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Effects of cow's and goat's milk as fermentation media on the microbial ecology of sugary kefir grains

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ABSTRACT

In the present study, we have investigated the importance of fermentation media on grain formation and the microbial characteristics of sugary kefir. The sugary kefir grains were fermented in brown sugar, cow's milk or goat's milk. Using culture-dependent and culture-independent methods, we identified the microorganisms present in both the grains and filtrate and then evaluated their distribution. The structure of the grains was also observed by scanning electronic microscopy (SEM). The identification results indicated that there were remarkable changes in microbial ecological profiles of the sugary kefir grains and their filtrates when brown sugar and milk were compared as fermentation media. Three lactic acid bacteria (LAB) species (*Leuconostoc mesenteroides*, *Lactobacillus mali* and *Lactobacillus hordei*) were found in the grains fermented using brown sugar. However, four species, named *Leu. mesenteroides*, *Lactococcus lactis*, *Bifidobacterium psychraerophilum* and *Enterococcus faecalis*, were identified in the grains fermented using either cow's or goat's milk. The size and structure of the kefir grains were also significantly influenced by the culture medium. We hypothesize that the grains originally may contain many different microorganisms and the identified changes are an adaption to each specific medium during grain formation and growth. The distribution of strains thus may vary depending on the carbon and energy sources available for grain fermentation and these microbial changes will further affect the granulation and growth of the grains. This study is important to our understanding of the mechanism of kefir grain formation and growth because it explores the relationship between fermentation media and kefir microorganisms.

1. Introduction

Milk kefir is commonly regarded as a dairy drink, the fermentation of which is induced by small, white, cauliflower-shaped masses named 'kefir grains' (Chen et al., 2008). The microbial population of these grains includes various bacterial and yeast species, the cells of which are firmly embedded in these grains. However, there is another fermented beverage, which is made from sugar and water with or without added fruit (usually figs and lemon), the fermentation of which is induced by grains of a different type. These grains are called sugary kefir grains. Sugary kefir grains are small transparent mucilaginous masses that consist of a polysaccharide gel containing embedded lactic acid bacteria (LAB) and yeasts. In contrast to milk kefir grains, which are made up of a complex of heteropolysaccharides namely kefiran, sugary kefir grains mainly consist of dextran (α 1–6 linked glucose polymer) (Waldherr et al., 2010). The first scientific description was published in nineteenth century where the granules were called 'ginger beer plants', which was a similar system as Tibi with a Mexican origin, associated to *Opuntia* plants (Leroi and Pidoux, 1993a). Sugary kefir is a popular health promoting beverage. Its health benefits include antimicrobial activities (Rodrigues et al., 2005a; Silva et al., 2009), anti-inflammatory effects (Diniz et al., 2003; Moreira et al., 2008) and cicatrizing activities (Moreira et al., 2008; Rodrigues et al., 2005b).

Several investigations have explored the microorganisms present in sugary kefir. The bacteria present include *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Enterobacter* spp. and *Gluconobacter* spp. and the yeasts present include *Saccharomyces* spp., *Zygosaccharomyces* spp., *Hahsenaspora* spp., *Hanseniaspora* spp., *Honsenizspora* spp. and *Candida* spp.; these species were identified across various different groups of sugary kefir grains (Waldherr et al., 2010). The yeast and LAB strains present in sugary kefir grains are in symbiosis. Leroi and Pidoux (1993a) indicated that this symbiotic relationship is confirmed by the fact that the LAB strains in the kefir are stimulated to grow by the release of glucose and fructose by the yeasts present in the kefir. Furthermore, among the microorganisms found in the sugary kefir grains, *Lactobacillus higardii* has been reported to be very

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important for grain formation (Waldherr et al., 2010). A glucansucrase, which was purified from *Lb. higardii*, is responsible for the production of dextran and this then plays an important part in maintaining the ecological niche associated with the grains.

Microbial and chemical properties may change depending on the culture media used and the country where the grain is produced. Silva et al. (2009) indicated that sugary kefir grains fermented with brown sugar show the greatest antimicrobial activity and inhibit *Candida albicans*, *Salmonella typhi*, *Shigella sonnei*, *Staphylococcus aureus* and *Escherichia coli* when compared to kefir fermented using molasses and demerara sugar. The interactions between yeasts and LAB also significantly change depending on the sugar source (Leroi and Pidoux, 1993b). However, few studies have reported on the effect of culture media on the microbial profile of sugary kefir and its grains.

Both sugar and milk kefir grains, although they are associated with the microbial action of mesophilic LAB and yeast strains, differ in terms of the materials used for the fermentation and the microbial characteristics of the grains (Pidoux et al., 1990). In our previous study, we intensively investigated the microbial profiles in various different milk kefir grains (Chen et al., 2008; Wang et al., 2008, 2011). In the present study, in order to further clarify the differences between milk kefir grains and sugary kefir grains to study the importance of the fermentation medium to the microbial characteristics of grain formation, we fermented sugary kefir grains in media consisting of brown sugar, cow's milk or goat's milk. We then identified the microorganisms present and evaluated the microbial distribution within the grains/filtrate from these kefirs using culture-dependent and culture-independent methods. In addition, grain structure was also investigated by scanning electronic microscopy (SEM).

2. Materials and methods

2.1. Sugary kefir grains

The sugar kefir grains were collected in Taipei in northern Taiwan. In the laboratory, the grains (5%, w/v) were transferred into 5% sterilized brown sugar (Taiwan Sugar Cooperation, Taipei, Taiwan) solution incubated at 20 °C for 24 h. After repeating the incubation procedure five times, the grains were ready for use in this study. To study the effect of different fermentation materials on microbial dynamics, the grains (5%, w/v) were transferred into sterilized cow's or goat's milk (National Taiwan University Dairy Farm, Taipei, Taiwan) and incubated at 20 °C for 24 h. This procedure was repeated for 4 weeks.

2.2. Kefir manufacturing

To manufacture kefir samples, the active grains (5%, w/v) were inoculated into various different fermentation media and incubated at 20 °C for 24 h. After fermentation, each kefir sample was analyzed to explore the kefir's microbial, chemical and physical properties.

2.3. Isolation and enumeration of microorganisms in the grains and the filtrates

A total of 10 g of grains and filtrate from each sample was homogenized in 90 mL of sterile saline solution (0.85% sodium chloride solution, pH 7.2–7.4) in a laboratory blender (Stomacher 400, Seward, England) for 15 min. The isolation and enumeration of microorganisms were carried out using a modification of the methods described by Chen et al. (2008) and Wang et al. (2008). Briefly, yeast isolates were examined on potato dextrose agar (PDA, Difco, Detroit, MI, USA), with 100 ppm chlortetracycline (Sigma, St. Louis, MO, USA). The plates were incubated at 25 °C for three days and enumerated. For LAB isolates, de Man, Rogosa and Sharpe (MRS) agar (Acumedia, Lansing, MI, USA) was used under aerobic conditions and 200 ppm cycloheximide (Sigma) was added to inhibit the growth of yeasts. The plates were incubated at 30 °C for two days and the resulting colonies were counted. After counting, single colony purification was carried out. The procedures to isolate and enumerate the microorganisms were repeated four times.

2.4. The Harrison disc method

The Harrison disc method, adopted from Harrigan (1998), was used to select representative colonies from each plate in a random statistical valid manner for further purification and identification in this study. This method was also used to study the microbial distribution of the various microorganisms present in the samples (Chen et al., 2008; Wang et al., 2008; Witthuhn et al., 2005). The microbial distribution was based on the ratio of identified examples of a specific strain to the total isolates.

2.5. DNA extraction from lactic bacteria and yeast

This part of the study followed the procedures described by Wang et al. (2008) and Chen et al. (2008). The purified LAB and yeast strains isolated by the Harrison disc method were inoculated into MRS and potato dextrose broths, respectively. Cells from 1 mL of each late exponential phase culture were then collected by centrifugation at 7500×*g* (10 min, 4 °C). The microbial pellets were subjected to DNA extraction using a blood and tissue genomic DNA extraction system (Viogene-Biotek Corp., Taipei, Taiwan). In the culture-independent studies, 0.5 g homogenized sample was directly handled as described above. DNA extraction was based on the manufacturer's instructions including protein digestion and isolation of genomic DNA. The isolated genomic DNA was resuspended in sterilized ddH_2O and stored at −20 °C until further analysis.

2.6. Carbohydrate fermentation and assimilation test

Lactic acid bacteria and yeast isolates were examined to establish their ability to ferment various different carbohydrates using API 50 CHL strips and API 20C AUX strips, respectively (BioMérieux, Marcy-l'Etoile, France).

2.7. DNA amplification

DNA amplification used on the various LAB and yeast isolates was adopted from Chen et al. (2008) and Wang et al. (2008), respectively. Two primers were used in this study. For the LAB isolates, the V3 region of the 16S rDNA gene was amplified by PCR using the forward primer 338fGC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGA GGG G ACT CCT ACG GGA GGC AGC AG-3′) (the GC clamp is underlined) and the reverse primer 518r (5′-ATT ACC GCG GCT GCT GG-3′) (Cocolin et al., 2001). For the yeast isolates, the D1 region of the 26S rRNA gene was amplified by PCR using the forward primer NL1GC (5′-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GGC CAT ATC AAT AAG GGG AGG AAA AG-3′) (the GC clamp is underlined) and a reversed primer NL4 (5′-GGTC CGT GTT TCA AGA CGG-3′) (O'Donnell, 1993).

2.8. PCR-DGGE

The PCR fragments were separated by DGGE using the DCode™ universal mutation detection system (Bio-Rad, Hercules, CA, USA) and 16×16×0.01 cm gels. The procedures were adopted from Chen et al. (2008) and Wang et al. (2008). Separation of the PCR amplicons was obtained by direct application of 20 μL of PCR products onto the denaturing gradient polyacrylamide gel. The denaturation gradient ranged from 30% to 50% for the lactic acid bacteria and yeasts [100%

corresponds to 7 M urea (J. T. Baker, Philipsburg, NJ, USA) and 40% w/v formamide (J. T. Baker)].

2.9. DNA sequencing

To verify the PCR-DGGE results and ensure that all isolates within each group belonged to just one species, randomly selected LAB from each group were further identified by 16S rDNA gene sequencing. The full length of the 16S rDNA was amplified using the forward primer 8f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and the reverse primer 1512r (5′-AAG GAG GTG ATC CAG CCG CA-3′) (Coenye et al., 1999). For yeast isolates, the identification was performed by sequencing the D1 and D2 expansion domains of the 26S rDNA using the forward primers NL1 (5′-GCAT ATC AAT AAG CGG AGG AAA AG-3′) and the reversed primer NL4 (5′-GGTC CGT GTT TCA AGA CGG-3′) (O'Donnell, 1993). The PCR amplicons were purified by the Concert Rapid PCR Purification system (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA, USA) and sent for sequencing. Sequencing was carried out at the Genomics Co. (Taipei, Taiwan) using an ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA). The DNA sequence identities were determined using BLAST from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

2.10. Scanning electron microscopy

The microstructures of the kefir grains were explored by scanning electron microscopy (SEM) according to the method of Chen et al. (2009).

2.11. Statistical analysis

Data were analyzed using analysis of variance (ANOVA). Statistical significance was judged at $p<0.05$ level and differences between treatments were tested using Duncan's multiple range tests. All statistical analyses of the data were performed based on four replications and were carried out on Statistical Analysis Systems software (SAS Institute Inc., 2001).

3. Results

3.1. LAB and yeast strain identification in grains using a culture dependent method

Using the Harrison disc method, 118, 120 and 112 colonies were isolated from sugary kefir grains fermented with brown sugar, cow's milk or goat's milk, by growth on MRS agar. The isolates, when classified by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), were found to form seven different groups of bacteria across the different kefir grain samples (Fig. 1(A)). Three groups of bacteria were found in the sugary kefir grains and four groups of bacteria were isolated from cow's or goat's milk. A similar approach on PD agar yielded 118, 120 and 112 yeast colonies from the sugary kefir grains fermented with brown sugar, cow's milk or goat's milk, respectively. After PCR-DGGE, a total of five different groups of yeasts were found to be present in the different kefir grain samples (Fig. 1(B)), with three found in the sugary kefir grains fermented on brown sugar and four groups isolated from cow's or goat's milk.

The identification results (Table 1) indicated that the strains in the grains were significantly affected by the fermentation material used. Three LAB species (*Leuconostoc mesenteroides*, *Lactobacillus mali*, *Lactobacillus hordei*) were found in the grains fermented on brown sugar. In contrast, four LAB species (*Leu. mesenteroides*, *Lactococcus lactis*, *Bifidobacterium psychraerophilum* and *Enterococcus faecalis*) were identified in grains fermented on either cow's or goat's milk. The PDA results (Table 1) showed that three yeasts (*Zygosaccharomyces*

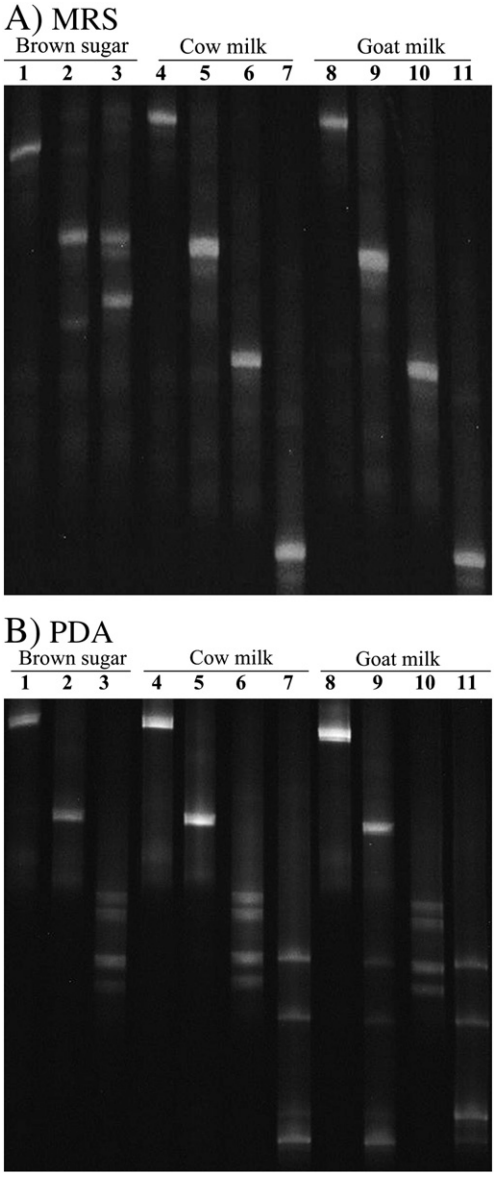

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) profiles obtained from strains isolated from the various sugary kefir grains using different culture media (A) de Man, Rogosa Sharpe (MRS) and (B) Potato Dextrin Agar (PDA).

Table 1
Sequencing results for the microorganisms isolated from the kefir grains.

Band(s)[a]	Closest relative	% Identity	Source[b]
(A) MRS			
1	*Leuconostoc mesenteroides*	99	HM058977
2	*Lactobacillus mali*	100	FM878596
3	*Lactobacillus hordei*	100	EU074850
4, 8	*Leuconostoc mesenteroides*	100	HM058977
5, 9	*Enterococcus faecalis*	100	HM007580
6, 10	*Lactococcus lactis*	100	HM007591
7, 11	*Bifidobacterium psychraerophilum*	100	AB437351
(B) PDA			
1, 4, 8	*Zygosaccharomyces fermentati*	100	EF554824
2, 5, 9	*Saccharomyces cerevisiae*	99	FN393983
3, 6, 10	*Dekkera bruxellensis*	100	DQ406716
7	*Pichia fermentans*	100	GQ458040
11	*Pichia fermentans*	99	GQ458040

[a] Bands are numbered as indicated on the DGGE gels shown in Fig. 1.
[b] Accession number of the sequence of the closest relative found by BLAST search.

fermentati, *Saccharomyces cerevisiae*, and *Dekkera bruxellensis*) were present in all three types of grains. However, *Pichia fermentans* was only identified as only present in grains grown on either cow's or goat's milk. In addition, different signals belonging to *Leu. mesenteroides* (Fig. 1(A), Lanes 1, 4, 8) and *P. fermentans* (Fig. 1(B), Lanes 7, 11) could be linked with the same GenBank accession numbers (Table 1).

3.2. LAB and yeasts identification in the kefir filtrate using a culture dependent method

From the kefir filtrate samples (Fig. 2(A)), 112, 118 and 114 bacterial colonies were isolated from the kefir fermented on brown sugar, cow's milk or goat's milk, respectively, using MRS agar. In total, only four different species of LAB were identified with three species in each filtrate sample, which is fewer than those found in the grains. A similar approach on PD agar yielded 116, 74 and 94 yeast colonies from the filtrates fermented on brown sugar, cow's milk or goat's milk, respectively. After PCR-DGGE, a total of four different groups of yeasts were present in the different kefir grain samples (Fig. 2(B)), with three groups found in the sugary kefir grains and two groups isolated from the cow's or goat's milk.

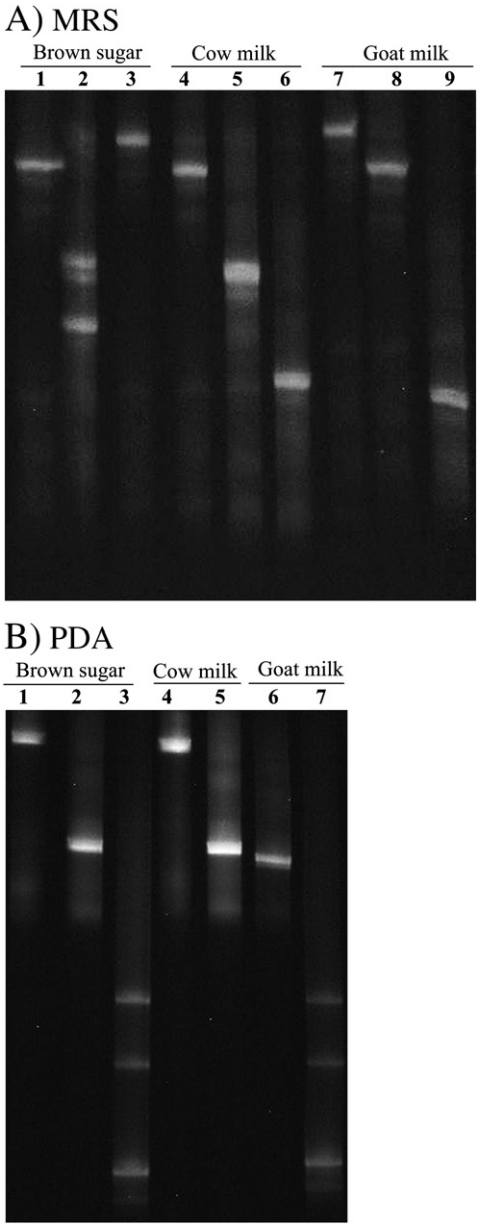

Fig. 2. Denaturing gradient gel electrophoresis (DGGE) profiles obtained from the strains isolated from the various sugary kefir filtrates using different culture media (A) de Man, Rogosa Sharpe (MRS) and (B) Potato Dextrin Agar (PDA).

The identification results (Table 2) indicated that two LAB species (*Leu. mesenteroides*, *Lb. hordei*), three LAB (*Leu. mesenteroides*, *Lc. lactis*, *Ec. faecalis*) and two LAB (*Leu. mesenteroides*, *Lc. lactis*) were found in the filtrates fermented on brown sugar, cow's milk or goat's milk, respectively. For the yeasts (Table 2), three yeasts (*Z. fermentati*, *S. cerevisiae*, *P. fermentans*), two yeasts (*Z. fermentati*, *S. cerevisiae*) and two yeasts (*S. cerevisiae*, *P. fermentans*) were found in the filtrates fermented on brown sugar, cow's milk or goat's milk, respectively.

3.3. The carbohydrate utilization of the isolated species

API 50 CHL results were used to evaluate the carbohydrate utilization of the LAB strains and the results are shown in Table 3. All six strains isolated from the different grain samples were able to utilize D-glucose, D-fructose, D-mannose, esculin, salicin, D-maltose, D-trehalose and gentiobiose. In contrast, only *Leu. mesenteroides*, *Ec. faecalis* and *Lc. lactis* were able to utilize D-galactose and D-lactose, which are the major carbon sources in milk. API 20C AUX results were used to evaluate the carbohydrate utilization of the yeast strains and the results (Table 4) show that all strains isolated from grain samples were able to hydrolyze D-glucose, D-xylose, D-galactose, sucrose and D-trehalose. However, only *Z. fermentati* was able to digest D-lactose.

We also conducted the API ZYM to evaluate the enzyme activities of the isolated strains (data not shown). The results showed that *Leu. mesenteroides*, *Lc. lactis* and *Lb. hordei* possess β-galactosidase, which is able to convert lactose into galactose and glucose but none of the yeasts isolated from the grain samples was able to produce β-galactosidase.

3.4. LAB and yeast identification in grains and filtrates using a culture-independent method

To negate the potential biases inherent when using microbial enrichment, the total DNA of the bacteria and yeasts in the different kefir grain and filtrate samples were extracted and directly identified by PCR-DGGE. The profiles obtained by culture-independent DGGE (Fig. 3) generally agreed with the results obtained by culture-dependent methods. *Leu. mesenteroides* and *Lb. hordei* were identified in both sugary kefir grains and filtrates, whereas, *Leu. mesenteroides*, *Ec. faecalis* and *B. psychraerophilum* were only recognized in the milk samples. Five additional DGGE bands in the sugary kefir samples were found. Further identification by DNA sequencing revealed that they were *Pseudomonas* spp., *Zymomonas mobilis* and *Sporolactobacillus* spp. An additional four bands were also found in the milk samples and these were *Pseudomonas* spp., *Enterobacterium* spp. and *Enterobacter sakazakii*.

Table 2
Sequencing results for the microorganisms isolated from the filtrates.

Band(s)[a]	Closest relative	% Identity	Source[b]
(A) MRS			
1, 4, 8	*Leuconostoc mesenteroides*	99	HM058977
2	*Lactobacillus hordei*	100	EU074850
3, 7	*Leuconostoc mesenteroides*	100	HM058977
5	*Enterococcus faecalis*	100	HM007580
6, 9	*Lactococcus lactis*	100	HM007591
(B) PDA			
1, 4	*Zygosaccharomyces fermentati*	100	EF554824
2, 5, 6	*Saccharomyces cerevisiae*	99	FN393983
3	*Pichia fermentans*	100	GQ458040
7	*Pichia fermentans*	99	GQ458040

[a] Bands are numbered as indicated on the DGGE gels shown in Fig. 2.
[b] Accession number of the sequence of the closest relative found by BLAST search.

Table 3
API 50 CHL results for the evaluation of carbohydrate utilization by the various lactic acid bacteria species.

Active ingredient	Species					
	Leu. mesenteroides	*Lb. mali*	*Lb. hordei*	*Ec. faecalis*	*Lc. lactis*	*B. psychraerophilum*
D-Ribose	+	−	−	+	+	−
D-Xylose	+	−	+	−	−	−
D-Galactose	+	−	−	+	+	−
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
L-Sorbose	−	+	+	+	+	+
D-Mannitol	+	−	+	+	+	−
D-Sorbitol	−	−	−	+	−	−
Methyl-αD-mannopyranoside	−	−	+	−	−	−
Methyl-αD-glucopyranoside	+	−	+	−	−	−
N-Acetylglucosamine	−	+	+	+	+	+
Amygdalin	−	+	+	+	−	−
Arbutin	−	−	+	+	−	−
Esculin	+	+	+	+	+	+
Salicin	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	−
D-Maltose	+	+	+	+	+	+
D-Lactose	+	−	−	+	+	−
D-Melibiose	+	−	+	−	−	−
Sucrose	+	+	+	−	−	+
D-Trehalose	+	+	+	+	+	+
Gentiobiose	+	+	+	+	+	+
D-Turanose	+	−	+	−	−	−
D-Tagatose	+	−	−	+	−	+
Potassium gluconate	−	−	−	−	+	−

A similar approach to explore the yeast ecology (Fig. 4) showed that only *S. cerevisiae* could be identified in all samples. However, *P. fermentans* was also found to be present in the goat milk filtrate. No additional bands were observed using the culture-independent method to identify yeast strains.

3.5. Microbial dynamic study

The effects of different types of fermentation materials on the microbial cell counts of the grain samples are indicated in Table 5. The yeast counts in the samples ranged from 5.12 to 5.84 log CFU/g with no significant difference ($p > 0.05$) across the different types of fermentation material. The LAB counts, on the other hand, showed a significant difference. Kefir grain samples made from goat's milk kefir

Table 4
API 20C AUX results for the evaluation of carbohydrate utilization by the various yeast species.

Active ingredients	Species			
	Z. fermentati	*S. cerevisiae*	*D. bruxellensis*	*P. fermentans*
D-Glucose	+	+	+	+
Glycerol	−	+	+	+
Calcium-2-ketogluconate	+	+	+	−
L-Arabinose	−	−	+	−
D-Xylose	+	+	+	+
Adonitol	−	+	+	−
Xylitol	+	+	+	−
D-Galactose	+	+	+	+
Inositol	−	−	−	−
D-Sorbitol	+	+	+	−
Methyl-αD-glucopyranoside	−	+	+	+
N-Acetylglucosamine	−	−	−	+
D-Cellobiose	+	+	+	−
D-Lactose	+	−	−	−
D-Maltose	−	+	−	+
Sucrose	+	+	+	+
D-Trehalose	+	+	+	+
D-Melezitose	−	+	−	+
D-Raffinose	−	+	−	−
H/PH+	−	−	−	−

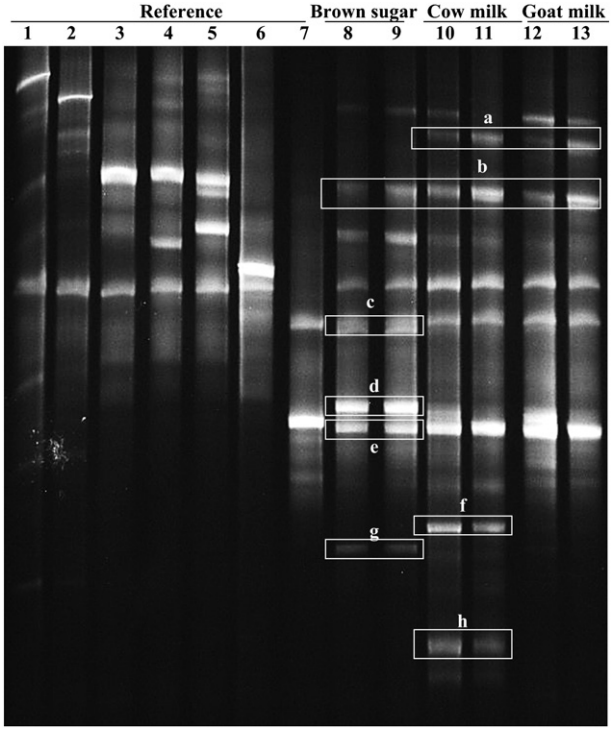

Fig. 3. Denaturing gradient gel electrophoreses profile obtained from seven MRS isolates and DNA extracted directly from the sugary kefir grains (Lanes 8, 10 and 12) and filtrates (Lanes 9, 11 and 13) using different fermentation materials (Lanes 8 and 9, brown sugar; Lanes 10 and 11, cow's milk; Lanes 12 and 13, goat's milk) after amplification by 338fGC/518r. Lanes 1 and 2, *Leuconostoc mesenteroides*; Lane 3, *Enterococcus faecalis*; Lane 4, *Lactobacillus mali*; Lane 5, *Lactobacillus hordei*; Lane 6, *Lactococcus lactis*; Lane 7, *Bifidobacterium psychraerophilum*. a and b, *Pseudomonas* spp.; c, d and e, *Zymomonas mobilis*; f, *Enterobacterium* spp.; g, *Sporolactobacillus* spp.; h, *Enterobacter sakazakii*.

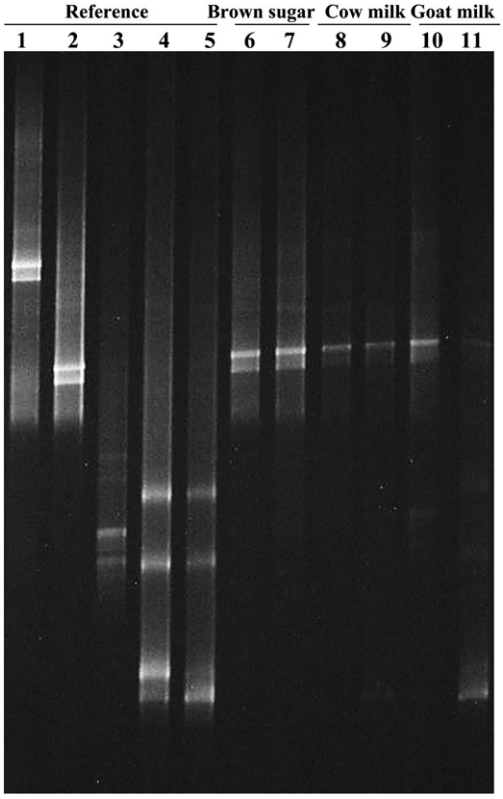

Fig. 4. Denaturing gradient gel electrophoreses profile obtained from five PDA isolates and DNA extracted directly from various sugary kefir grains (Lanes 6, 8 and 10) and filtrates (Lanes 7, 9 and 11) using different fermentation materials (Lanes 6 and 7, brown sugar; Lanes 8 and 9, cow's milk; Lanes 10 and 11, goat's milk) after amplification by NL1GC/LS2. Lane 1, *Zygosaccharomyces fermentati*; Lane 2, *Saccharomyces cerevisiae*; Lane 3, *Dekkera bruxellensis*; Lanes 4 and 5, *Pichia fermentans*.

had the highest ($p<0.05$) cell count (9.19 log CFU/g), followed by cow's milk kefir (8.10 log CFU/g) and then brown sugar kefir (7.26 log CFU/g).

The effect of different types of fermentation materials on the microbial ecology is also shown in Table 5. When grains from brown sugar kefir were examined, *Leu. mesenteroides* (62%) was the most dominant LAB species, followed by *Lb. hordei* (24%) and *Lb. mali* (14%). In contrast, for the grains from cow's milk kefir, *Lc. lactis* accounted for 88% of the total LAB isolates, which made this the most prevalent species. A similar ecological profile was detected in the kefir grains grown in the goat's milk, which was also dominated by *Lc. lactis* (92%). In terms of yeast distribution, *S. cerevisiae* was the most abundant yeast in brown sugar kefir, followed by *D. bruxellensis* (8%) and *Z. fermentati* (7%). Similarly, *S. cerevisiae* was the most prevalent yeast in kefir grains from cow's or goat's milk, followed by *P. fermentans*, *D. bruxellensis* and *Z. fermentati*.

Table 5
The effects of different fermentation media on microbial distribution in sugary kefir grains.

Strains	% (Identified number/total isolates)		
	Brown sugar	Cow's milk	Goat's milk
MRS			
LAB counts (log cfu/g)	7.26 ± 0.03	8.10 ± 0.01	9.19 ± 0.02
Leuconostoc mesenteroides	62 (73/118)	6 (7/120)	5 (6/112)
Lactobacillus mali	14 (17/118)	–	–
Lactobacillus hordei	24 (28/118)	–	–
Enterococcus faecalis	–	3 (4/120)	2 (2/112)
Lactococcus lactis	–	88 (105/120)	92 (103/112)
Bifidobacterium psychraerophilum	–	3 (4/120)	1 (1/112)
PDA (log cfu/g)			
Yeast counts	5.84 ± 0.07	5.12 ± 0.07	5.24 ± 0.06
Zygosaccharomyces fermentati	7 (9/132)	4 (5/120)	2 (2/120)
Saccharomyces cerevisiae	85 (112/132)	67 (80/120)	66 (79/120)
Dekkera bruxellensis	8 (11/132)	14 (17/120)	2 (2/120)
Pichia fermentans	–	15 (18/120)	30 (37/120)

Table 6
The effects of different fermentation media on microbial distribution in sugary kefir filtrates.

Strains	% (Identified number/total isolates)		
	Brown sugar	Cow's milk	Goat's milk
MRS			
Leuconostoc mesenteroides	98 (110/112)	18 (21/118)	21 (24/114)
Lactobacillus hordei	2 (2/112)	–	–
Enterococcus faecalis	–	2 (2/118)	–
Lactococcus lactis	–	80 (94/118)	79 (90/114)
PDA			
Zygosaccharomyces fermentati	8 (9/116)	3 (2/74)	–
Saccharomyces cerevisiae	86 (100/116)	97 (72/74)	33 (31/94)
Pichia fermentans	6 (7/116)	–	67 (63/94)

When filtrates were examined (Table 6), *Leu. mesenteroides* and *S. cerevisiae* accounted for 98% and 86% of the total isolates, respectively, which made them the most dominant LAB and yeast species in the brown sugar samples. For cow's milk filtrate, *Lc. lactis* and *S. cerevisiae* were the most dominant LAB and yeasts, respectively. However, *Lc. lactis* and *P. fermentans* were the main strains in goat's milk kefir. These microbial ecological profiles reveal that the fermentation material used to make the kefir significantly affected the microbial distribution in both grains and filtrates.

3.6. Grain structure

When the grains were examined by SEM (Fig. 5), the outer layers were covered by a network of exopolysaccharides, which helped the yeast and bacteria (cocci and bacilli) to adhere to each other. The microbiological composition of the grains in brown sugar (Fig. 5(A)) consists of a high density of yeast, lactococci and lactobacilli on the surface. At a higher magnification (Fig. 5(B)), the outer layer of the grain was found to contain pseudomycelia. However, the grain structure in milk had a different microbiological composition. The kefir grains cultivated in milk had higher numbers of bacterial cells compared to yeast cells. Cocci filled the grains together with the exopolysaccharide (EPS) matrix in grains from milk (Fig. 5(C)–(F)) and we also noticed that the grain size became smaller compared to the brown sugary kefir grains.

4. Discussion

In this study, we compared the microbial characteristics of sugary kefir grains cultivated in brown sugar, cow's milk or goat's milk. The identification results indicated that the fermentation material used caused remarkable differences in the microbiota of both the grains (Fig. 1 and Table 1) and the filtrates (Fig. 2 and Table 2). Since we autoclaved all the fermentation media and equipment to exclude possible external contamination, these isolated strains must have originally existed in the sugary kefir grains. *Leu. mesenteroides*, *S. cerevisiae* and *Z. fermentati* were found in both brown sugar and milk samples and have been found to be the most common strains observed in sugary kefir grains (Bergmann et al., 2010; da CP Miguel et al., 2011; Waldherr et al., 2010). *Leu. mesenteroides*, a hetero-fermentative, is able to utilize the major carbon sources in milk (lactose and galactose) and in brown sugar (sucrose) (Table 3) in order to produce aroma. This strain was the only LAB found in both the brown sugar and milk samples. It was also the most abundant LAB strain in the brown sugar samples (Table 5). Zhou et al. (2009) indicated that *Leu. mesenteroides* is able to degrade lactose to lactic acid, acetic acid, ethanol and carbon dioxide

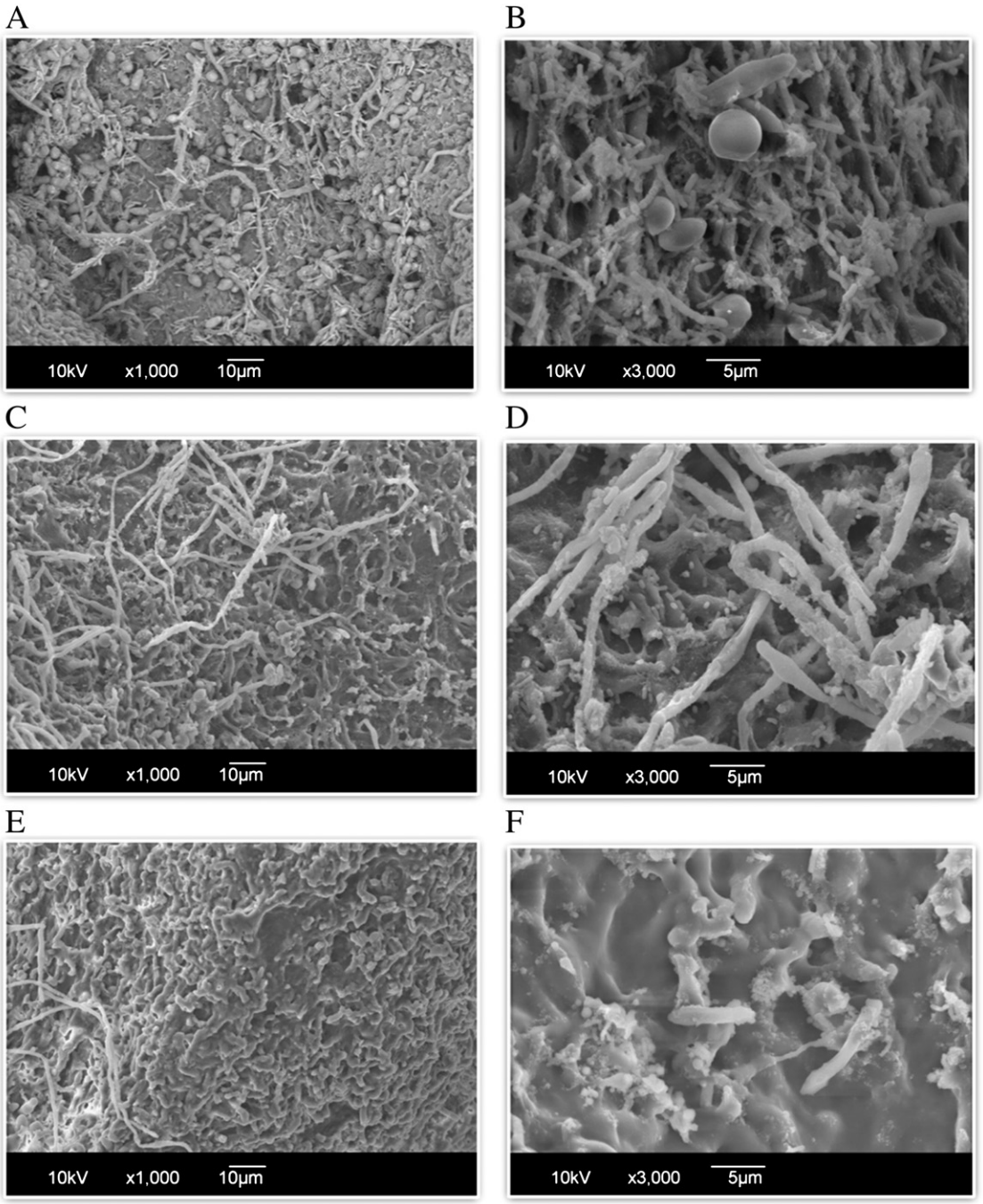

Fig. 5. Scanning electron micrographs showing the effect of different fermentation materials on sugary kefir grains; (A) and (B) brown sugar; (C) and (D) cow's milk; (E) and (F) goat's milk. 1, yeast; 2, cocci; 3, streptococci and 4, lactobacilli.

and also to degrade citric acid to diacetyl, which endows fermented foods with their flavor.

Unusual microorganisms were also observed in the brown sugar samples and these included *Lb. hordei*, *Lb. mali* and *D. bruxellensis*. *Lb. hordei* and *Lb. mali* are Gram-positive rods, about 0.5–1 μm in length and non-motile. This is the first time they have been found in sugary kefir. The lactobacilli were found to be able to utilize most of the carbon sources listed in Table 3 except for ribose, galactose, sorbitol, lactose and tagatose. It is likely that their limited carbohydrate utilization affected their propagation in the milk samples. The studies on *Lb. hordei* and *Lb. mali* are few in the literature. Rouse et al. (2008) isolated a bacteriocinogenic *Lb. hordei* from malted barley. *Lb. mali* is an EPS producing strain. Seto et al. (2006) indicated that there was stimulation of EPS production when *Lb. mali* co-cultured with *Gluconacetobacter xylinus* in the presence of sucrose. *D. bruxellensis*, which was found in both the brown sugar and milk samples, is a sucrose-positive, galactose-positive and lactose-negative yeast. This yeast produces 4-ethyl phenols and volatile acidity is considered to be a major cause of wine spoilage (Comitini and Ciani, 2011).

In a manner that is probably dependent on carbon and energy sources availability during grain fermentation, four other strains, *B. psychraerophilum*, *Ec. faecalis*, *Lc. lactis* and *P. fermentans*, were only observed in the milk samples. *B. psychraerophilum* is Gram-positive, catalase-negative, oxidase-negative, non-motile, non-spore-forming rod. It has been found in animal gastrointestinal tracts (Simpson et al., 2004) and dairy products (Watanabe et al., 2009). *Ec. faecalis* is considered to be a species commonly present in food and also plays an important role in the flavor development of certain cheeses

(Zamfir et al., 2006). *Lc. lactis* was the richest LAB strain in the milk samples (Table 5). It is also the most common strain found in most dairy products. A previous study (Zamfir et al., 2006) has indicated that the presence of this strain in the fermented dairy products may derive from raw milk. However, our milk samples had been autoclaved before inoculation. We speculated that the *Lc. lactis* and similar strains, which were not detectable in the brown sugar samples, may originally exist in the sugary kefir grains in very low numbers and that they remain too few to be isolated when the sugary kefir grains are fermented on brown sugar.

It is worth noting that the strains present as well as the distributions of the strains were different between the grains and filtrates (Tables 1, 2, 4, 5). Several studies have revealed that the microorganism profile of the final product may not be essentially equivalent to that of the grains due to fermentation conditions and microbial characteristics (Chen et al., 2009). In the present study, the filtrates contained fewer strains than their grains, which suggest strong attachment between certain microorganisms and the kefir grain matrix; this will result in them not being released into the filtrates. Additionally, the location and shape of the microorganisms in the grains may be the factors affecting their releases to the filtrate. In the sugary kefir grains, cocci were found on the periphery of the grains (Fig. 5). Therefore, it is probable that large numbers of cocci are released into all types of fermentation media (brown sugar or milk) and these results in being the most numerous strains in the final kefir products. This finding also agrees with other studies (Beshkova et al., 2002; Chen et al., 2009). Grain size was also affected by the fermentation material. Using milk as a fermentation medium might result in the loss to the grains of EPS producing strains such as *Lb. mali* because of changes in sugar availability. One consequence of this may be that the grains in milk are smaller than in brown sugar.

Since culture-free method excludes the requirement for strain isolation, we expected to find more strains in the samples using this approach. *Pseudomonas* spp. was identified in all of the sugary kefir grain samples. Chen et al. (2008) and Kourkoutas et al. (2006) also found *Pseudomonas* spp. by DGGE fingerprinting in kefir grains and in a feta-type cheese that used kefir as a starter. *Zm. mobilis* and *Sporolactobacillus* were only found in grains fermented on brown sugar. *Zm. mobilis* is a Gram-negative, facultative anaerobic bacterium that ferments glucose, fructose and sucrose as carbon sources (Viikari and Berry, 1988). It was originally isolated from alcoholic beverages. *Zm. mobilis* is receiving current attention due to its capability to produce exopolysaccharide (de Oliveira et al., 2007) and for its bioethanol-producing capability. *Enterobacter* spp. and *Enterobacter sakazakii* were identified in the cow milk sample. *Eb. sakazakii* and *Enterobacterium* spp. have been reported in a number of isolated environments including soil, rats, milk powder factories, chocolate factories and households (Kandhai et al., 2004). These strains are commonly found in foods and environment. However, the varieties of the LAB and yeast strains identified by cultured-free PCR-DGGE were lower than those identified using enrichment on nutritive media (Figs. 3 and 4). The cell numbers of certain species, various culture ages and the presence of proteins may be the factors that affect the detection limit of PCR-DGGE (Beh et al., 2006; de Barros Lopes et al., 1996; Prakitchaiwattana et al., 2004; Theunissen et al., 2005).

Since sugary kefir is a popular health promoting beverage, the microorganisms in its grains might relate to its health benefits. *Leu. mesenteroides*, isolated from both brown sugar and milk samples in this study, was found to produce bacteriocin against several strains of *Listeria monocytogenes* (Wang, 1993). *Lc. lactis*, observed in the milk samples, has been reported to suppress hyper-sensitive reaction and inhibit *E. coli*, *Ec. faecalis*, *Listeria innocua*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (Todorov et al., 2006). However, investigations of other LAB (*B. psychraerophilum*, *Lb. hordei*, *Lb. mali*), also found in the sugary kefir grains, on their functional properties were very few. Further study is necessary to evaluate their probiotic effects.

Based on our findings, we propose that the grains originally contain many different microorganisms that are able to adapt to different environments during grain formation and growth. Thus, the distribution of strains during grain fermentation may vary depending on the carbon and energy sources available. Moreover, the fermentation medium is likely to significantly affect the proliferation of certain EPS producing strains that are very important to grain granulation and growth.

In conclusion, in the present study, we successfully showed the importance of the fermentation media to the microbial ecological profiles of sugary kefir grains, which originally are likely to contain a great variety of different microorganisms. Changes to the microbial ecology of the grains affect both granulation and growth of the grains. This study is important in understanding the mechanism of kefir grain formation and growth because it explores the relationship between fermentation media and the kefir microorganisms. Finally, during this study, two unusual LAB strains, *Lb. hordei* and *Lb. mali*, were identified for the first time in the brown sugar kefir grain samples.

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