and Sharpe (MRS) broth at 30°C, and *Lactococcus* and *Enterococcus* in M17 broth (Merck, Darmstadt, Germany) at 30°C. DNA from pure cultures was extracted from 1·5-ml overnight cultures by GelElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St Louis, MO, USA), according to the manufacturer's instructions.

Dairy samples

Cheese was made from 350-kg milk as described by Skeie *et al.* (2001) with some modifications. The starter (ST) used was Probat Visbyvac 505 (Danisco, Copenhagen, Denmark), and the cheese milk was inoculated at 1%. Two adjunct lactobacilli strains were inoculated (1%) in MRS broth (Difco, Sparks, NV, USA) and grown at 30°C for 20 h and subsequently inoculated at 0·3% to the cheese milk. Cheeses denoted 70- were produced with adjunct *Lact. paracasei* INF448, while cheeses denoted 77- were produced with adjunct *Lact. plantarum* INF15D. The rennet used was ChyMax Plus (Chr. Hansen, Hørsholm, Denmark), plastic cheese moulds giving 5-kg cheese were used (Laude b.v., Ter Apel, the Netherlands), and salting was for 10 h. The temperature in the curing room was 19°C, and the cheese was kept there for 14 days and then stored at 4°C. The cheese was sampled according to IDF-standard 50c (1995) and analysed 24 h (time 0) after cheese making and then after 4 and 7 weeks of ripening. Samples of starter (ST) and of cheese milk before rennetting (CMBR) were also included in this study. The kefir analysed was a commercial product from the dairy company TINE (Oslo, Norway). The samples were first analysed the day after the fermented product was filled in cartons and then analysed again at the end of shelf life after 3 weeks of storage at 4°C. The kefir grains analysed were of two different origins, Russia (RIV) and Romania (Rm), and were provided by the same dairy company.

Extraction of total DNA from dairy samples

Cheese milk and cheese samples were collected for total DNA extraction at the sampling times described earlier. Cheese samples (10 g) were added to 90-ml sterile 2% w/v sodium citrate solution and homogenized for 2 min in an Omni mixer (Omni International, Waterbury, CT, USA). Thirty millilitres of this homogenized cheese was then centrifuged for 5 min at 180 ***g*** at 4°C. The fat layer was removed with sterile pipette tips, and 10 ml of supernatant was collected in a 15-ml tube. For the milk samples, dilution 1 : 10 of fresh or fermented milk was also collected in a 15-ml tube. All tubes were then centrifuged for 10 min at 4800 ***g*** at 4°C. The supernatant was discarded, and the cell pellet collected in a new 1·5-ml tube and washed with sodium citrate solution. The DNA isolation from cheese and milk samples was performed by GelElute™ Bacterial Genomic DNA kit (Sigma-Aldrich), according to the manufacturer's instructions with minor changes; lysozyme and proteinase K treatment were prolonged to 60 and 40 min, respectively. For kefir, DNA was extracted directly from 2 ml of product, whereas for kefir grains, one whole grain of *c.* 3 g was used. DNA was extracted according to the protocol described by Alegría *et al.* (2010) with some modifications. Briefly, one kefir grain was added sodium citrate solution (2% w/v) in a weight ratio of 1 : 10 and homogenized in an Omni mixer at level 4 for 1 min. Two millilitres of the mixture was centrifuged at 3300 ***g*** for 15 min, and the pellet added 200 μl of enzymatic solution [20 mmol l^{-1} Tris HCL pH 8, 2 mmol l^{-1} EDTA, 1·2% Triton X-100, 20 U mutanolysine (Sigma-Aldrich)] and lysed at 37°C for 1 h. DNA was then extracted and purified using a

commercial kit (QIAamp DNA Stool Mini Kit; Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol, with the addition of a mechanical disruption step by bead-beating with 0·5 g of glass beads (Ø ≤ 106 μm; Sigma-Aldrich) at 6 m s^{-1} for 20 s following the enzymatic lysis.

PCR and HRM conditions

The PCR was performed in a final volume of 20 μl containing 1× LightCycler® 480 HRM MasterMix (Roche, Mannheim, Germany), 2 mmol l^{-1} of $MgCl_2$, 0·4 μmol l^{-1} of each primer and 1 μl of extracted DNA from dairy samples or 1 μl of DNA standardized to 10 ng from pure cultures. The primer pair LAC1 (5′-AGCAGTAGGGAATCTTCCA-3′) and LAC2 (5′- ATTTCACCGCTACACATG-3′) was used to amplify DNA from the LAB genera *Lactobacillus*, *Leuconostoc* and *Pediococcus*, whereas primer pair LAC3 (5′-AGCAGTAGGGAATCTTCGG-3′) and LAC2 was used for the genera *Lactococcous*, *Enterococcus* and *Streptococcus* (Walter *et al.* 2001; Endo and Okada 2005). The primers were synthesized by Invitrogen Ltd (Paisley, UK). PCR amplification was performed in a 96-multiwell Light Cycler® 480 Real-Time instrument (Roche) with initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s and elongation at 72°C for 1 min. The HRM analysis was performed after PCR amplification, with an increase of 0·01°C per s from 70 to 95°C and 40 acquisitions per degree. The HRM profiles were subjected to gene scanning analysis by LightCycler® 480 software ver. 1.5 (Roche), whereby the instrument software identifies changes in the shape of the curves, which indicates the presence of sequence variations in the PCR product.

DGGE and bands identification by HRM and sequencing

The INGENYphorU system (Ingeny International BV, Goes, the Netherlands) was used for DGGE analysis. PCR products (20 μl) were applied to 9% (v/v) polyacrylamide gels with a 30–55% urea–formamide denaturing gradient (100% denaturant corresponding to 7 mol l^{-1} urea and 40% [v/v] formamide), in 1× TAE buffer (40 mmol l^{-1} Tris base, 20 mmol l^{-1} acetic acid, 1 mmol l^{-1} EDTA [pH 8]). Gels were run at a constant voltage of 75 V for 16 h at 60°C. Gels were stained in 1× TAE containing 0·5 mg l^{-1} ethidium bromide and analysed under UV light. Selected DGGE bands were excised from the gel with a sterile scalpel blade and incubated overnight at 4°C with 50 μl of 0·1× TE buffer to allow diffusion of the DNA. The PCR amplification was performed as described earlier with the same primers, adding 2 μl of the solution of extracted DNA. After PCR and HRM analysis, the selected bands were purified using the QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions, and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing chemistry and an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis

Raw data from the HRM melting profiles were aligned using temperature, scaled from 0 to 100 and smoothed by a locally weighted scatterplot smoother (LOWESS). First-derivative spectra were calculated, and a data set was constructed with temperatures from 77 to 88°C, using temperature values as variables. The HRM profiles were then clustered by the single linkage method of Euclidian distances. Statistical analysis was carried out using the r software (http://www.r-project.org).

Results

Band identification of the pure strains

To identify the DGGE bands by HRM, DNA from the 14 reference strains were amplified with the genera-specific primer pairs (LAC1–LAC2 or LAC3–LAC2). The primers amplified around the hypervariable V3 region of the 16S rRNA gene, giving a PCR product of about 350 bp. The DGGE analysis of the reference strains separated the PCR products according to sequence composition, and more than one band was generated for some references strains. The bands were excised, reamplified and subjected to HRM. Cluster analysis and gene scanning analysis of the melting profiles of the bands demonstrated how they clearly clustered in relation to the reference strains, allowing band identification at species level (data not shown). Of the 14 reference strains belonging to 13 different species, 13 different melting profiles were identified. The *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* strains showed similar melting profiles. The *Lact. rhamnosus* strain gave four bands on DGGE, and as previous analysis of all four *Lact. rhamnosus* bands displayed the same HRM profile and sequence identification (data not shown), only two bands were excised in this work. The cluster analysis of these bands showed similarity to *Lact. paracasei* and *Lact. rhamnosus*, both part of the *Lactobacillus casei* group.

Application to dairy samples

Samples of cheeses produced with two different adjunct *Lactobacillus* spp., kefir grains and samples of fresh and stored kefir were analysed with the two different primer pairs. From the DGGE gels, 13 bands (Fig. 1) were

excised, re-amplified along with the reference strains and analysed by HRM. The melting profiles of the gel bands and the reference strains generated by HRM were compared using gene scanning analysis and cluster analysis, and for validation, the bands were sequenced and identified by comparison to the sequences reported in the GenBank using the BLAST algorithm. The resulting dendrogram and sequence alignments showed how bands A1 and A2 clustered with and thus could be identified as *Lc. lactis* (Fig. 2 cluster IV). When using the primer pair LAC3–LAC2 on the dairy products, all of their DGGE patterns were similar to that of the reference *Lc. lactis* subsp. *lactis* INF1005, demonstrating how this species was the dominant within the genera for which the primers were selective (Fig. 1). The primer pair LAC1–LAC2 showed more variation in fingerprint profiles among the samples in DGGE. The DGGE band identification by HRM showed similar melting profiles for *Lact. kefiri* and bands A5 and A9 (Fig. 2 cluster I), for *Lact. plantarum* and bands A12 and A13 (Fig. 2 cluster II), for *Lact. kefiranofaciens* and bands A3 and A4 (Fig. 2 cluster III), for *Lact. rhamnosus* and band A6 (Fig. 2 cluster V) and for *Lact. paracasei* and band A11 (Fig. 2 cluster VI). Sequencing of the bands validated these results. Bands A7, A8, A10 and *Leuc. mesenteroides* constituted a separate cluster (Fig. 2 cluster VII), and owing to the high dissimilarity between the three bands and the reference strain, the bands were consequently identified by sequencing as *Leuconostoc* spp. (A7) and *Leuc. mesenteroides* (A8, A10).

Use of HRM as fingerprinting of total LAB community

The melting profiles of DNA from the microbial communities of the dairy samples were subjected to clustering analysis (Fig. 3a,b). Melting profiles generated with the LAC3–LAC2 primer pair showed a lower dissimilarity (<2·5%) compared to LAC1–LAC2 (10%) owing to the presence of the same species in all the samples. This difference between the two primer pairs was also demonstrated in the DGGE analysis, where the band patterns created by the *Lactobacillus–Leuconostoc*-specific primers showed greater variety. In the LAC1–LAC2 primer pair dendrogram, samples with high similarity in DGGE patterns were also clustered together by their HRM melting profile, as found for the cheeses at 4 and 7 weeks, for the two starters, for the kefir grains, and for the kefirs, respectively (Fig. 3b). Sample 77-CMBR clusters together with the cheeses 77-T4 and 77-T7, and displayed similar DGGE patterns. When reference strains are included in the cluster analyses of the dairy sample melting profiles, it gives a preview of the possible major species present in the total DNA, prior to DGGE. However, a more com-

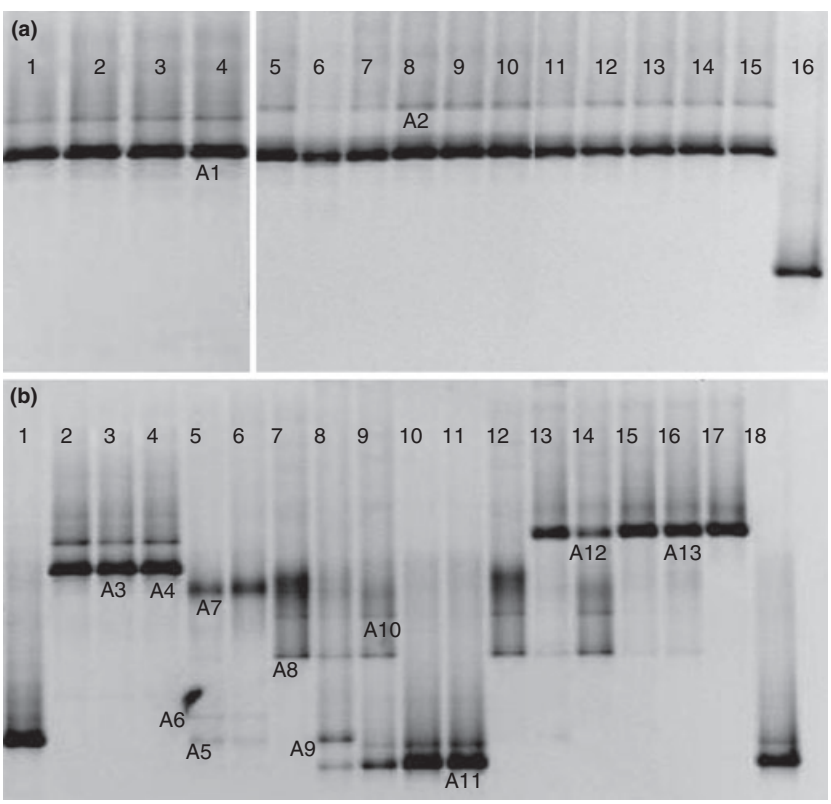

Figure 1 (a) Denaturing gradient gel electrophoresis (DGGE) profiles of kefir, cheese and pure strains with LAC3–LAC2 primers. Lane (L)1: kefir grain RIV, L2: kefir grain Rm, L3: kefir (fresh), L4: kefir (3 weeks), L5: 70-ST, L6: 70-CMBR, L7: cheese 70-T0, L8: cheese 70-T4, L9: 70-T7, L10: 77-ST, L11:77-CMBR, L12: 77-T0, L13: cheese 77-T4, L14: 77-T7, L15: *Lactococcus lactis* subsp. *lactis* INF1005, L16: *Enterococcus faecalis* NG0005. (b) DGGE profiles of kefir, cheese and pure strains with LAC1–LAC2 primers. Lane (L)1: *Lactobacillus kefiri MRS RIIx8*, L2 *Lactobacillus kefiranofaciens MRS RVI9*, L3: kefir grain RIV, L4: kefir grain Rm, L5: kefir (fresh), L6: kefir (3 weeks), L7: 70-ST, L8: 70-CMBR, L9: cheese 70-T0, L10: cheese 70-T4, L11: 70-T7, L12: 77-ST, L13:77-CMBR, L14: 77-T0, L15: cheese 77-T4, L16: 77-T7, L17: *Lactobacillus plantarum* INF15d, L18: *Lactobacillus paracasei* INF448.

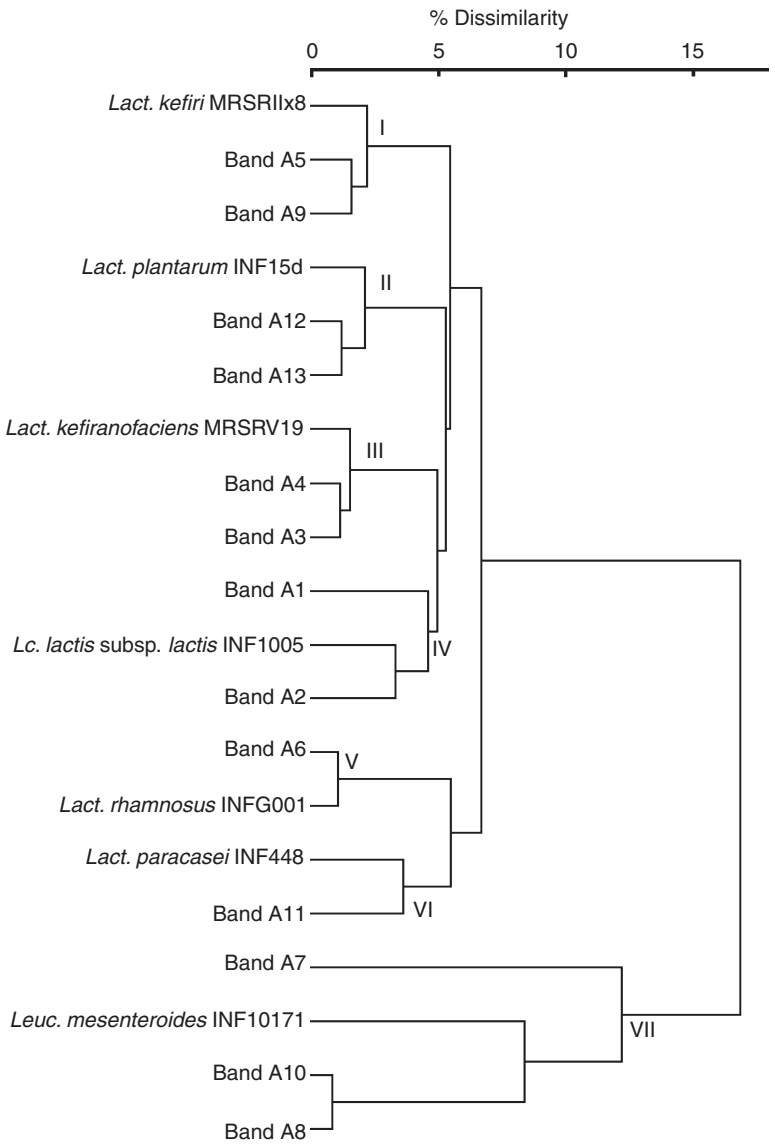

Figure 2 Cluster dendrogram of the high-resolution melt profile of reference species and denaturing gradient gel electrophoresis (DGGE) band excised from cheese and kefir DGGE gels. Roman numbers identify the different clusters.

plete overview is achieved when combining HRM with DGGE analysis (data not shown).

Discussion

DGGE is a well-established method that has been used to analyse the ecology of many different bacterial communities, including dairy products. Identification by comparison of DGGE band migration distance with reference strains is based on subjective interpretation of band positioning by visual comparison or use of gel analysis software. Here, an additional approach for the identification of DGGE bands is proposed, based on high-resolution melting profiles comparison of known reference strains with melting profiles of DGGE bands. The HRM system only requires around 20-min extra running time connected to the band reamplification PCR, and the 96-well format allows the analysis of a high sample number. This may be particularly useful when species give more than one band, as is the case for many LAB (e.g. *Lact. rhamnosus* and *Lc. lactis* in the 16S rRNA V3 region). The analysis relies on a preliminary construction of a collection of reference species that has to be included in the analysis. To maximize the potential of the HRM method, some foreknowledge about the expected microbial composition of the sample analysed is thus required. Analysis of a new sample type should therefore start with identifying expected species, through literature searches and isolation of pure cultures from the samples, to use as references. For initial validation of the method, as well as for those bands not identified by HRM reference strain comparisons, sequencing is still required. The largest

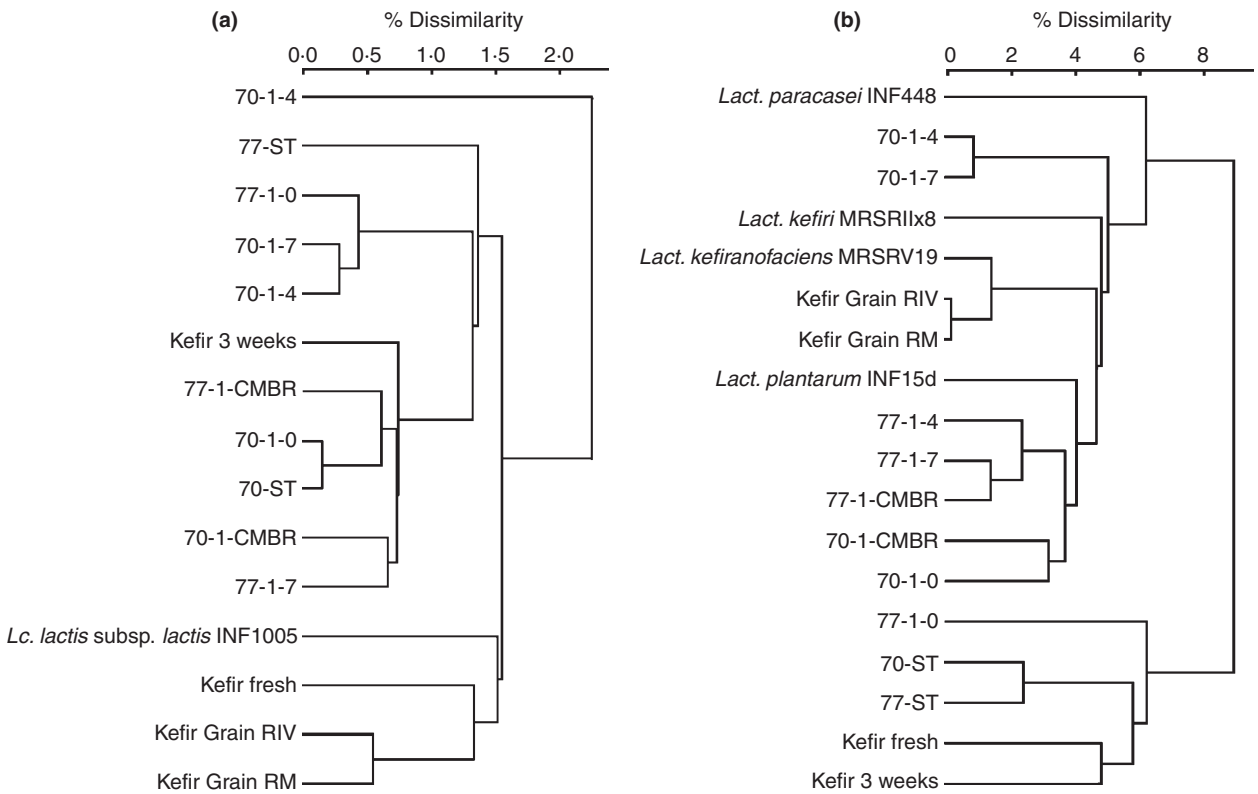

Figure 3 Cluster dendrograms of high-resolution melt profiles of kefir, cheese and reference species with (a) LAC3–LAC2 and (b) LAC1–LAC2 primer pairs.

time-saving potential with this method will be when working with samples of somewhat similar character and making use of a constructed reference collection covering the most common species. As in this case for fermented milks and cheeses, it has been possible to build an adequate library within a short time, and this is continuously expanded as unknown bands are identified. In the present work, 10 of 13 bands were identified using HRM melting curve comparison and gene scanning analysis, while only three needed sequencing for an unambiguous identification.

For both DGGE and HRM, it is possible that amplicons with same sequence will produce similar bands and melting profiles as the two methods are sequence-based techniques. For instance, it is well known that the two subspecies of *Lc. lactis*, *lactis* and *cremoris*, are similar in the V3 region but may be differentiated using primers targeting the V1 region of the 16S rRNA gene (Ward *et al.* 1998). Again, some foreknowledge about the species which are expected to be present has to be taken into consideration when choosing primers and planning the experiment.

Normally, during PCR amplification for DGGE analysis, it is common to add a 40-bp G+C-rich sequence, a GC-clamp, to the 5′ end of one of the primers. This is included in all DGGE protocols, as Muyzer *et al.* (1993) reported that without it, stable and partially melted molecules were not formed. Instead, the amplicons quickly progressed to two single strands, differing in the mobility. In our experiments, addition of a GC-clamp led to incomplete melting profiles when applying HRM (data not shown). In all runs described in this work, GC-clamps were therefore omitted. However, separation was successful on the DGGE gels, with clear and identifiable bands.

Of the cheeses investigated here, samples taken at 4 and 7 weeks showed dominance of the adjunct species, which remained stable throughout ripening, whereas *Leuc. mesenteroides* was detected in the cheese starter, cheese milk and cheese after 1 day. In cheese with *Lact. paracasei* adjunct, *Leuc. mesenteroides* was not detected after 4 and 7 weeks of ripening, whereas in cheese with *Lact. plantarum*, adjunct weak bands of *Leuc. mesenteroides* could also be found during ripening. Presence of *Lact. kefiri* was only seen in cheese milk before rennetting, together with *Lact. paracasei.*

For both kefir grains and kefir, the presence of *Lc. lactis* subsp. *lactis* was demonstrated, a species commonly found

in kefir and kefir grains of many different geographical origins (Farnworth 2005; Kesmen and Kacmaz 2011; Magalhaes *et al.* 2011). In both kefir grain cultures, the only other species detected was *Lact. kefiranofaciens*, also earlier reported as a major constituent of kefir grain microflora and the producer of the exopolysaccharide kefiran, an important part of the kefir grain matrix (Vancanneyt *et al.* 2004; Kesmen and Kacmaz 2011). *Lact. kefiranofaciens* was not found in either of the kefir fermentates, indicating no transfer of this species from the kefir grain starter to the fermented milk. In both fresh and 3-week-old kefir, strong bands representing *Leuconostoc* spp. were found, as well as two somewhat weaker bands identified as *Lact. kefiri* and *Lact. rhamnosus*. *Leuconostoc* spp. and *Lact. kefiri* are often reported as kefir and kefir grain constituents and have been demonstrated in, for example, Taiwanese, Argentinean, Turkish, Brazilian, Canadian and Portuguese samples (Pintado *et al.* 1996; Garrote *et al.* 2001; Chen *et al.* 2008; Miguel *et al.* 2010; Kesmen and Kacmaz 2011).

Furthermore, HRM and cluster analyses of sample melting profiles without performing DGGE may also offer interesting industrial applications, as the method could be used to investigate changes in microbial balance. One application could be the comparisons of dairy starter culture profiles or monitoring the microbial composition of products over time, as has earlier required DGGE (Nielsen *et al.* 2007; Dolci *et al.* 2009).

This work showed how identification of DGGE bands was possible by comparison of HRM melting profiles with known reference strains, and HRM profiling is thus suggested as an additional approach for identification of DGGE bands. However, bands not identified by comparison with reference strains still need sequencing for unambiguous identification. As the HRM is based on real-time PCR equipment, a further improvement of the present method could involve combining identification with quantification protocols.

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Paper III

Evaluation of the use of single- and co-cultures of lactic acid bacteria and yeasts isolated from kefir grains as a starter culture for kefir

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Keywords: Kefir fermented milk; lactic acid bacteria; yeasts; organic acids; volatile compounds

Abstract

Kefir grains contain lactic acid bacteria and yeasts in symbiotic relationships embedded in a protein and polysaccharide matrix. They constitute the starter culture for kefir, a fermented milk drink with a complex sensory profile containing high amounts of lactic acid, ethanol, CO_2, acetaldehyde and diacetyl. In the commercial production of large volumes of kefir, direct fermentation with kefir grains is impractical as this would demand a correspondingly large quantity of grains, and so a 2- or 3-step fermentation process is employed. The objective of this work was to investigate whether co-culture inoculums of microbial isolates from kefir grains could be used as a starter in a single-step fermentation process to obtain a kefir with an aroma and flavour profile similar to the existing commercial product. Milk fermented with single- and co-cultures of isolated lactic acid bacteria and yeasts from kefir grains was compared to kefir grain fermentate and kefir. The single- and co-culture inoculum compositions were of proportions corresponding to the amounts earlier determined to be present in kefir grains. Analysis of organic acids, carbohydrates and volatile compounds in all fermentates showed that the metabolite profiles of the kefir and kefir grain fermentates were more complex and contained significantly more aroma- and flavour contributing components than the single- and co-culture fermentates, underlining the crucial role of kefir grains for the manufacure of authentic kefir.

Introduction

Kefir is a traditional fermented milk drink originating in the Caucasian mountains. The starter culture for kefir is kefir grains, which consist of a protein and polysaccharide matrix embedding a poorly defined microbiota consisting mainly of lactic acid bacteria (LAB) and yeasts (Farnworth 2005). Kefir grains are retrieved after fermentation for use as new inoculations, and their activity can be sustained for years if maintained under appropriate conditions (Simova et al. 2002). For the commercial production of large volumes of kefir, a 2- or 3-step fermentation process is employed, in which the product (fermentate) of kefir grain fermentation is used as starter in a second fermentation step. This second fermentate can then either be considered the end-product kefir, or be used as bulk starter in a third fermentation step to obtain the kefir end-product. As each fermentation step takes 18-20 h at 20-22°C, the commercial kefir manufacture process is time-consuming. If a defined standardized starter in a convenient form, e.g. a freeze-dried culture, was available, this could be used in a single-step fermentation process, aiming to obtain a kefir of similar aroma and flavour profile as with the existing manufacturing process. Whether kefir grains are imperative to kefir fermentation, or if it is possible to make authentic kefir by co-culturing pure cultures of bacteria and yeast species demonstrated to be present in kefir grains has also been the subject of earlier investigations. However, consensus has not been reached. Some results indicate that a typical kefir product with the correct physicochemical and organoleptic qualities can be achieved (Chen et al. 2009; Duitschaever et al. 1987; Gobbetti and Rossi 1994), but it is more often concluded that the sensory properties achieved by the use of kefir grain starter cannot be reproduced by using mixtures of pure strains originating from kefir grains as inoculum (Assadi et al. 2000; Beshkova et al. 2002; Simova et al. 2002).

63 Kefir's desirable organoleptic properties result from both lactic acid fermentation by LAB
64 and alcohol fermentation by yeasts, and the major fermentation end products to be
65 expected are lactic acid, acetic acid, acetaldehyde, acetoin, diacetyl, ethanol and CO_2
66 (Güzel-Seydim et al. 2000). The development of the flavor compounds can derive from
67 fermentation of lactose and citrate, from degradation of milk proteins and fat, and from
68 amino acids and free fatty acids metabolism (Wszolek et al. 2006; Mayo et al. 2010). The
69 microbiota of seven kefir grain cultures used in commercial kefir production in Norway
70 have all been found to be dominated by the lactic acid bacteria *Lactobacillus*
71 *kefiranofaciens* and the yeasts *Kluyveromyces marxianus* and *Kazachstania humatica*.
72 Furthermore, a secondary microflora mainly consisting of lactic acid bacteria is also
73 present, some of which produce aroma and flavour components, and the composition of
74 species varies somewhat between the different kefir grain cultures (Grønnevik et al., paper
75 I). In the commercial kefir production, all the cultures are used in combination, as sensory
76 evaluations have shown that omitting some cultures gives a product with divergent and
77 less complex organoleptic characteristics. The combined kefir grain fermentates are thus
78 subsequently used as mother culture in a second fermentation to make kefir.
79
80 The aim of this work was to investigate whether co-culture inoculums of kefir grain
81 isolates, in proportions corresponding to the amounts present in kefir grains, could be used
82 as a starter for a single-step fermentation process to obtain a kefir of similar aroma and
83 flavour profile as with the existing manufacture process. To evaluate, the contents of
84 carbohydrates, organic acids and volatile compounds were analysed and compared.
85

Materials and methods

Sample acquisition of kefir and kefir grain fermentates

Fermentates of seven different cultures of kefir grains denoted RII, RII_x, RIII, RIV, RVI, Rm and CH were provided by the Norwegian dairy company TINE Meieriet Oslo (Oslo, Norway). The fermentates were products of a 5% (w/v) kefir grain inoculation in milk (1.2% fat) and incubation at 20 °C for 18 hours. At the end of incubation, the kefir grains were separated from the fermentates using sterile sieves. Samples of fresh fermented kefir from five different productions were also provided by the dairy, along with samples of the inoculation milk.

Preparation of concentrated cultures of bacteria and yeasts

Actively growing pure cultures of four lactic acid bacteria and two yeasts previously isolated from kefir grains (Grønnevik et al., paper I) were inoculated separately in 200 mL liquid growth medium using an inoculation of 1% (v/v). The growth media and incubation conditions used were M17 (Merck, Darmstadt, Germany) at 30 ^{o}C for 2 d for *Lactococcus (Lc.) lactis* subsp. *lactis* 44; MRS (Merck) at 30 ^{o}C for 3 d for *Lactobacillus (Lb.) kefiranofaciens* 119, *Lb. kefiri* 88, and *Leuconostoc (Leuc.) mesenteroides* 141; and yeast extract glucose broth (YGB) (Brugnoni et al. 2007) at 22 °C for 5 d for *Kluyveromyces (K.) marxianus* 182 and *Kazachstania (Kaz.) humatica* 228. Following incubation, the cultures were centrifuged at 5000 x *g* at 4 °C for 15 min in a Beckman J2-MC centrifuge (Beckman Coulter, Inc., Brea, CA, USA). Cell pellets were resuspended in 20 mL UHT milk (1.5% fat) (TINE Meieriet Oslo) before distribution into 4 mL aliquotes and addition of 15% (v/v) sterile glycerol and storage at -80 °C.

Inoculation of UHT milk with single- and co-cultures

The thawed concentrated cultures had viable LAB and yeast counts ranging between 7.8–10.1 and 6.4–7.6 log cfu·mL^{-1}, respectively (results not shown). The fermentation volumes were 40 mL UHT milk (1.5% fat), and the inoculum amounts were calculated to ensure inoculation rates corresponding to that of a 5% (w/v) kefir grain inoculation. For 40 mL, this was equvivalent to 2 g of kefir grains, and previous analyses (Grønnevik et al., paper I) have determined the amounts of microorganisms per g of kefir grain to be 7.6, 7.7 and 7.9 log cfu·g^{-1} kefir grain for yeasts, lactobacilli and lactococci, respectively. Thus, volumes of the individual concentrated cultures calculated to obtain a standard initial inoculum of yeasts, lactobacilli and lactococci of 7.9, 8.0 and 8.2, log cfu·mL^{-1}, respectively, were added to the 40 mL milk samples. Each bacteria and yeast strain was inoculated as a single culture in milk, as well as in 8 different co-culture combinations (CC 1-8, Table 1). All samples were incubated under normal kefir fermentation conditions at 20 °C for 18 h.

pH and metabolite analysis

For pH measurements, a Radiometer (PHM92) pH meter with a combined glass electrode and temperature probe (Radiometer, Copenhagen, Denmark) was used. The pH meter was calibrated using standard buffer solutions at pH 4.0 and 7.0 (Merck). For all fermentates, the volatile compounds were measured in 10.0 g samples using headspace gas chromatography (HSGC) as described by Grønnevik et al. (2011). Organic acids and carbohydrates were analysed in 1.00 g samples using high performance liquid chromatography (HPLC), according to the method of Narvhus, Østeraas, Mutukumira, and Abrahamsen (1998), with modifications in the column temperature (32°C) and concentration of H_2SO_4 in the mobile phase (5 mM). The presented results are average of

2 different batches for the kefir grain fermentates and the UHT milk, and of 5 different batches for the kefir and the 1.2% fat kefir inoculation milk. For the single strain and co-culture combinations, results are average of two analysis replicates of one fermented milk sample, except for *K. marxianus 182* and CC8, where results are single analyses.

Statistical analysis

Analyses results were compared using principal component analysis (PCA) with cross-validation and Non-linear Iterative Partial Least Squares algorithm (NIPALS), using Unscrambler® X 10.1 (Camo Software AS, Oslo, Norway). Each variable was weighted by dividing with the standard deviation of that variable. Interpretation of the data was made by inspection of the scores and loadings plots. The significance of differences in analysed variables between the different samples and sample groups were tested by performing ANOVA one-way Tukey 95% Simultaneous Confidence Intervals using Minitab v. 16.2.1 (Minitab Inc., State College, PA, USA).

Results and discussion

Lactic acid production

The single-strain inoculations made it possible to evaluate the contribution of each isolate alone in milk. It has been stated that pure cultures of kefir bacteria and yeasts either do not grow in milk or have a low biochemical activity (Garrote et al. 2010), and here *Lb. kefiri* 88 did not produce measurable metabolites whereas *Leuc. mesenteroides* 141 and *Kaz. humatica* 228 grew poorly in single-culture with little production of lactic acid and reduction of pH. Both alone and in combinations, *Lc. lactis* subsp. *lactis* 44 was the main lactate producer, generating more than 6 $g \cdot kg^{-1}$, whereas the homofermentative *Lb. kefiranofaciens* 119 and the heterofermentative *Leuc. mesenteroides* 141 alone produced

161 smaller amounts, 0.82±0.04 g·kg^{-1} and 0.55±0.02 g·kg^{-1}, respectively. In the co-culture
162 combinations (hereafter denoted CC) without *Lc. lactis* subsp. *lactis* 44, lactic acid
163 amounts were below 2 g·kg^{-1}. CC8 was the only CC to contain the lactate-utilising yeast
164 *K. marxianus* 182 (Kurtzman and Fell 1998), and its fermentate consequently showed a
165 much lower level of lactic acid (3 g·kg^{-1}). CC7 differed from CC8 by the lack of *K.*
166 *marxianus* 182 and 6.7 g·kg^{-1} of lactate was produced by this CC. In kefir and the kefir
167 grain fermentates, the lactic acid amounts were significantly higher (P<0.05) than in all
168 CCs, ranging between 7.9 and 9.0 g·kg^{-1}.
169
170 *Citrate and its degradation products*
171 The degradation of the milk component citrate and the corresponding formation of
172 3.3±0.2 mg·kg^{-1} of diacetyl, 289±6.1 mg·kg^{-1} of acetoin and 686±10 mg·kg^{-1} of acetic acid
173 showed how the *Lc. lactis* subsp. *lactis* 44 strain belonged to the biovariant *diacetylactis*
174 (Tamime 2002). Diacetyl has a low flavour threshold since 1.5-5 mg·kg^{-1} is enough to
175 give the desired aroma in fermented milk (Hemme and Foucaud-Scheunemann 2004). In
176 the kefirs, diacetyl was present in the lower range of the flavour threshold at 1.0±0.5
177 mg·kg^{-1} and this was not significantly different from any of the CCs (Table 4). In the kefir
178 grain fermentates the level of diacetyl ranged from 0.9±0.1 to 2.2±0.5 mg·kg^{-1}. Also
179 *Leuconostoc* is able to utilise citrate, however, only within a pH range from 6.3 to 4.5
180 (Hemme and Foucaud-Scheunemann 2004). As *Leuc. mesenteroides* 141 in single-culture
181 grew poorly in milk, only a limited citrate metabolism had occurred. Citrate was reduced
182 by >95% by *Lc. lactis* subsp. *lactis* 44 and the kefir grain cultures, whereas only a 42%
183 reduction was seen in the fermentate of single-culture *Leuc. mesenteroides* 141. Some
184 lactobacilli may utilise citrate, and a 37% citrate reduction was seen in the *Lb.*
185 *kefiranofaciens* 119 single-culture, but hardly any reduction by *Lb. kefiri* 88 or the yeast

cultures (<10%) (Table 3). In single-culture and in CC3, *Lc. lactis* subsp. *lactis* 44 was responsible for significantly (P<0.05) higher production of acetoin than any other CC, in amounts close to those seen in kefir and kefir grain fermentates (Tabels 3 and 4). In all other CCs containing *Lc. lactis* subsp. *lactis* 44, *Leuc. mesenteroides* 141 was also present. *Leuconostoc* can further convert acetoin to 2,3 butanediol (Hemme and Foucaud-Scheunemann 2004), and this was likely the reason for the lower acetoin levels seen in these CCs, ranging from 5.8±1.4 to 13±6.4 $mg \cdot kg^{-1}$. In the HSGC assay employed here, 2,3 butanediol was not analysed.

Products of alcohol fermentation

When comparing the single- and co-cultures, it is evident that presence of yeasts lead to the highest production of ethanol (Table 2). In single-culture, *K. marxianus* 182 produced significantly higher ethanol amounts than *Kaz. humatica* 228, 1870 $mg \cdot kg^{-1}$ compared to 80±33 $mg \cdot kg^{-1}$. Of the LAB, the heterofermentative *Leuc. mesenteroides* 141 produced ethanol at about the same level as *Kaz. humatica* 228, 54±0.6 $mg \cdot kg^{-1}$. *K. marxianus* is among the only 2% of yeasts that possess the lactose-hydrolysing enzyme β-galactosidase and thus can utilise lactose for energy production (Roostita and Fleet 1996). The other yeast present in the kefir grains, *Kaz. humatica*, lacks this enzyme, but is able to metabolise galactose and so will be able to grow in milk when co-cultured with *K. marxianus* or LAB (Mikata et al. 2001). *Kaz. humatica* 228 was present in all CCs except CC5, and here an extensive accumulation of galactose was found, close to 1200 $mg \cdot kg^{-1}$ (Table 3). Galactose accumulation has earlier been found during LAB fermentation in yogurt (Robinson et al. 2002), and has been suggested to contribute in LAB-yeast interaction in kefir grains, favouring the growth of lactose-negative yeasts (Koroleva 1988). This seems to be the case in the kefir grains investigated here, as *Kaz. humatica*

228 utilised the galactose excreted by LAB and the lactose-positive *K. marxianus* 182 when co-cultured.

The highest ethanol amount of 1.7 $g \cdot kg^{-1}$ was found in CC8, which contained both *K. marxianus* 182*, Kaz. humatica* 228 and *Leuc. mesenteroides* 141. This was similar to the amounts found in some kefir grain fermentates, RII and RIV, and significantly higher than the ethanol content of kefir (Table 4). CC8 also showed significantly ($P<0.05$) higher reduction of lactose and corresponding higher production of acetic and formic acid compared to kefir and kefir grain fermentates (Table 4), in addition to the significantly ($P<0.05$) lower lactate levels due to utilisation by *K. marxianus* 182. CC8 also contained significantly lower amounts of acetoin compared to kefir and kefir grain fermentates (Table 4). These characteristics and comparison with the single-culture fermentates indicated high activity and dominance of *Lc. lactis* subsp. *lactis* 44 and *K. marxianus* 182 in CC8. CC7, without *K. marxianus* 182, showed lower yeast metabolism activity, with significantly ($P<0.05$) lower production of acetaldehyde, ethanol and 2-methyl butanal, and less utilisation of lactose and galactose compared to CC8.

In the CCS where *Kaz. humatica* 228 was the only yeast present, ethanol production was highest when co-cultured with *Leuc. mesenteroides* 141. The latter accumulated high amounts of both glucose and galactose that could be available for utilisation by *Kaz. humatica* 228 (Table 3). In kefir, one fermentation step later, ethanol amounts were only around 30 $mg \cdot kg^{-1}$, compared to between 640±127 and 1603±14 $mg \cdot kg^{-1}$ in the kefir grain fermentates. During kefir grain fermentation, microorganisms are transferrred from the grains to the milk, called shedding, and this has earlier been found to be higher for LAB than for yeasts (Grønnevik et al. 2011). In the second fermentation step, there are thus

fewer yeasts cells present and a correspondingly lower ethanol production will take place. This was also seen for acetaldehyde, a metabolite formed during growth of both yeasts and LAB, that can be reduced to ethanol (Axelsson 2004; Van Dijken et al. 1993). In single-culture, *K. marxianus* 182 produced 15 $mg \cdot kg^{-1}$ of acetaldehyde, and in the kefir grain fermentates the amounts ranged between 7.8±0.2 and 26±12 $mg \cdot kg^{-1}$. Beshkova et al. (2003) have earlier demonstrated a concentration of 18.3 and 9.5 $mg \cdot kg^{-1}$ in kefir produced with kefir starter culture and kefir grains, respectively, and Güzel-Seydim et al. (2000) reported 5 $mg \cdot kg^{-1}$ in kefir from kefir grains. In the kefir here, acetaldehyde amounts were much lower, at 0.4±0.1 $mg \cdot kg^{-1}$. In earlier investigation of this kefir (Porcellato et al. 2012), *Leuconostoc* spp. has been demonstrated present, and its ability to reduce acetaldehyde to ethanol (Hemme and Foucaud-Scheunemann 2004), could be a likely explanation.

Sample grouping by principal component analysis (PCA)

In the PCA plot, the kefir grain fermentates and kefirs grouped in separate clusters (Fig. 1, clusters B and C, respectively). Cluster B was positioned on the right hand side of the plot with the compounds of sensory influence, such as diacetyl, ethanol, ethyl acetate and lactic and acetic acids, due to the high content of these components. On the opposite side of the PCA plot, the majority of the single-strain fermentates were grouped (cluster A) due to low levels of aroma and flavour compounds, but higher levels of carbohydrates and citrate were found, indicating a much lower level of general and specific metabolism. Single-strain *K. marxianus* 182 positioned closer to cluster C due to large lactose reduction and corresponding production of ethanol and 2-methylbutanal (Table 2), whereas *Lc. lactis* subsp. *lactis* 44 positioned closer to cluster B due to extensive citrate reduction and lactate production (Table 3). CCs 4, 5, 6 and 7 clustered in the middle of the

PCA plot (cluster D), whereas CC1 and CC2 were in cluster A due to significantly (P<0.05) lower conversion of citrate and production of lactate compared to the other CCs. CC8, the only CC containing all pure cultures including *K. marxianus* 182, was positioned separate from all other samples, due to high amounts of formic acid and ethanol, as well as extensive lactose degradation. Of all CCs, CC3 showed most similarity with both the kefir grain fermentate group as well as the kefir group when all compounds analysed were compared using ANOVA (Table 4), and was positioned between cluster B and C in the PCA plot (Fig. 1). Adding *Leuc. mesenteroides* 141 to the combination, as in CC4, led to increased ethanol amounts and galactose accumulation, as well as significantly (P<0.05) lower amounts of the aroma metabolites acetoin, diacetyl and acetaldehyde.

Comparison of the kefir grain fermentates using ANOVA showed significant differences (P<0.05) only for ethyl acetate, one of the main volatile compounds produced by kefir yeasts (Liu et al. 2004), which was significantly highest in Rm (Table 2, Fig. 1). Rm is the only of the kefir grain cultures with a definite different origin, acquired from Romania, whereas the remaining cultures all are believed to be of Russian origin. From these results, the necessity of including all seven fermentates in the present manufacture process could be questioned.

The complexity of the kefir grain microbiota composition and its physical arrangement in the kefir grain matrix complicates the understanding of the pathways employed in the collective metabolism (Robinson et al. 2002; Rudi 2008). As seen here, it is likely that several kinds of interrelationships occur, and they could be both of LAB-yeast, LAB-LAB and yeast-yeast character. These interactions may have significant influence on the activities of the different strains, e.g. by the interaction of complementary metabolisms,

where a compound produced by one organism may be metabolised further by another (Narvhus and Gadaga 2003). It is known that yeasts produce compounds essential for LAB growth, such as vitamins, amino acids and purines (Viljoen 2006). In addition, some yeasts can utilise lactic acid and other organic acids, thus increasing the pH and allowing continued growth of LAB (Simova et al. 2006; Viljoen 2001). It is probable that the microorganisms are less exposed to stress conditions such as low pH or suboptimal temperatures when inside the kefir grain matrix (Garrote et al. 2010), and there could also be presence of uncultivable organisms requiring symbiotic interactions that are difficult to reconstruct (Garrote et al. 2010; Kowalczyk et al. 2012), all adding to the difficulty of replacing grains with pure cultures.

Conclusion

Milk fermented with co-cultures of kefir grain isolates of LAB and yeasts gave different profiles of aroma and flavour compounds compared to kefir grain fermentates or commercial 2-step fermentation kefir. Lower amounts of the aroma and flavour compounds, as well as of lactic acid were formed under similar inoculation rates and incubation conditions.

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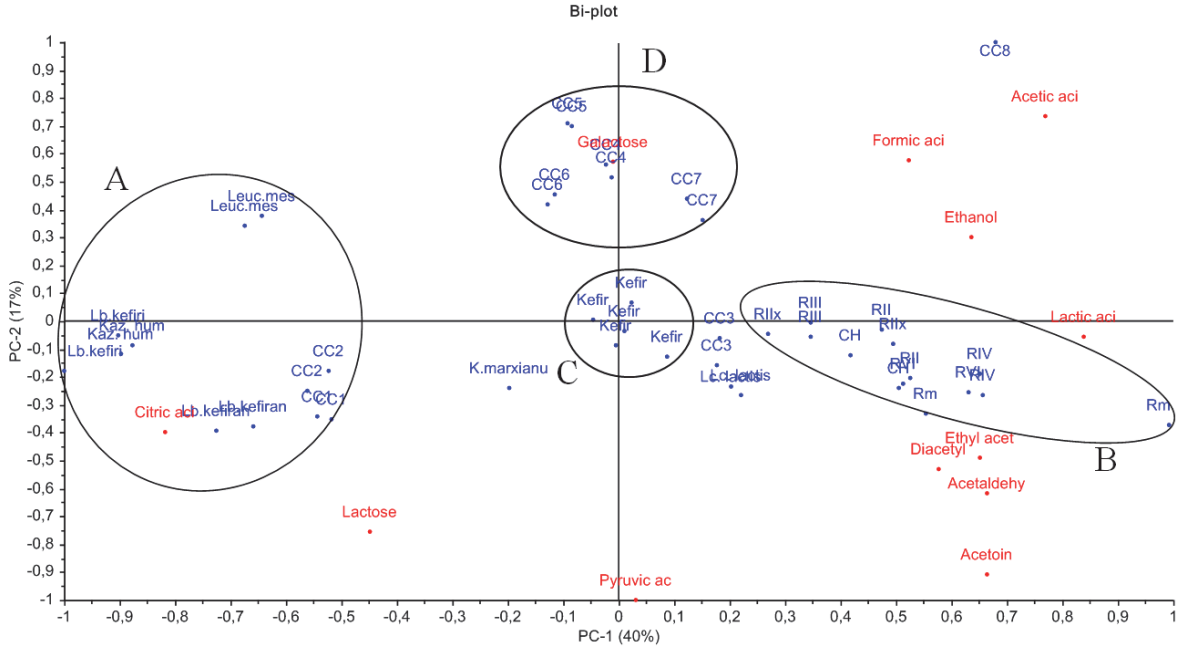

Fig. 1 The scores and loadings bi-plot for volatile compounds, organic acids and carbohydrates analysed in milk fermented with kefir grains and single and co-cultures of *Lactobacillus (Lb.) kefiranofaciens* (in all CCs), *Kazachstania humatica* (in all CCs except 5), *Lb. kefiri* (in CC2 and CC5-8), *Lactococcus lactis* subsp. *lactis* (in CC3-5, CC7 and CC8), *Leuconostoc mesenteroides* (in CC4-8) and *Kluyveromyces marxianus* (CC8), and in the untreated milk and the kefir end product

Table 1 Composition of the CCs. The bacteria and yeast species were isolates from kefir grains, and inoculum sizes were corresponding to the amounts added when inoculating with 5% of kefir grains, as determined previously (Grønnevik et al., paper I)

Bacteria and yeast strains	CC							
	1	2	3	4	5	6	7	8
Lb. kefiranofaciens 119	.	.	.	.	.	.	.	.
Lb. kefiri 88		.			.	.	.	.
Lc. lactis subsp. *lactis* 44			.	.	.		.	.
Leuc. mesenteroides 141				.	.	.	.	.
K. marxianus 182								.
Kaz. humatica 228	.	.	.	.		.	.	.

Table 2 Analysis of volatile compounds of single- and co-cultures of isolated bacteria and yeasts therefrom. The presented results are average of 2 different batches for the kefir grain fermentates and the UHT milk, and of 5 different batches for the kefir and the 1.2% fat kefir inoculation milk. For the single strain and co-culture combinations, results are average of two analysis replicates of one fermented milk sample, except for *K. marxianus* 182 and CC8, where results are single analyses

		Volatile compounds					
		Acetaldehyde mg kg^{-1}	Ethanol mg kg^{-1}	Diacetyl mg kg^{-1}	Acetoin mg kg^{-1}	Ethyl acetate mg kg^{-1}	2-methyl-butanal mg kg^{-1}
Single cultures							
Lc. lactis subsp. *lactis* 44	**Mean**	**5.7**	**4.7**	**3.3**	**289**	**-**	**-**
	St.d.	0.0	0.0	0.2	6.1	-	-
Lb. kefiranofaciens 119	**Mean**	**1.0**	**1.0**	**0.6**	**73**	**-**	**-**
	St.d.	0.0	0.2	0.1	6.0	-	-
K. marxianus 182		**15**	**1870**	**-**	**16**	**0.99**	**0.38**
Kaz. humatica 228	**Mean**	**1.0**	**80**	**-**	**4.3**	**-**	**-**
	St.d.	0.0	33	-	1.4	-	-
Leu. mesenteroides 141	**Mean**	**0.1**	**54**	**-**	**1.8**	**-**	**-**
	St.d.	0.0	0.6	-	0.8	-	-
Lb. kefiri 88	**Mean**	**0.0**	**4.4**	**-**	**1.0**	**-**	**-**
	St.d.	0.0	0.8	-	1.4	-	-
Co-culture combinations							
CC1	**Mean**	**1.3**	**89**	**0.5**	**151**	**-**	**-**
	St.d.	0.0	4.9	0.1	17	-	-
CC2	**Mean**	**0.4**	**87**	**0.5**	**140**	**-**	**-**
	St.d.	0.0	1.2	0.0	0.5	-	-
CC3	**Mean**	**2.9**	**89**	**1.9**	**312**	**-**	**-**
	St.d.	0.0	2.4	0.1	6.5	-	-
CC4	**Mean**	**0.6**	**858**	**-**	**13**	**-**	**0.02**
	St.d.	0.1	30.7	-	6.4	-	0.01
CC5	**Mean**	**0.1**	**386**	**-**	**5.8**	**-**	**-**
	St.d.	0.0	1.2	-	1.4	-	-
CC6	**Mean**	**0.5**	**1344**	**0.2**	**5.8**	**0.04**	**-**
	St.d.	0.0	9.0	0.0	1.2	0.00	-
CC7	**Mean**	**0.6**	**1296**	**1.4**	**6.5**	**0.03**	**0.01**
	St.d.	0.1	28	0.4	1.0	0.00	0.00
CC8		**4.9**	**1744**	**0.9**	**11**	**1.12**	**0.06**
Kefir grain fermentates							
RII	**Mean**	**7.8**	**1603**	**2.2**	**278**	**0.75**	**0.02**
	St.d.	0.2	14	0.5	8.1	0.08	0.00
RIIx	**Mean**	**7.6**	**1153**	**1.5**	**297**	**0.81**	**0.03**
	St.d.	0.7	692	0.7	10	0.32	0.01
RIII	**Mean**	**7.1**	**1047**	**1.3**	**267**	**1.11**	**0.03**
	St.d.	1.6	324	0.1	19	0.11	0.01
RIV	**Mean**	**16**	**1503**	**1.9**	**316**	**2.40**	**0.07**
	St.d.	2.5	142	0.6	19	0.49	0.04
RVI	**Mean**	**16**	**1149**	**1.4**	**337**	**2.52**	**0.07**
	St.d.	6.2	34	0.7	4.9	1.08	0.04
Rm	**Mean**	**26**	**1188**	**0.9**	**332**	**4.50**	**0.11**
	St.d.	11.9	749	0.1	41	1.58	0.04
CH	**Mean**	**12**	**640**	**1.1**	**335**	**2.71**	**0.07**
	St.d.	1.6	127	0.4	3.6	0.17	0.01
Kefir	**Mean**	**0.4**	**29**	**1.0**	**203**	**0.09**	**-**
	St.d.	0.1	1.6	0.5	98	0.02	-
Milks							
Milk [a]	**Mean**	**0.1**	**0.6**	**-**	**5.2**	**0.12**	**-**
	St.d.	0.0	0.2	-	8.8	0.04	-
UHT milk [b]	**Mean**	**0.0**	**0.6**	**-**	**0.0**	**0.01**	**-**
	St.d.	0.0	0.0	-	0.0	0.01	-

- = not detected

[a] used for kefir grain inoculation (1.2% fat)

[b] used for single and co-culture inoculations (1.5% fat)

Table 3 Analysis of organic acids and carbohydrates in kefir grain fermentates and fermentates of single- and co-cultures of isolated bacteria and yeasts therefrom. The presented results are average of 2 different batches for the kefir grain fermentates and the UHT milk, and of 5 different batches for the kefir and the 1.2% fat kefir inoculation milk. For the single strain and co-culture combinations, results are average of two analysis replicates of one fermented milk sample, except for *K. marxianus 182*, where the result is a single analysis

		Organic acids					Carbohydrates			pH
		Citric mg kg^{-1}	Lactic mg kg^{-1}	Acetic mg kg^{-1}	Pyruvic mg kg^{-1}	Formic mg kg^{-1}	Lactose %	Glucose mg kg^{-1}	Galactose mg kg^{-1}	
Single cultures										
Lc. lactis subsp. *lactis 44*	**Mean**	**83**	**6314**	**686**	**10**	**11**	**3.9**	**-**	**286**	**4.57**
	St.d.	1	107	10	1.0	3.5	0.1	-	3.5	
Lb. kefiranofaciens 119	**Mean**	**1256**	**822**	**166**	**18**	**-**	**4.4**	**-**	**171**	**6.22**
	St.d.	44	37	235	0.6	-	0.2	-	4.3	
K. marxianus 182		**1614**	**25**	**122**	**32**	**12**	**2.7**	**-**	**89**	**6.20**
Kaz. humatica 228	**Mean**	**1729**	**-**	**-**	**-**	**10**	**4.3**	**-**	**-**	**6.62**
	St.d.	43	-	-	-	0	0.1	-	-	
Leu. mesenteroides 141	**Mean**	**1145**	**550**	**387**	**-**	**-**	**3.9**	**2072**	**1119**	**6.32**
	St.d.	27	16	0	-	-	0.1	61	33	
Lb. kefiri 88	**Mean**	**1787**	**-**	**-**	**-**	**-**	**4.4**	**98**	**139**	**6.68**
	St.d.	144	-	-	-	-	0.4	6.8	15	
Co-culture										
CC1	**Mean**	**1070**	**928**	**341**	**18**	**9**	**4.2**	**-**	**62**	**6.14**
	St.d.	7.9	0.1	22	0.7	0.3	0.0	-	14	
CC2	**Mean**	**1046**	**897**	**329**	**12**	**4**	**4.1**	**-**	**75**	**6.13**
	St.d.	33	34	22	0.6	5.5	0.1	-	6.1	
CC3	**Mean**	**91**	**6572**	**733**	**8.7**	**22**	**3.6**	**-**	**-**	**4.45**
	St.d.	13	316	1.7	1.0	3.3	0.2	-	-	
CC4	**Mean**	**85**	**6180**	**897**	**-**	**18**	**3.5**	-	**272**	**4.53**
	St.d.	6.4	366	62	-	1.6	0.2	-	28	
CC5	**Mean**	**85**	**6318**	**871**	**-**	**10**	**3.5**	-	**1159**	**4.49**
	St.d.	0.8	41	29	-	0.1	0.0	-	20	
CC6	**Mean**	**179**	**2202**	**875**	**-**	**20**	**3.8**	-	**31**	**5.57**
	St.d.	11	121	50	-	1.5	0.2	-	44	
CC7	**Mean**	**97**	**6712**	**998**	**-**	**25**	**3.9**	**-**	**81**	**4.56**
	St.d.	5.8	197	39	-	3.4	0.2	-	9.3	
CC8	**Mean**	**68**	**3176**	**1083**	**-**	**181**	**2.7**	**-**	**-**	**4.97**
	St.d.	3.3	97	38	-	7.2	0.1	-	-	
Kefir grain fermentates										
RII	**Mean**	**124**	**9064**	**807**	**11**	**32**	**3.9**	**-**	**276**	**4.5**
	St.d.	23	430	19	4.4	1	0.1	-	11	
RIIx	**Mean**	**108**	**8079**	**759**	**8.8**	**33**	**3.9**	**-**	**263**	**4.5**
	St.d.	8	61	14	2.0	7	0.2	-	8.9	
RIII	**Mean**	**109**	**8338**	**767**	**6.2**	**32**	**4.0**	**-**	**310**	**4.5**
	St.d.	8	36	27	0.1	3	0.0	-	2.8	
RIV	**Mean**	**111**	**8409**	**766**	**10**	**33**	**3.8**	-	**291**	**4.5**
	St.d.	10	179	9	6.3	4	0.1	-	1.9	
RVI	**Mean**	**108**	**8186**	**737**	**8.3**	**31**	**3.9**	-	**303**	**4.5**
	St.d.	7	77	36	3.0	2	0.1	-	12	
Rm	**Mean**	**112**	**8733**	**746**	**7.2**	**27**	**3.8**	-	**299**	**4.5**
	St.d.	0	738	57	2.8	4	0.2	-	34	
CH	**Mean**	**108**	**8091**	**743**	**5.2**	**35**	**4.0**	**-**	**294**	**4.5**
	St.d.	10	167	3	2.5	4	0.0	-	1.9	
Kefir	**Mean**	**111**	**7897**	**680**	**13**	**25**	**4.0**	**56**	**300**	**4.5**
	St.d.	6	258	9	1.3	8	0.1	6.0	40	
Milks										
Milk [a]	**Mean**	**1873**	**-**	**-**	**1.3**	**-**	**4.9**	**182**	**72**	**6.6**
	St.d.	46	-	-	0.3	-	0.1	12	27	
UHT milk [b]	**Mean**	**1990**	**-**	**-**	**-**	**-**	**4.7**	**81**	**42**	**6.6**
	St.d.	340	-	-	-	-	0.4	89	27	

- = not detected

[a] used for kefir grain inoculation (1.2% fat)

[b] used for single and co-culture inoculations (1.5% fat)

Table 4 The significance of differences between the co-culture fermentates, the group of kefir grain fermentates and the group of kefirs analysed using ANOVA one-way Tukey 95% Simultaneous Confidence Intervals. Means that do not share a letter were significantly different, and component amounts were descending with letter order in the alphabet

	P-value	CC1	CC2	CC3	CC4	CC5	CC6	CC7	CC8	Kefir grain ferm.	Kefir
Acetaldehyde	0.002	AB	AB	AB	AB	AB	AB	AB	AB	A	B
Ethanol	0.000	C	C	C	ABC	BC	AB	AB	AB	AB	C
Diacetyl	0.000	AB	AB	A	B	B	B	AB	AB	A	AB
Acetoin	0.000	CD	CD	AB	D	D	D	D	D	A	BC
Ethyl acetate	0.006	AB	AB	AB	AB	AB	AB	AB	AB	A	B
2-metyl-butanal	0.003	AB	AB	AB	AB	AB	AB	AB	AB	A	B
Citric acid	0.000	A	A	CD	CD	CD	B	CD	D	C	C
Lactic acid	0.000	D	D	B	B	B	C	B	C	A	A
Acetic acid	0.000	F	F	DE	BC	C	C	AB	A	D	E
Formic acid	0.000	DE	E	BCD	CDE	DE	BCDE	BCD	A	B	BC
Lactose	0.000	A	AB	BCD	CD	D	ABCD	ABCD	E	ABC	AB
Galactose	0.000	C	C	C	B	A	C	C	C	B	B

Paper IV

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Microbiological and chemical properties of Norwegian kefir during storage

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ABSTRACT

Five commercial productions of Norwegian kefir were investigated for development in microbiology, volatile compounds, organic acids, carbohydrates and free amino acids during cold storage for 8 weeks. Lactic acid bacteria numbers decreased during the first 4 weeks of storage, whereas yeast numbers increased throughout the storage period. The important yeast metabolites CO_2 and ethanol both increased throughout the storage period. The amino acid glutamic acid was reduced during storage, and a consequent increase in its decarboxylation product γ-aminobutyric acid (GABA) was found. GABA has earlier been found to have blood-pressure-lowering effect in mild hypertensives when consumed in fermented milk in amounts of 10 mg daily over a 12-week period.

1. Introduction

Kefir is a fermented milk product with a long history. From its origin in the Caucasian mountains of the former USSR, the making and consumption of kefir has spread to many parts of the world. In Norway, kefir has been commercially produced since the 1930s.

Kefir grains comprise the starter culture for kefir. Resembling small cauliflower florets in appearance, they vary in size from approximately 3 to 30 mm, and contain a complex mixture of lactic acid bacteria (LAB), acetic acid bacteria and yeasts in a protein and polysaccharide matrix. For traditional kefir production, kefir grains are added to cows' milk in a 1:20 ratio, and left to ferment at 18–20 °C for about 20 h. At the end of the fermentation, the kefir grains are retrieved by sieving and re-used for new fermentations. However, for the commercial production of large volumes of kefir, direct fermentation with kefir grains is impractical as this would demand a correspondingly large quantity of grains. In addition, the vigorous fermentation gives a product that contains a high level of CO_2 and the liberation of gas disturbs the gel, causing whey separation. Commercial production of kefir thus often involves several fermentation steps, including the making of a mother culture and a bulk starter (Wszolek, Tamime, Muir, & Barclay, 2001).

After fermentation, kefir is viscous and slightly carbonated with a sharp acidic and slight yeasty flavour. When consuming kefir, a prickling sensation on the tongue is typical, due to CO_2 produced by the yeast microflora (Farnworth, 2005; Irigoyen, Arana, Castiella, Torre, & Ibáñez, 2005).

The microbiota of kefir grains is poorly defined and complex and varies accordingly to different grain origins or grain cultivation methods (Witthuhn, Schoeman, & Britz, 2005). The microbiological profile of the kefir product is different from the grains and is considerably affected by the origin of the kefir grains used and also the proportion of the initial kefir grain inoculum (Farnworth, 2005; Garrote, Abraham, & De Antoni, 1998; Koroleva, 1988a; Simova et al., 2002).

Overall, there are few reports on changes occurring during storage of kefir, and no reports found in the available literature document results from more than 4 weeks of storage. The production procedures reported for the different kefirs vary in the number of fermentation steps, inoculation rate and starter culture used, and this must be kept in mind when comparing results. In addition, most published studies concern milk fermented directly with kefir grains, i.e. equivalent to a mother culture, whereas commercial kefir production uses at least one more fermentation step. The aim of this work was to use a comprehensive analysis setup to investigate the development in the microbiological profile and changes in the metabolites present in Norwegian commercial kefir during fermentation and 8 weeks of cold storage.

2. Materials and methods

2.1. Kefir

Due to in-house factory challenges, the bulk starter step of the fermentation was omitted for all kefir productions examined in this work. Direct inoculation with 0.2% (v/v) of mother culture was used as starter, fermenting at 20–22 °C for approximately 20 h to pH 4.5. Fifteen samples (1 L cartons) from each of 5 commercial productions

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of kefir (denoted replicates A–E) were collected on the day of production from the Norwegian dairy company TINE Meieret Øst (Oslo, Norway) over a period of 2 weeks. The samples were stored at 5.5–6 °C until analysis. All productions were analysed when freshly fermented, and then after 1, 2, 3 (commercially stated shelf life), 4 and 8 weeks of storage. At each sampling point, two new cartons were opened; one for microbiological and one for chemical analysis. In addition, samples of the original homogenised and heat treated milk before fermentation were collected and analysed.

2.2. pH

For pH measurements, a Radiometer (PHM92) pH metre with a combined glass electrode and temperature probe (Radiometer, Copenhagen, Denmark) was used. The pH metre was calibrated using standard buffer solutions at pH 4.0 and 7.0 (Merck, Darmstadt, Germany).

2.3. Carbon dioxide

Carbon dioxide production was determined using an infra red (IR) gas analyser (ADC 225 MK3, Analytical development Co. Ltd., Hoddesdon, Hertfordshire, UK), as described by Østlie, Helland, and Narvhus (2003). A 1 mL sterile disposable plastic syringe with a sterile 5/8″ needle (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used to puncture the top of the kefir carton and withdraw the headspace gas for analysis. The amount of CO_2 in the newly fermented kefir was set as index value 1, and the results are given as a relative indexed increase according to this.

2.4. Microbiological analysis

The development in microflora was investigated by making serial dilutions of kefir in 0.1% peptone water (LP0040, Oxoid Ltd, Basingstoke, England) (Mian, Fleet, & Hocking, 1997). Dilutions were plated on M17 (Merck) and MRS (Merck) for presumptive *Lactococcus* spp. and presumptive *Lactobacillus* spp., respectively. For yeasts, yeast extract glucose chloramphenicol (YGC) agar (Merck) was used, whereas for presumptive *Leuconostoc* spp. LD-agar with the following composition was used: 2% (w/w) tryptone (LP0042, Oxoid), 0.5% (w/w) yeast extract (Difco Laboratories, Sparks, MI, USA), 0.25% (w/w) gelatine (Merck), 1% (w/w) lactose (BDH Laboratory Supplies, Poole, England), 0.4% (w/w) sodium chloride (Merck), 0.2% (w/w) tri-sodium citrate dihydrate (Merck), 0.8% (w/w) calcium lactate pentahydrate (Sigma–Aldrich Chemie GmbH, Steinheim, Germany), 1.5% (w/w) agar (Merck) and 200 mg L^{-1} vancomycin hydrochloride (Sigma–Aldrich). VRBA (Oxoid) was used to check for coliforms. To avoid growth of yeasts on the bacterial plates and vice versa, 200 mg L^{-1} cycloheximide (Calbiochem, La Jolla, CA, USA) was added to M17 and MRS, and 100 mg L^{-1} oxytetracycline hydrochloride (Calbiochem) was added to YGC (Chen, Wang, & Chen, 2008; Irigoyen et al., 2005). Plates were incubated aerobically at 30 °C for 2 d for M17, at 20 °C for 4 d for LD, at 25 °C for 5–7 d for YGCA, at 37 °C for 1 d for VRBA, and in a CO_2 incubator (W.C. Heraeus GmbH, Hanau, Germany) at 10% CO_2 at 30 °C for 4 d for MRS. The results were expressed as log colony forming units per mL (cfu mL^{-1}) [of kefir].

2.5. Volatile compounds

Volatile compounds were measured using headspace gas chromatography (HSGC) according to the method of Narvhus, Østeraas, Mutukumira, and Abrahamsen (1998), with the following modifications regarding equipment: The headspace sampler used was a HP 7694 with a 6890 GC system (Agilent, Santa Clara, CA, USA), Series 900 interface connector clips (Perkin Elmer, Shelton, CT, USA), a hydrogen generator (Model 75-32, Whatman, Haverhill, MA, USA) with 1.6 bar pressure and TotalChrom LC software (Perkin Elmer). Volatile compounds were separated on a CP-SIL 5CB GC column: 25 × 0.53 mm I.D., film thickness 5 μm (Varian, Middelburg, The Netherlands). The GC was fitted with a flame ionisation detector at 200 °C. Peaks were identified according to their retention times and quantified using external standard solutions of the following compounds: acetaldehyde, 2-butanone, ethyl acetate, 2-methyl-1-propanol, 2-methyl-butanal, 3-methyl-butanal, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanal, diacetyl (Sigma–Aldrich); 2-butanol, acetoin, acetone, 2.3-pentadion (Merck), ethanol (Arcus, Oslo, Norway).

2.6. Organic acids and carbohydrates

Organic acids and carbohydrates were analysed using high performance liquid chromatography (HPLC), by a modification of the method of Marsili, Ostapenko, Simmons, and Green (1981). The procedure used was as described by Narvhus et al. (1998), with the following modifications: The temperature of the column was 32 °C, and the concentration of H_2SO_4 in the mobile phase was 15 mM. Standard solutions for external calibration were prepared the same way as the samples, and organic acids and carbohydrates were identified according to their retention times compared with the standard solutions. The organic acids used for standard solutions were citric, orotic, pyruvic, succinic, lactic, formic, acetic, uric and propionic acids (Sigma–Aldrich), and the carbohydrates were lactose, glucose and galactose (Merck).

2.7. Free amino acids

Free amino acids were analysed using high performance liquid chromatography (HPLC) as described by Skeie, Feten, Almøy, Østlie, and Isaksson (2006), with the following modifications: The auto-injector used was a 1200 series, the LC terminal was TotalChrom (Perkin Elmer) and the column was an XTerra RP 150 × 4.6 mm column with particle size 3.5 μm (Waters Corp., Milford, MA, USA). Derivatisation with *o*-phthaldialdehyde (OPA) and fluorenylmethyl chloroformate (FMOC) was done according to Bütikofer and Ardö (1999), with the modification that the internal standard contained 0.2 μmol mL^{-1} L-norvalin (Merck).

2.8. Statistical analysis

The significance of differences in analysed variables with respect to storage time was tested by Student's t test (unequal variances) using Unscrambler® X (Camo Software AS, Oslo, Norway).

3. Results and discussion

3.1. Lactic acid bacteria

Earlier bacteria isolation experiments in our laboratory from kefir grains have shown through 16S rRNA sequencing that all isolates from M17 plates were *Lactococcus* spp. From MRS plates, 95% of all isolates were *Lactobacillus* spp., whereas the remaining 5% were *Lactococcus* spp. (Grønnevik, unpublished results). In this experiment, presumptive lactobacilli and presumptive lactococci were both present at levels of 8 $\log_{10}$ cfu mL^{-1} in the newly fermented kefir (Fig. 1), and these results are in agreement with findings in Spanish, Turkish, South African, Scottish and Polish kefir (García Fontán, Martínez, Franco, & Carballo, 2006; Güzel-Seydim, Twyffels, Seydim, & Greene, 2005; Irigoyen et al., 2005; Loretan, Mostert, & Viljoen, 2003; Wszolek et al., 2001). After 4 weeks of

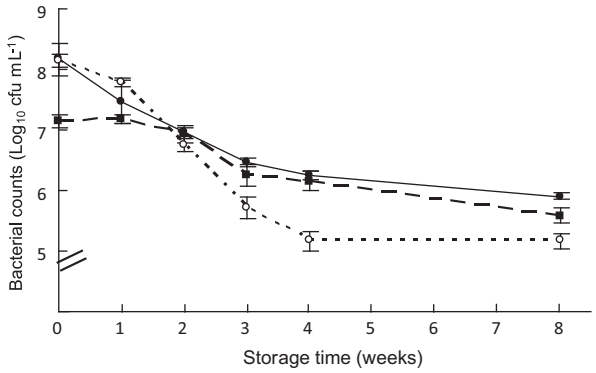

Fig. 1. Development of presumptive *Lactococcus* spp. (○, M17), presumptive *Lactobacillus* spp. (●, MRS) and presumptive *Leuconostoc* spp. (■, LD-agar) in kefir during 8 weeks of storage (time 0 = after incubation). The presented results are average values from 5 kefir productions; error bars show standard deviation.

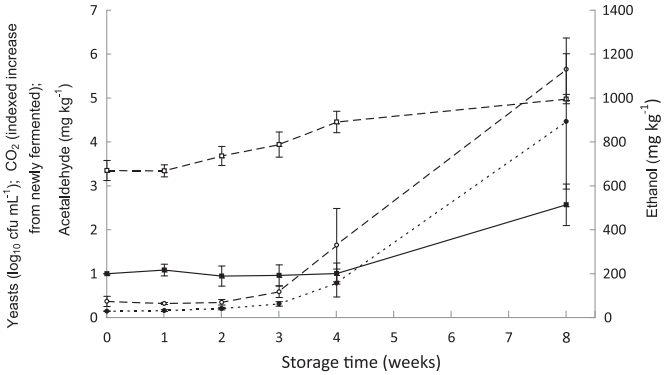

Fig. 2. Development in yeast growth (□), CO_2 (■), ethanol (●) and acetaldehyde (○) in kefir during 8 weeks of storage (time 0 = after incubation). The presented results are average values from 5 kefir productions; error bars show standard deviation.

storage, the numbers had decreased significantly ($P < 0.01$) by 2 and 3 $\log_{10}$ units, respectively, and were at the same level after 8 weeks of storage. The growth medium chosen for *Leuconostoc* spp. contained vancomycin for inhibition of *Lactococcus* spp., as well as some *Lactobacillus* species (Danielsen & Wind, 2003; Elliott & Facklam, 1996). Earlier experiments in our laboratory showed that approximately 50% of the bacterial isolates from kefir grains on LD-agar were *Leuconostoc* spp., whereas the other 50% belonged to the species *Lactobacillus kefiri* (Grønnevik, unpublished results). *L. kefiri* thus tolerates vancomycin concentrations of 200 mg L^{-1}, and could also constitute a part of the bacterial numbers found on LD-agar in this experiment. Like presumptive lactobacilli and lactococci, the amounts of presumptive *Leuconostoc* spp. also decreased significantly ($P < 0.01$) during storage, from 7 $\log_{10}$ cfu mL^{-1} in newly fermented kefir to 5.6 $\log_{10}$ cfu mL^{-1} after 8 weeks of storage (Fig. 1). No coliform bacteria were found in any of the samples at any time of analysis. pH decreased significantly ($P < 0.05$) during the first week of storage from 4.50 ± 0.05 to 4.41 ± 0.05 and remained at that level throughout storage (results not shown), whereas no significant change was found in levels of lactic acid during storage (Fig. 4b).

3.2. Yeasts, carbon dioxide and ethanol

For yeasts, the Fermented Milks Codex (Codex Alimentarius Commission, 2003) lists 10^4 cfu g^{-1} as minimum yeast content for kefir, however the amounts found in the newly made kefir analysed in this study were somewhat lower, 3.3 $\log_{10}$ cfu mL^{-1} (Fig. 2). Yeast amounts in kefir vary, and reported values range from 10^3 to10^6 (Duitschaever, Kemp, & Emmons, 1988; Farnworth, 2005; Güzel-Seydim et al., 2005; Irigoyen et al., 2005; Kuo & Lin, 1999; Simova et al., 2002; Zajsek & Gorsek, 2010). In South African household kefir, yeast levels as high as 8 $\log_{10}$ cfu mL^{-1} have been found (Loretan et al., 2003). Yeast numbers continuously increased during storage; a significant increase to 4 $\log_{10}$ cfu mL^{-1} occurred after 3 weeks and a further significant increase to 5 $\log_{10}$ cfu mL^{-1} occurred after 8 weeks of storage (Fig. 2).

As yeasts numbers increased during storage, correspondingly significant increases in ethanol and CO_2 levels occurred between 4 and 8 weeks of storage (Fig. 2). This was an expected result as ethanol and CO_2 are the main end products of yeast fermentation (Beshkova, Simova, Frengova, Simov, & Dimitrov, 2003; Latorre-García, del Castillo-Agudo, & Polaina, 2007). Ethanol amounts reached 890 ± 300 mg kg^{-1} after 8 weeks, which was within the expected range as the alcohol content seldom exceeds 0.1% in modern commercial kefir production (Koroleva, 1988b).

3.3. Carbohydrate metabolism

The glucose in the original milk was reduced to <60 mg kg^{-1} after fermentation, and completely metabolised after 3 weeks of storage (Fig. 3). Lactose decreased during fermentation, as expected as lactose is hydrolysed to glucose and galactose moieties early in LAB lactose metabolism. Also, *Kluyveromyces marxianus* is one of the few yeast species that possesses β-galactosidase, making utilisation of lactose for pyruvate production possible (Dickinson & Kruckenberg, 2006; Graciano Fonseca, Heinzle, Wittmann, & Gombert, 2008), and the presence of this yeast has earlier been demonstrated in Norwegian kefir grains (Grønnevik, unpublished results). During storage, no significant change was found in lactose in weeks 0–4, whereas a significant decrease occurred between weeks 4 and 8. The significantly higher yeast numbers in the last half of the storage period compared to the first (Fig. 2), and the presence of *K. marxianus* indicate that lactose was utilised by yeast metabolism in weeks 4–8 of storage.

During the first 4 weeks of storage, galactose increased significantly. Such galactose accumulation has earlier been found during LAB fermentation in yogurt (Robinson, Tamime, & Wszolek, 2002), and has been suggested to contribute in LAB-yeast interaction, as it could favour the growth of lactose-negative yeasts (Koroleva, 1988a). However, in the kefir investigated in this work it is more likely to be a consequence of yeast growth and lactose hydrolysis, as yeast numbers also increased significantly in the same period (Fig. 2). In weeks 4–8 of storage, galactose decreased significantly whereas yeast numbers increased. Along with the lactose decrease

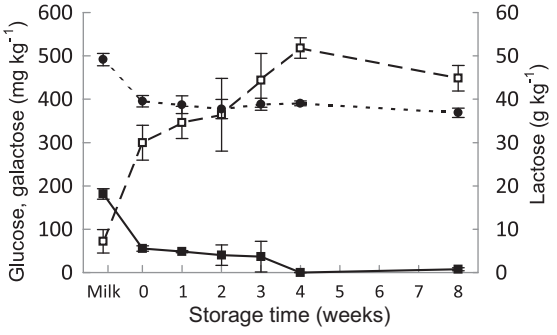

Fig. 3. Development of lactose (●), glucose (■) and galactose (□) in kefir during 8 weeks of storage (time 0 = after incubation). The presented results are average values from 5 kefir productions; error bars show standard deviation.

found in the same storage period, this could indicate alterations in carbohydrate metabolism of the kefir microorganisms.

3.4. Volatile compounds and organic acids

Acetaldehyde can be a metabolite from pyruvate metabolism, as well as from threonine conversion in LAB or a secondary metabolite from yeast growth (Christensen, Dudley, Pederson, & Steele, 1999; Lees & Jago, 1976; Romano, Capece, & Jespersen, 2006). Until 4 weeks of storage, little change in acetaldehyde concentration was found, then between 4 and 8 weeks of storage the concentration increased significantly to 6 ± 1 mg kg^{-1} (Fig. 2), corresponding to the increase in yeast growth and ethanol concentration during the same period. This is a lower level of acetaldehyde than found in e.g. Turkish kefir inoculated directly with 5% kefir grains, which was found to reach between 11 and 25 mg kg^{-1} after fermentation and up to 3 weeks of storage (Güzel-Seydim, Seydim, & Greene, 2000; Güzel-Seydim, Seydim, Greene, & Bodine, 2000). The optimum flavour balance is reported to be achieved when the ratio of diacetyl to acetaldehyde in the kefir is 3:1 (Muir, Tamime, & Wszolek, 1999). This ratio was only found in one of the newly fermented kefirs in this work. During storage the ratio was always 0.7:1 or lower, due to the low diacetyl levels.

The citrate present in the original milk decreased by more than 90% during fermentation presumably due to citrate metabolism by LAB, and a concurrent increase was found in the concentrations of acetate and the volatile compounds diacetyl and acetoin (Fig. 4a and b). The subsequent decrease in amounts of diacetyl and acetoin after the first week of storage was probably due to further reduction to 2,3-butanediol (Hugenholtz, 1993), although this could not be confirmed as this component was not measurable on the HSGC used.

Acetic acid is an intermediate in citrate metabolism, as well as one of the products of heterofermentative lactose metabolism and it can be produced by acetic acid bacteria by oxidation of ethanol (Drysdalge & Fleet, 1989; Hugenholtz, 1993; Østlie et al., 2003). Acetic acid increased during kefir fermentation to 800 mg kg^{-1} (Fig. 4b). This was well in agreement with reported amounts produced from citrate metabolism by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* (Verhue & Tjan, 1991). Furthermore, the acetic acid concentration remained stable throughout the storage period, indicating neither production by acetic acid bacteria nor production or utilisation by yeasts (Deak, 2008, pp. 59–70; Dickinson & Kruckenberg, 2006).

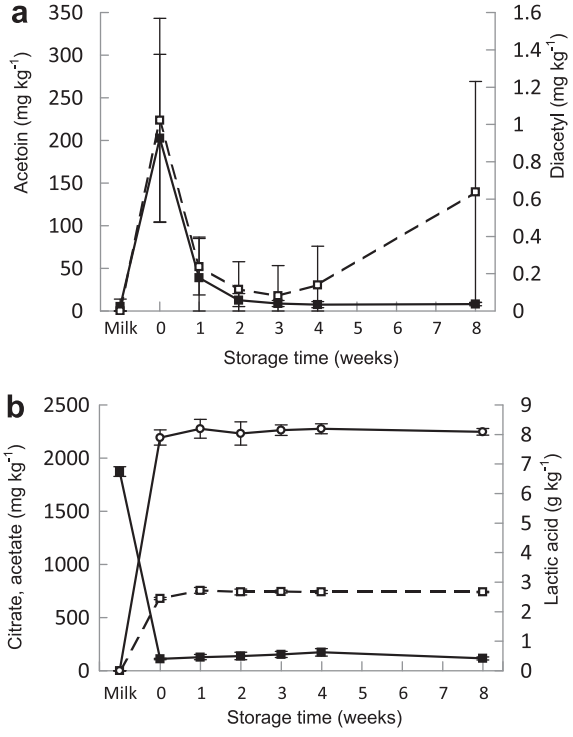

Fig. 4. Development in a) acetoin (■) and diacetyl (□) and b) citrate (■), acetate (□) and lactic acid (○) in kefir during fermentation and 8 weeks of storage (time 0 = after incubation). The presented results are average from 5 kefir productions; error bars show standard deviation.

3.5. Free amino acids

LAB require a complex mix of nutrients for growth, among them different amino acids. Milk contains only trace amounts of amino acids, so LAB need proteolytic systems to obtain sufficient amounts (Robinson et al., 2002). In kefir, LAB might be provided with essential growth factors such as amino acids and small peptides resulting from yeast metabolism (Leroi & Pidoux, 1993; Narvhus & Gadaga, 2003; Paramithiotis, Gioulatos, Tsakalidou, & Kalantzopoulus, 2006; Roostita & Fleet, 1996). The concentrations of free amino acids measured in the kefir at each time of analysis may be considered a result of a combination of proteolytic activity, assimilation of peptides and release of amino acids from the cells.

In the kefirs in this study, the most prominent of the free amino acids at all times was glutamic acid, and the amounts increased significantly ($P < 0.01$) during fermentation from 658 ± 20 mmol kg^{-1} in the original milk to 1644 ± 297 mmol kg^{-1} in the newly fermented kefir (Fig. 5a). Glutamic acid can be formed from proteolytic activity, from deamination of glutamine, or from transamination of other amino acids as the preferred amino group acceptor α-ketoglutarate is converted to glutamic acid (Fernández & Zúñiga, 2006; Smit, Smit, & Engels, 2005). The increase during fermentation was likely not a consequence of transamination, as α-ketoglutarate amounts also increased during fermentation. Glutamic acid can be decarboxylated to γ-aminobutyric acid (GABA) (Fernández & Zúñiga, 2006), and during storage glutamic acid continuously decreased, whereas GABA

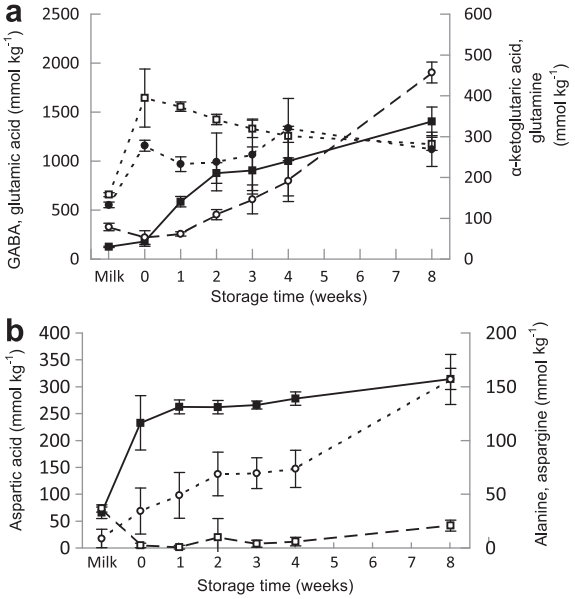

Fig. 5. Development in a) α-ketoglutarate (●), γ-aminobutyric acid (■, GABA), glutamic acid (□) and glutamine (○) and b) aspartic acid (■), alanine (□) and asparagine (○) in kefir during fermentation and 8 weeks of storage (time 0 = after incubation). The presented results are average values from 5 kefir productions; error bars show standard deviation.

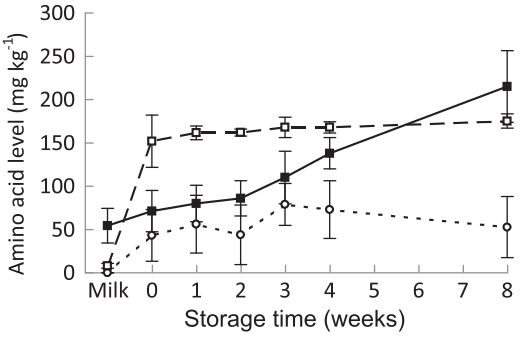

Fig. 6. Development of histidine (○), tyrosine (□) and serine (■) in kefir during fermentation and 8 weeks of storage (time 0 = after incubation). The presented results are average values from 5 kefir productions; error bars show standard deviation.

continuously increased, ending at 1175 ± 67 mmol kg^{-1} and 1405 ± 146 mmol L^{-1}, respectively, after 8 weeks of storage (Fig. 5a). GABA has been found to have blood-pressure-lowering effect in mild hypertensives when amounts of 10 mg in a fermented milk were consumed daily over a 12-week period (Inoue et al., 2003). Approximately 530 g of newly fermented kefir corresponds to 10 mg of GABA, but as GABA levels increased during storage, the amount of kefir necessary for a 10 mg intake of GABA would decrease to 100 g after 2 weeks.

During fermentation, alanine levels decreased significantly ($P < 0.01$) from 37 ± 3 mmol kg^{-1} to 2.4 ± 2.2 mmol kg^{-1} (Fig. 5b), likely due to catabolism to pyruvate (Fernández & Zúñiga, 2006; Le Bars & Yvon, 2008). Aspartic acid levels increased significantly ($P < 0.01$) during fermentation, from 66 ± 11 mmol kg^{-1} to 233 ± 50 mmol kg^{-1}, probably due to proteolytic activity. The increased yeast numbers in the last part of the storage period (weeks 4–8) would be expected to lead to increased proteolytic activity in the kefir, possibly explaining the increase in asparagine during this period (Fig. 5b).

Serine and aromatic amino acids histidine and tyrosine increased during fermentation (Fig. 6), probably due to proteolytic activity. Little net change was found in tyrosine levels during storage, whereas serine continued increasing. This could either be a result of dynamic and continuous reactions keeping levels stable, or more likely accumulation, if metabolic energy could be generated in other ways (Christensen et al., 1999). Histidine levels during storage either remained constant, or decreased. The decrease found in some samples could be due to decarboxylation to histamine, as regulation of intracellular pH and the generation of metabolic energy (Christensen et al., 1999), or to deamination to glutamic acid (Fernández & Zúñiga, 2006), although glutamic acid levels continuously decreased during storage due to decarboxylation to GABA.

4. Conclusions

To the best of our knowledge, this study has been the first to investigate kefir during a storage period of 8 weeks, and the first to report on storage of commercial kefir fermented with mother culture made from kefir grains as starter. The changes in kefir properties were less pronounced during weeks 0–4 than during weeks 4–8 of storage and the decrease in LAB and increase in yeasts throughout the storage period indicate that the changes were probably due to yeasts and their metabolism.

Acknowledgements

The authors are grateful to Kari R. Olsen for assistance with the HSGC and HPLC analyses, and to Professor Arne Tronsmo and Associate Professor Hilde Østlie for proof-reading and comments.

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Corrigendum

Corrigendum to "Microbiological and chemical properties of Norwegian kefir during storage" [Int Dairy J 21 (2011) 601–606]

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Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway

The authors regret that there was a calculation error in the data concerning amino acid analysis in their paper "Microbiological and chemical properties of Norwegian kefir during storage" published in International Dairy Journal. The necessary corrections to the article are given below.

The authors would like to apologise for any inconvenience caused.

Figure corrections:

New version of Fig. 5 (figure text unchanged):

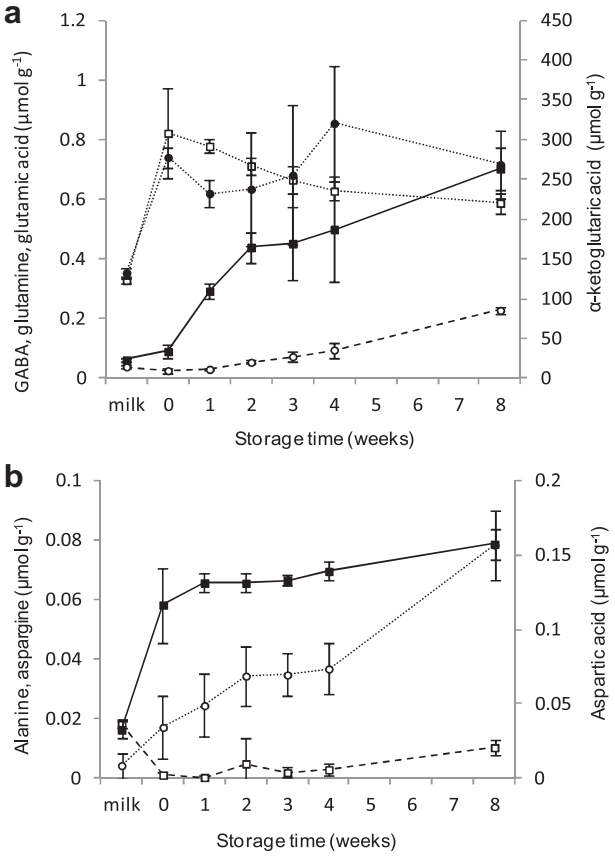

Fig. 5. Development in a) α-ketoglutarate (●), γ-aminobutyric acid (■, GABA), glutamic acid (□) and glutamine (○) and b) aspartic acid (■), alanine (□) and asparagine (○) in kefir during fermentation and 8 weeks of storage (time 0 = after incubation). The presented results are average values from 5 kefir productions; error bars show standard deviation.

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0958-6946/$ – see front matter
doi:10.1016/j.idairyj.2012.03.005

New version of Fig. 6, (figure text unchanged):

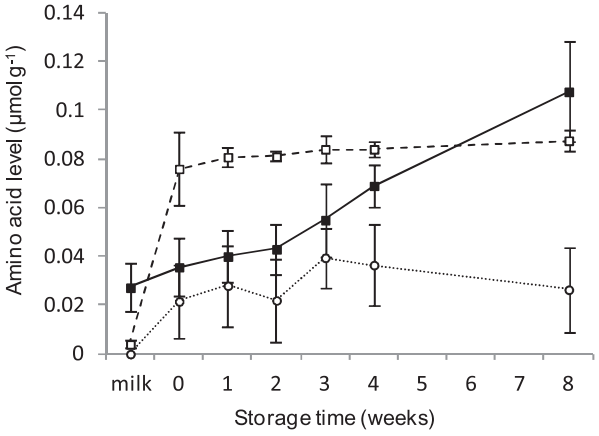

Fig. 6. Development of histidine (○), tyrosine (□) and serine (■) in kefir during fermentation and 8 weeks of storage (time 0 = after incubation). The presented results are average values from 5 kefir productions; error bars show standard deviation.

Text corrections:

- Page 604, section 3.5. *Free amino acids*
  Lines 12–16 (second paragraph)
  Changes from:

  "In the kefirs in this study, the most prominent of the free amino acids at all times was glutamic acid, and the amounts increased significantly ($P < 0.01$) during fermentation from 658 ± 20 mmol kg^{-1} in the original milk to 1644 ± 297 mmol kg^{-1} in the newly fermented kefir (Fig. 5a)."

  Changes to:

  "In the kefirs in this study, the most prominent of the free amino acids at all times was glutamic acid, and the amounts increased significantly ($P < 0.01$) during fermentation from 0.33 ± 0.01 μmol g^{-1} in the original milk to 0.82 ± 0.15 μmol g^{-1} in the newly fermented kefir (Fig. 5a)."

- Page 605, lines 1–2
  Changes from:

  "… continuously increased, ending at 1175 ± 67 mmol kg^{-1} and 1405 ± 146 mmol L^{-1}, respectively, after 8weeks of storage (Fig. 5a)."

  Changes to:

  "… continuously increased, ending at 0.59 ± 0.03 μmol g^{-1} and 0.7 ± 0.07 μmol g^{-1}, respectively, after 8weeks of storage (Fig. 5a)."

- Page 605, lines 5–14
  Changes from:

  "Approximately 530 g of newly fermented kefir corresponds to10 mg of GABA, but as GABA levels increased during storage, the amount of kefir necessary for a 10 mg intake of GABA would decrease to 100 g after 2 weeks. During fermentation, alanine levels decreased significantly ($P < 0.01$) from 37 ± 3 mmol kg^{-1} to 2.4 ± 2.2 mmol kg^{-1} (Fig. 5b), likely due to catabolism to pyruvate (Fernández & Zúñiga, 2006; Le Bars & Yvon, 2008). Aspartic acid levels increased significantly ($P < 0.01$) during fermentation, from 66 ± 11 mmol kg^{-1} to 233 ± 50 mmol kg^{-1}, probably due to proteolytic activity."

  Changes to:

  "Approximately 1 kg of newly fermented kefir corresponds to10 mg of GABA, but as GABA levels increased during storage, the amount of kefir necessary for a 10 mg intake of GABA would decrease to 220 g after 2 weeks. During fermentation, alanine levels decreased significantly ($P < 0.01$) from 0.018 ± 0.002 μmol g^{-1} to 0.001 ± 0.001 μmol g^{-1} (Fig. 5b), likely due to catabolism to pyruvate (Fernández & Zúñiga, 2006; Le Bars & Yvon, 2008). Aspartic acid levels increased significantly ($P < 0.01$) during fermentation, from 0.033 ± 0.005 μmol g^{-1} to 0.116 ± 0.025 μmol g^{-1}, probably due to proteolytic activity."