Bioresource Technology 101 (2010) 8843–8850

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Production of fermented cheese whey-based beverage using kefir grains as starter culture: Evaluation of morphological and microbial variations

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ARTICLE INFO

Article history:
Received 26 March 2010
Received in revised form 9 June 2010
Accepted 17 June 2010
Available online 8 July 2010

Keywords:
Kefir
Cheese whey
Milk
PCR-DGGE
CSLM

ABSTRACT

Whey valorization concerns have led to recent interest on the production of whey beverage simulating kefir. In this study, the structure and microbiota of Brazilian kefir grains and beverages obtained from milk and whole/deproteinised whey was characterized using microscopy and molecular techniques. The aim was to evaluate its stability and possible shift of probiotic bacteria to the beverages. Fluorescence staining in combination with Confocal Laser Scanning Microscopy showed distribution of yeasts in macro-clusters among the grain's matrix essentially composed of polysaccharides (kefiran) and bacteria. Denaturing gradient gel electrophoresis displayed communities included yeast affiliated to *Kluyveromyces marxianus, Saccharomyces cerevisiae, Kazachatania unispora*, bacteria affiliated to *Lactobacillus kefiranofaciens* subsp. *Kefirgranum, Lactobacillus kefiranofaciens* subsp. *Kefiranofaciens* and an uncultured bacterium also related to the genus *Lactobacillus*. A steady structure and dominant microbiota, including probiotic bacteria, was detected in the analyzed kefir beverages and grains. This robustness is determinant for future implementation of whey-based kefir beverages.

1. Introduction

Cheese whey is the liquid remaining after the precipitation and removal of milk casein during cheese-making. This byproduct represents approximately 85–90% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5–5.0% w/v), soluble proteins (0.6–0.8% w/v), lipids, and mineral salts (Dragone et al., 2009 and references there in). Cheese whey represents an important environmental problem because of the high volumes produced and its high organic matter content, exhibiting a COD of 60,000–80,000 ppm. Worldwide production of whey is estimated to be in the order of 160 million tonnes per year, showing a 1–2% annual growth rate (Smithers, 2008). The pressure of antipollution regulations together with whey nutritional value challenges the dairy industry to face whey surplus as a resource and not only as a waste problem (Guimarães et al., 2010).

Several methods have been proposed for whey valorization (Guimarães et al., 2010; Koutinas et al., 2009 and references there in). Besides potable ethanol production by lactose converting microorganisms (reviewed by Guimarães et al. (2010)) and genetically-engineered *Saccharomyces cerevisiae* cells (Domingues et al., 2001; Guimarães et al., 2008; Domingues et al., 2010), the production of alcoholic beverages from whey has also been pointed as an alternative (Holsinger and Posati, 1974), including distilled beverages (Dragone et al., 2009) and kefir-like whey beverages (Paraskevopoulou et al., 2003).

Kefir is made by inoculating milk with kefir grains. These grains are irregular granules that vary in size from 3 to 35 mm in diameter (Güzel-Seydim et al., 2005) contain lactic acid bacteria (*Lactobacillus, Lactococcus, Leuconostoc*), acetic acid bacteria and yeast mixture coupled together with casein and complex sugars by a matrix of polysaccharides denominated kefiran (Güzel-Seydim et al., 2005). Yeasts are important in kefir fermentation because of the production of ethanol and carbon dioxide. Kefir grains usually contain lactose-fermenting yeasts (*Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Torula kefir*), as well as non lactose-fermenting yeasts (*S. cerevisiae*) (Farnworth, 2005). This mixed culture of kefir yeast, which ferments lactose, seems to have the potential for beverage production using cheese whey.

Cheese whey utilization by kefir grains has been studied for potable alcohol production (Koutinas et al., 2009) indicating the ability of this biocatalyst to produce high yields in alcoholic

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doi:10.1016/j.biortech.2010.06.083

fermentations. In addition, the production of kefir-like whey beverages using a cheese whey–milk mixture as substrate has also been reported (Paraskevopoulou et al., 2003). Reports on single cell protein production (using kefir yeasts; Koutinas et al., 2005) and more recently, on starter culture production from whey for use in cheese ripening (Koutinas et al., 2009) can also been found. All these studies show promising perspectives for kefir grains application in whey valorization strategies. Nevertheless, one important aspect has to be clarified for fully application of kefir grains to whey fermentations. Namely, if the microbiota present in the grains change when using whey instead of the traditional milk as substrate. Another relevant issue is whether the kefir probiotic bacteria are present in the beverages. Therefore, the motivation of the present work was to elucidate the stability, organization and identification of the dominant microbiota present in Brazilian kefir grains and correspondent beverages.

2. Methods

2.1. Milk and whey-based fermentation media

Three different substrates with a lactose concentration of 46 g/l were used as fermentation media: pasteurized full cows' milk (M), cheese whey (CW) and deproteinised cheese whey (DPW). Cheese whey powder, obtained from a regional dairy industry (Quinta dos Ingleses, Caíde de Rei, Portugal), was dissolved in sterile distilled water to the desired lactose concentration. Deproteinised cheese whey was made by autoclaving at 115 °C for 10 min the cheese whey solution, followed by aseptic centrifugation (2220*g* for 20 min) to remove fines and cream.

2.2. Milk kefir and cheese whey kefir production

Brazilian kefir grains were used in the present study. Inoculum was grown in pasteurized whole milk during 7 days. The substrate was changed daily. Later the grains (12.5 g) were washed with sterile distilled water and inoculated in 250 ml of each substrate. Erlenmeyers containing kefir grains were statically incubated for 48 h and 72 h at 25 °C. Samples of the beverage were aseptically taken in begin and end of the fermentation. Determination of total reducing sugars was used to assess the depletion of substrate. Replicates were used in each fermentation. Lactose and ethanol were further quantified by high-performance liquid chromatography (HPLC), using Jasco chromatograph equipped with the refractive index (RI) detector (Jasco 830-RI).

2.3. Fluorescence staining and CLSM examination of kefir grains

Samples of the grains used as inoculum and collected after fermentation of milk, cheese whey and deproteinised cheese whey were washed in phosphate buffered saline (PBS) and fixated in 3% formaldehyde (v/v in PBS) for 24 h at 4 °C. The grains were washed again in PBS and stored in a solution of 50% ethanol and PBS. To visualize the internal surface, fixed grains were embedded for cryosectioning according to Batstone et al. (2004). The grains in blocks were sectioned into 10 μm thick slices using a cryostat CM 1900 (Leica, Germany) with the knife temperature of −20 °C and cabinet temperature of −18 °C. Intact grains and sections were stained with SYTO 9 (20 ng/μl, Molecular Probes, Spain) to visualize cellular nucleic acids, followed by Calcofluor white (25 μM, Sigma, Spain) to stain chitin in cell walls of fungi, and finally Concanavalin A (ConA) conjugated with Alexa Fluor 594 (1 mg/ml, Molecular Probes, Spain) to stain alpha-linked sugar in polysaccharides. The structure of both external (intact grains) and internal (sections) surface of the grains was examined in Confocal Laser Scanning Microscopy (CLSM) (FluoView 1000, Olympus, Germany). The collection wavelengths of all stains were listed in Table 1.

2.4. DNA extraction and PCR-DGGE analysis

Kefir grains and fermented product, collected at the end of fermentations, were frozen at the time of sampling and stored at −20 °C. Samples of the grains used as inoculum were also collected. Approximately 1.5 ml of each liquid sample (i.e. beverage) was centrifuged at 13000 rpm for 5 min for five times. Pellets were resuspended in 400 μl of sterile demineralised water. Each sample (grains and beverage) was transferred into a plastic tube and was subjected to DNA extraction using a NucleoSpin Tissue kit (Macherey–Nagel, Düren, Germany), according to the manufacturer's instructions. The extracted DNA was stored at −20 °C.

Genomic DNA was used as template for PCR amplification of bacterial or fungal ribosomal target regions, for denaturing gradient gel electrophoresis (DGGE) analyses. Two primers sets were used for the analysis of each microbial community. Table 2 presents information about the primers and conditions of PCR and DGGE. All PCRs were performed in mix (50 μl) containing: 0.625 U Taq DNA polymerase (Invitrogen, Barcelona, Spain), 2.5 μl buffer 10×, 0.1 mM dNTP, 0.2 μM of each primer, 1.5 mM $MgCl_2$ and 1 μl of extracted DNA. Aliquots (2 μl) of the amplification products were analyzed by electrophoresis on 1% agarose gels and ethidium bromide staining. The size of the products was estimated using a 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania).

The PCR products were analyzed by DGGE using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, Richmond, CA, USA). Samples were applied to 8% (w/v) polyacrylamide gels in 0.5 × TAE. Optimal separation was achieved with a 30–55% urea-formamide denaturing gradient for bacteria community and 12–60% for the yeast community (100% correspondent to 7 M urea and 40% [v/v] formamide). Gels were run according to the conditions displayed in Table 2. DGGE gels were stained with $AgNO_3$ as described by Sanguinetti et al. (1994) and scanned in an Epson Perfection V750 PRO (Epson, USA).

2.5. Cloning and sequencing

Bacterial 16S rRNA genes were amplified from genomic DNA with the primer pair 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-CGGCTACCTTGTTACGAC-3′). For amplification of fungal ITS region, the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used. PCR was performed according to the method described by Wang et al. (2006) (bacteria) and Naumova et al. (2004) (yeast). The amplification products were visualized by electrophoresis in 0.5% agarose gel at 60–65 V in 0.5× TAE for 1 h. The purification was made using the Kit QIAquick PCR Purification (QIAGEN). The purified products were ligated into the pGEM®-T vector using the vector pGEM®-T vector system I (PROMEGA) and subsequently transformed in competent cells of *Escherichia coli* (JM109) according to the manufacturer's instructions. Fifty white colonies (positive recombinants) were collected for each transformation and screened by PCR-DGGE

Table 1
Stains used in the proposed staining scheme.

Dye	Excitation (nm)	Emission (nm)	Targets
Syto 9	470	510–540	Cellular nucleic acids
Calcofluor White	405	Maximum 500	Cellulose and chitin in cell walls of fungi
ConA – Alexa 594	590	Maximum 617	Alpha-linked sugar in polysaccharides

Table 2
DGGE-PCR primers used to detect yeasts and bacteria in grains and kefir beverage of milk, cheese whey and deproteinised cheese whey.

Primer	Sequence (5′ – 3′)	Community	Target	PCR conditions	DGGE conditions	References
968fGC	AAC GCG AAG AAC CTT AC GC clamp connected to the 5′ end of 968f	Bacteria	V6–V8 region of the 16S rRNA gene	Condition 1	16 h at 85 V at 60 °C	[a]
1401r	CGG TGT GTA CAA GAC CC					
ITS1fGC	TCC GTA GGT GAA CCT GCG G GC clamp connected to the 5′ end of ITS1gc	Yeast	ITS region of the rDNA	Condition 1	16 h at 85 V at 60 °C	[b]
ITS2r	GCT GCG TTC TTC ATC GAT GC					
338fGC	AC TCC TAC GGG AGG CAG CAG GC clamp connected to the 5′ end of 338fgc	Bacteria	V3 region of the 16S rRNA gene	Condition 1	8 h at 85 V at 60 °C	[c]
518r	ATT ACC GCG GCT GCT GG					
NS3fGC	GCA AGT CTG GTG CCA GCA GCC GC clamp connected to the 5′ end of NS3gc	Yeast	18S region of the rDNA	Condition 2	16 h at 85 V at 60 °C	[d]
YM951r	TTG GCA AAT GCT TTC GC					

GC clamp – CGC CCG CCG CGC GCG GCG GGC GGG GCG GG, f – forward primer; r – reverse primer.
Condition 1 – Denatured for 5 min at 95 °C. Thirty cycles: denaturing at 92 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s. Final extension for 10 min at 72 °C. Condition 2–35 cycles instead of 30.
[a] Randazzo et al. (2002).
[b] White et al. (1990).
[c] Ovreas et al. (1997).
[d] Haruta et al. (2006).

using the primers 968fGC/1401r (bacteria) and ITS1GC/ITS2 (yeast). Clones whose DGGE mobility corresponded to bands in the community profile of kefir grains and beverage were selected for sequencing. Different clones exhibiting the same DGGE mobility were included as replicates for sequencing.

Inserts from the selected clones were amplified using pGEM®-T vector-targeting primers SP6 (5′-CAT ACG ATT TAG GTG ACA CTA TAG-3′) and T7 (5′-TAA TAC GAC TCA CTA TAG GGA GA-3′). Sequencing reactions were performed at BIOPREMIER (Lisboa, Portugal) using the same primer pair.

2.6. Phylogenetic analysis

The sequence information was imported into the BioEdit v7.0.9 software package (Hall, 1999) for assembly and the consensus sequences obtained were manually checked and corrected when necessary. Sequence similarity searches were performed in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) using the blast database.

3. Results and discussion

3.1. Kefir fermentation chemical analysis

Table 3 summarizes the main chemical characterization results of kefir beverages fermentation. Lactose was consumed and ethanol was produced during the fermentation. At 48 h the lactose concentration in the milk fermentation was residual while in the whey fermentations a lactose concentration of 15–20 g/l was observed. This likely reflects an adaptation period of the microbial community to the whole and deproteinised cheese whey as kefir grains were preserved in milk. Despite the higher lactose consumption during milk fermentation, the concentrations of ethanol did not show significant differences to those obtained during the cheese whey and deproteinised cheese whey fermentation.

As total consumption of lactose was not achieved in 48 h of whey fermentation, a second set of fermentations was performed under the same conditions. Total lactose consumption was attained within 72 h fermentation. During this time, ethanol concentration increased up to ~12 g/l and stabilized after lactose was totally consumed. No significant differences were found in the consumption of lactose and ethanol produced when using milk or whey as substrates.

3.2. Structure of kefir grains as revealed by fluorescence staining and CLSM imaging

Micro-scale examination of the structure of kefir grains was performed by fluorescently probing the distribution of cells (bacteria and yeast) and polysaccharides using a triple staining scheme, followed by CLSM examination (results in Supplementary Fig. S1). No significant difference was observed between the structure of kefir grains collected after fermentation of milk, cheese whey and deproteinised cheese whey. The microbial biomass visualized with the Fluor chrome SYTY9 (green), covered great portion of the external surface and was localized both within and between the ConA (red) stained regions, i.e. the polysaccharide matrix. This polysaccharide matrix, called kefiran, is produced by lactic acid bacteria and usually associated to the therapeutic properties of kefir (Tada et al., 2007). Kefiran has frequently been claimed to be effective against a variety of complaints and diseases. Several studies have investigated the antitumor activity, antibacterial

Table 3
Lactose and ethanol concentration in the performed fermentations.

Time	Milk		Cheese whey		Deproteinised cheese whey	
Fermentation (48 h)	Lactose (g/l)	Ethanol (g/l)	Lactose (g/l)	Ethanol (g/l)	Lactose (g/l)	Ethanol (g/l)
0 h	46.06 ± 0.18	n.d.	45.70 ± 0.711	n.d.	46.06 ± 0.18	n.d.
48 h	1.26 ± 0.02	8.65 ± 1.65	14.17 ± 2.16	8.3 0 ± 1.22	19.63 ± 0.36	7.81 ± 0.34
Fermentation (72 h)	Lactose (g/l)	Ethanol (g/l)	Lactose (g/l)	Ethanol (g/l)	Lactose (g/l)	Ethanol (g/l)
0 h	47.12 ± 0.00	n.d.	47.14 ± 0.00	n.d.	47.14 ± 0.00	n.d.
72 h	n.d.	12.26 ± 1.42	n.d.	11.72 ± 0.77	n.d.	11.86 ± 0.00

Data are average values of duplicate ± standard deviation.
n.d. – not detected.

and antifungal activities (Otles and Cagindi, 2003; Silva et al., 2009). Recently, the potential of kefiran to modulate key steps in the virulence of *Bacillus cereus* in the context of intestinal infections has been reported (Medrano et al., 2009). *Lactobacillus kefiranofaciens* and several other unidentified species of *Lactobacillus* have been pointed by several authors as the major producers of the kefiran polymer in kefir grains (Tada et al., 2007). Otles and Cagindi (2003) found that kefiran producing encapsulated *L. kefiranofaciens* are located all over the grain and increased in the center, while some species of *Lactobacilllus* populated only a small region at the surface layer.

Staining with Calcofluor white (blue) was used to highlight yeast cells in the microbial biomass. Blue stained regions were found as smaller portions randomly distributed among the grain's surface (Fig. S1). A similar distribution pattern was observed in the internal surface of the grains, with macro-clusters of yeasts distributed within the grain's matrix, essentially composed of polysaccharides and bacteria. Cells stained in red were also observed, likely due to ConA binding to mannose proteins on yeast surfaces.

Altogether, CLSM inspection of the grains revealed the maintenance of the structure and relative proportion of microbiota and polysaccharides in the different fermentation conditions. Interestingly, the structure of the grains was found to develop likewise when using cheese whey (whole and deproteinised) and milk as substrate. Therefore, this suggests that the main characteristics of kefir are maintained when using whole and deproteinised cheese whey instead of milk. To deeper evaluate the stability and composition of relevant microbial groups, the microbiota present in the different fermentations was further analyzed using a molecular approach.

3.3. Evaluation of different primers to assess bacterial and fungal communities in beverage and kefir grains

Although many studies have clearly demonstrated the broad applicability of PCR-DGGE to discriminate among target bacteria, the displayed community profiles can be highly dependent on the PCR primers used (Jianzhong et al., 2009). It has been shown that targeting different rDNA regions may, sometimes, lead to different results in terms of microbial composition. PCR bias (Kanagawa, 2003), co-migration of DNA from different species in the same band (Sekiguchi et al., 2001) and formation of multiple bands in amplification of genes from single genomes (Nübel et al., 1996), may provide incorrect information about dominance and diversity of certain ribotypes in the community.

In this study, four of the mostly used primers for PCR-DGGE, were selected to profile microbial communities in fermented products and kefir grains: two primer sets targeting different regions of bacterial 16S rDNA, namely 968fGC/1401r (V6–V8 region) and 338fGC/518r (V3 region), and the primer pairs ITS1/ITS2 and NS3/YM951 targeting fungal ITS (internal transcribed spacer) and 18S rDNA regions, respectively. All the analyzed primer pairs gave satisfactory amplification of the samples. For yeast community, both ITS and 18S rDNA PCR-DGGE analyses yielded the same microbial DGGE profile. Three predominant bands were observed in both gels (Fig. 1).

For bacteria, however, a different profile was generated by the two primer pairs tested. The primer pair 968fGC/1401r, targeting the 16S rDNA V6–V8 regions, yield patterns with two main bands (high intensity) in the microbial profile (Fig. 2a), whereas the pair 338fGC/518r generated profiles with five bands (high intensity), but of similar dominance in the profile (Fig. 2b).

Other authors tested the feasibility of different primers pairs for molecular detection of microbial communities. Ercolini et al. (2001) used the primer pair 338fGC/518r to differentiate and identify lactic acid bacteria (LAB) isolated from food. The analysis of the amplified variable V3 region of the 16S rDNA allowed to differentiate within species of the genera *Enterococcus, Lactococcus, Lactobacillus, Pediococcus*, and *Leuconostoc.* However, cases of co-migration were also observed, which made it impossible to achieve an unequivocal identification of some species. In another study, the presence of *Leuconostoc* in Stilton cheese could only be detected when targeting the V4–V5 region of the 16S rDNA and not when the V3 region was analyzed (Ercolini et al., 2003). Randazzo et al. (2002) used the 16S rDNA V6–V8 regions to examine the evolution of bacterial community during manufacturing of Ragusano cheese. This PCR-DGGE analysis was able to successfully identify and differentiate between species of *Lactococcus, Leuconostoc, Streptococcus, Lactobacillus* and *Macrococcus*. Van Beek and Priest (2002) monitored LBA communities during fermentation of Malt whisky by PCR-DGGE of V3 and RT-PCR-DGGE of V6–V8 regions of 16S rDNA. These authors optimized the separation of lactobacilli in DGGE by adopting the V6–V8 region as a target, giving better resolution of several species due to higher heterogeneity in sequences of species from *Lactobacillus.* In a recent study, Magalhães et al. (2010) could not differentiate some species of *Lactobacillus* by PCR-DGGE migration of fragments of the 16S rDNA V3 region. Additionally, some individual *Lactobacillus* spp. were found to correspond to more than one band in the DGGE profile, probably due to target sequence heterogeneity among multiple copies of the 16S rDNAs. Multiple bands were also observed in pure culture amplicons produced with the V3 primer pair, but not with the V6–V8, in DGGE profiles of other bacteria species, such as, *E. coli*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* (Araújo and Schneider, 2008).

Altogether, the results obtained in this work using different pairs of primers, show a stable DGGE profile either in kefir grains or correspondent beverage, under different fermentation conditions (time and/or substrate) suggesting the presence of a robust dominant microbial consortium. This has high industrial relevance in terms of preservation of the properties of the produced beverages.

3.4. Culture-independent analysis of bacterial and yeast communities

Traditionally, many plating procedures are only partially selective and exclude parts of the microbial community. Thus, in this study the composition of microbiota in kefir grains was evaluated using PCR-DGGE analysis. In addition, the microbial community present in the fermented beverages obtained from milk, cheese whey and deproteinised cheese whey was also assessed. Representative DGGE fingerprints are shown in Figs. 1 and 2. No differences in community structure were found in all the fermented beverages and kefir grains, suggesting the involvement of the same group of microorganisms in the different fermentations performed. As the ecological conditions remained unchanged, a stable microbiota without changes in species composition could be detected. Furthermore, kefir beverages constitutes an environment characterized by a relatively high pH, produced by LAB – the largest group of bacteria belonging to the kefir microbiota – inhibiting the growth of other groups of microorganisms due to the antimicrobial activity of kefiran. Therefore, only few strains are highly competitive under the prevailing ecological conditions and may persist for decades in continuously propagated fermentative processes (Cheirsilp et al., 2003). Interestingly, a recent study has reported antimicrobial activity of the broth fermented with kefir grains towards common pathogens such as *Candida albicans*, *Salmonella typhi*, *Shigella sonnei*, *Staphylococcus aureus* and *E. coli* (Silva et al., 2009).

To determine the composition of microbiota in grains and kefir beverages (milk, cheese whey and deproteinised cheese whey), nearly full-length bacterial 16S rRNA gene and fungal ITS rDNA

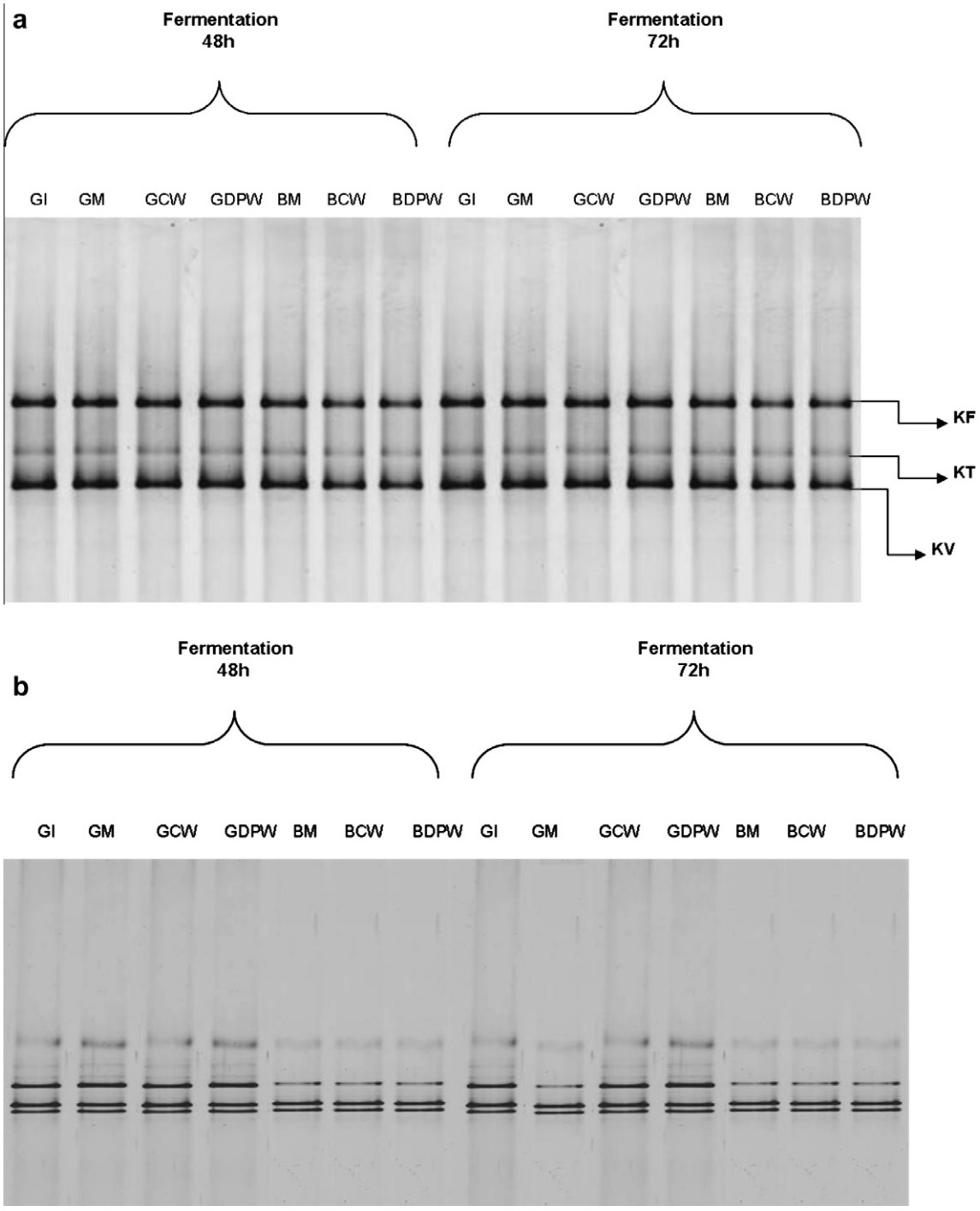

Fig. 1. DGGE profiles of fungal ITS (a) and 18S (b) rDNA fragments amplified from kefir beverages (milk, cheese whey, deproteinised cheese whey) and grains samples. GI = inoculo, GM = grain (fermentation of milk), GCW = grain (fermentation of cheese whey), GDPW = grain (fermentation of deproteinised cheese whey) BM = beverage (fermentation of milk), BCW = beverage (fermentation of cheese whey), BDPW = beverage (fermentation of deproteinised cheese whey).

fragments were amplified and used to construct clone libraries. Clones containing inserts corresponding to prominent bands in the DGGE profiles were sequenced and the obtained sequences further compared to sequences deposited in the GenBank database using the NCBI BLAST search program. Table 4 summarizes the obtained similarity search results. Bacterial clones kJ and KR were closest related to *Lactobacillus kefiranofaciens* subsp. *Kefirgranum* (98%) and *Lactobacillus kefiranofaciens* subsp. *Kefiranofaciens* (99%), respectively, whereas KX was affiliated to a yet uncultured bacterium also affiliated to *Lactobacillus*. Bacterial clones KE and KN were not found. They were not recovered for sequencing and may represent other bacterial ribotypes, however with lower PCR amplification efficiency. KE and KN are represented by bands of low intensity in DGGE gel (Fig. 2). Yeast clones, KF, KT and kV were closest related to *K. marxianus* (99%), *S. cerevisiae* (98%) and *Kazachatania unispora* (99%), respectively.

Jianzhong et al. (2009) identified similar species when investigating the microbiota of Tibetan kefir grains by culture independent methods. DGGE of partially amplified 16S rRNA for bacteria and 26S rRNA for yeasts, followed by sequencing of the most in-

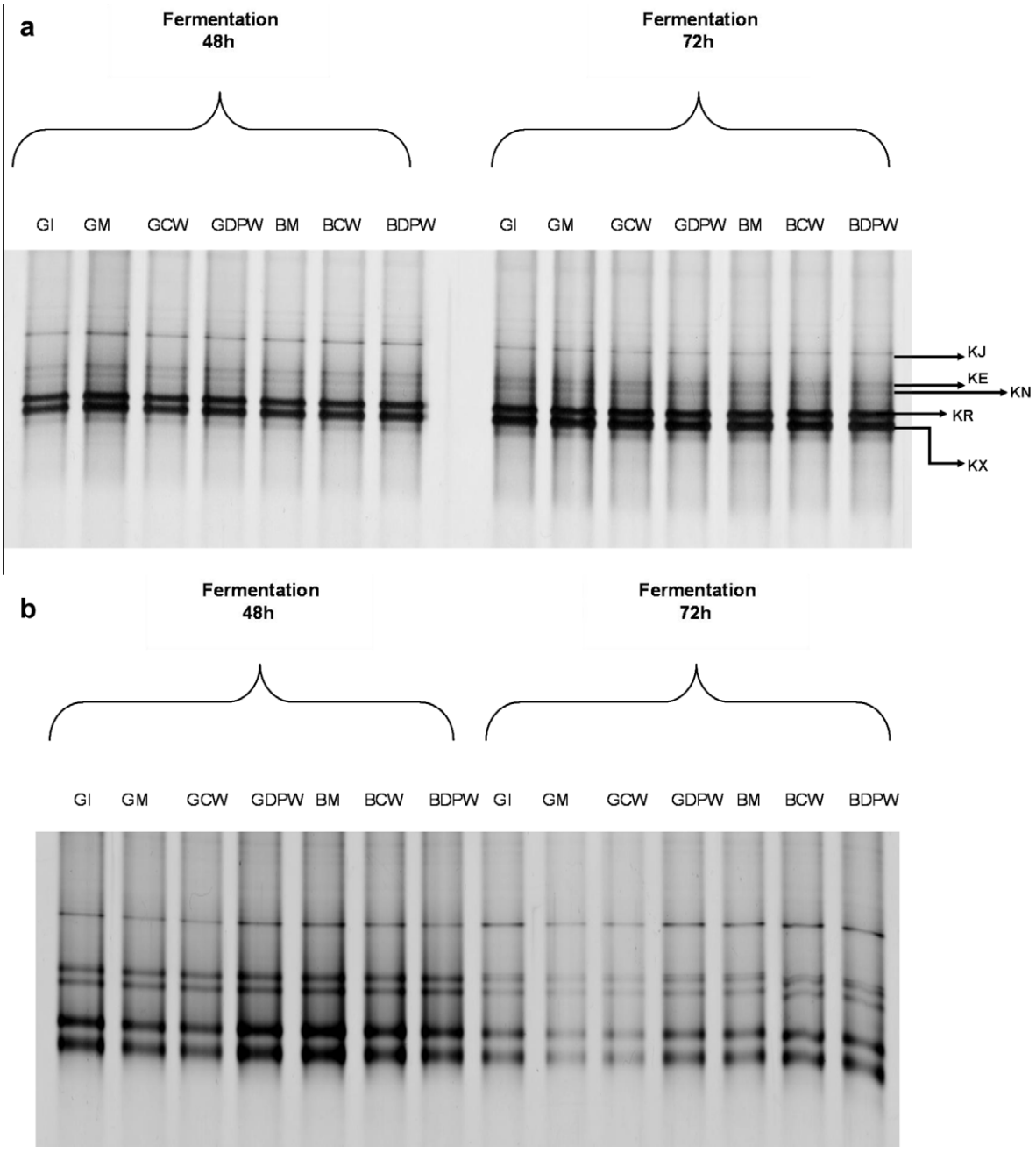

Fig. 2. DGGE profiles of bacterial 16S rDNA V6–V8 regions (a) and V3 region (b) amplified from kefir beverages (milk, cheese whey, deproteinised cheese whey) and grains samples. GI = inoculo, GM = grain (fermentation of milk), GCW = grain (fermentation of cheese whey), GDPW = grain (fermentation of deproteinised cheese whey) BM = beverage (fermentation of milk), BCW = beverage (fermentation of cheese whey), BDPW = beverage (fermentation of deproteinised cheese whey).

Table 4
Identification of representative bacterial and yeast clones by sequencing of portions of the 16S rRNA and ITS, respectively.

Clone	Species	GenBank Accession No.	% Similarity	*E* value
KJ	*Lactobacillus kefiranofaciens* subsp. *kefirgranum*	AB372208.1/FJ749467.1	98	$8e^{-}$ 87
KR	*Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*	AJ575260.1/AJ575259.1	99	0.0
KX	Uncultured bacterium clone IMAU 311/uncultured *Lactobacillus* sp. Clone 2c	GQ267936.1/EF593063.1	97	$1e^{-}$ 24
KF	*Kluyveromyces marxianus*	AF543841.1/EU019227.1	99	0.0
KT	*Saccharomyces cerevisiae*	AM262831.1/AM262824.1	98	0.0
KV	*Kazachatania unispora*	D89896.1/EU789404.1	99	0.0

tense bands, showed that the dominant microorganisms were *Pseudomonas* sp., *Leuconostoc mesenteroides*, *Lactobacillus helveticus*, *L. kefiranofaciens*, *Lactococcus lactis*, *Lactobacillus kefiri*, *Lactobacillus casei*, *K. unispora*, *K. marxianus* and *S. cerevisiae*. The bacterial and yeast communities present in three kinds of Tibetan kefir grains, obtained from different regions, showed 78–84% and 80–

92% similarity, respectively. The microorganisms associated with sugary kefir beverage were investigated by Magalhães et al. (2010) using a combination of culture-dependent and independent methods. Bacteria and yeasts were identified via phenotypic and genotypic methods. The bacterial community DNA was amplified with primers 338fGC and 518r spanning the V3 region of the 16S rRNA gene. The yeast community DNA was amplified using the primers NS3 and YM951r. The authors identified similar species when investigating the microbiota of sugary Brazilian kefir beverage. *Lactobacillus paracasei* was the major bacterial isolate identified, followed by *Acetobacter lovaniensis*, *Lactobacillus parabuchneri*, *Lactobacillus kefir* and *L. lactis*. *S. cerevisiae* and *K. lactis* were the most common yeast species isolated.

Our data show the presence of *Lactobacillus* in the kefir grains and correspondent fermented beverages. In addition, *L. kefiranofaciens* identified in this study is considered one of the main producers of kefiran polymer (Tada et al., 2007). Previous studies reported a variety of different species of *Lactobacillus* that have been isolated and identified in milk kefir grains from around the world (Jianzhong et al., 2009). *Lactobacillus* species are important producers of lactic acid. They are probiotics, good at improving the intestinal environment (Jianzhong et al., 2009). The presence of this group in the studied beverages confers a probiotic label to the kefir drinks highlighting its industrial relevance.

Based on the DGGE profiles of yeast, a closest relative of the lactose-fermenting yeast *K. marxianus*, was found in this study together with organisms affiliated to non-lactose-fermenting yeast, i.e. *S. cerevisiae* and *K. unispora*. Magalhães et al. (2010) identified similar yeasts species when investigating the microbiota of sugary Brazilian kefir by culture independent and dependent methods. The yeast flora of sugary kefir was dominated by lactose-negative strains. Among them, *S. cerevisiae* predominated, followed by *Kazachstania aerobia* and *Lachancea meyersii*.

K. marxianus-related yeast present in this study was, likely, using lactose as carbon source and producing ethanol and carbon dioxide endowing kefir good flavor (Magalhães et al., 2010 and references there in). *S. cerevisiae*-like yeast was detected in this study. The presence of these organisms contributes to the enhancement of organoleptic quality of the kefir beverage, promoting a strong and typically yeasty aroma as well as its refreshing, pungent taste (Magalhães et al., 2010). This yeast also reduces the concentration of lactic acid, removes the hydrogen peroxide and produces compounds that stimulate the growth of other bacteria, thus increasing the production of kefiran (Cheirsilp et al., 2003). *K. unispora*-like yeast was also detected in this study. Magalhães et al. (2010) affirm that the presence of *Kazachstania* genus yeasts in kefir could be connected with the assimilation of some acids produced by lactic acid bacteria.

In this study, differentiation of the DGGE displayed bacterial and yeast species was possible by using the chosen target rDNA regions. Furthermore, two *Lactobacillus* related sequences were differentiated at the subspecies' level, i.e. *Lactobacillus kefiranofaciens* subsp. *kefirgranum* and *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* by targeting the 16SrDNA V6–V8 regions. According to the DGGE profile, members of this specie, considered one of the main producers of kefiran polymer (Tada et al., 2007), were dominant in bacterial community. Compared to other reports, the DGGE displayed dominant bacterial community obtained in this study exhibited much lower diversity at the genus level. Some weaker bands observed on the generated DGGE may represent other bacterial ribotypes, present in lower numbers, or with lower PCR amplification efficiency. Clones with inserts yielding PCR-DGGE fragments corresponding to those faint bands were not found in the screened clone library, hindering further phylogenetic assignment.

In spite of specific differences in the microbiota of kefir grains obtained from different origins, the co-existence of a symbiotic association between lactic acid bacteria and yeasts, included in a polysaccharide–protein matrix, enabling lactic-alcoholic fermentation forms the core that characterizes the concept of kefir (Farnworth, 2005). An important probiotic group of bacteria, i.e. *Lactobacillus* spp., is constantly found. Being so, the probiotic properties from whey-based Brazilian kefir beverages found in this study is likely extensible to other kefir beverages.

4. Conclusions

The present study revealed a consistent grain structure and kefir microbiota when replacing milk with whole/deproteinised cheese whey as fermentation substrate. The dominant microbiota, as revealed by PCR-DGGE, was composed by yeast affiliated to *K. marxianus*, *S. cerevisiae*, *K. unispora*, and bacteria affiliated to the *Lactobacillus* genus. Interestingly, this dominant bacterial community was also found in the fermented beverages, conferring probiotic label to kefir beverages. In addition, the observed microbiota stability is determinant for the implementation of this type of kefir beverages and whey valorization. These results open up perspectives for this innovative application of kefir grains.

Acknowledgements

The authors acknowledge Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), CAPES-GRICES.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.06.083.

References

Araújo, J.C., Schneider, R.P., 2008. DGGE with genomic DNA: Suitable for detection of numerically important organisms but not for identification of the most abundant organisms. Water Res. 42, 5002–5010.

Batstone, D.J., Keller, J., Blackall, L.L., 2004. The influence of substrate kinetics on the microbial community structure in granular anaerobic biomass. Water Res. 38, 1390–1404.

Cheirsilp, B., Shimizu, H., Shioya, S., 2003. Enhanced kefiran production by mixed culture of *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae*. J. Biotechnol. 100, 43–53.

Domingues, L., Lima, N., Teixeira, J.A., 2001. Alcohol production from cheese whey permeate using genetically modified flocculent yeast cells. Biotechnol. Bioeng. 72, 507–514.

Domingues, L., Guimarães, P.M.R., Oliveira, C., 2010. Metabolic engineering of *Saccharomyces cerevisiae* for lactose/whey fermentation. Bioeng. Bugs. 1 (3), 1–8.

Dragone, G., Mussatto, S.I., Oliveira, J.M., Teixeira, J.A., 2009. Characterisation of volatile compounds in an alcoholic beverage produced by whey fermentation. Food Chem. 112, 929–935.

Ercolini, D., Hill, P.J., Dodd, C.E.R., 2003. Bacterial community structure and location in Stilton cheese. Appl. Environ. Microbiol. 69, 3540–3548.

Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001. Behavior of variable V3 region from 16S rDNA of important lactic acid bacteria in denaturing gradient gel electrophoresis. Curr. Microbiol. 42, 199–202.

Farnworth, E.R., 2005. Kefirda complex probiotic. Food Sci. Technol. Bull.: Funct. Foods 2, 1–17.

Guimarães, P.M.R., François, J., Parrou, J.L., Teixeira, J.A., Domingues, L., 2008. Adaptive evolution of a lactose-consuming *Saccharomyces cerevisiae* recombinant. Appl. Environ. Microbiol. 74, 1748–1756.

Guimarães, P.M.R., Teixeira, J.A., Domingues, L., 2010. Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnol. Adv. 28, 375–384.

Güzel-Seydim, Z., Wyffels, J.T., Seydim, A.C., Greene, A.K., 2005. Turkish kefir and kefir grains: microbial enumeration and electron microscopic observation. Int. J. Dairy Technol. 58, 25–29.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Haruta, S., Ueno, S., Egawa, I., Hashiguchi, K., Fujii, A., Nagano, M., Ishii, M., Igarashi, Y., 2006. Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. Int. J. Food Microbiol. 109, 79–87.

Holsinger, V.H., Posati, L.P., De Vilbiss, E.D., 1974. Whey beverages: a review. J. Dairy Sci. 57, 849–859.
Jianzhong, Z., Xiaoli, L., Hanhu, J., Mingsheng, D., 2009. Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis. Food Microbiol. 26, 770–775.
Kanagawa, T., 2003. Microvariation artifacts introduced by PCR and cloning. J. Biosci. Bioeng. 96, 317–323.
Koutinas, A.A., Athanasiadis, I., Bekatorou, A., Iconomopoulou, M., Blekas, G., 2005. Kefir yeast technology: scale-up in SCP production using milk whey. Biotechnol. Bioeng. 89, 788–796.
Koutinas, A.A., Papapostolou, H., Dimitrellou, D., Kopsahelis, N., Katechaki, E., Bekatorou, A., Bosnea, L.A., 2009. Whey valorisation: a complete and novel technology development for dairy industry starter culture production. Bioresour. Technol. 100, 3734–3739.
Magalhães, K.T., Pereira, G.V.M., Dias, D.R., Schwan, R.F., 2010. Microbial communities and chemical changes during fermentation of sugary Brazilian kefir. World J. Microbiol. Biotechnol. 33, 1–10.
Medrano, M., Hamet, M.F., Abraham, A.G., Pérez, P.F., 2009. Kefiran protects Caco-2 cells from cytopathic effects induced by *Bacillus cereus* infection. Anton. van Leeuw. 96, 505–513.
Naumova, E.S., Ivannikova, Yu.V., Naumov, G.I., 2004. Genetic differentiation of the sherry yeasts *Saccharomyces cerevisiae*. Appl. Biochem. Microbiol. 41, 578–582.
Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178, 5636–5643.
Otles, S., Cagindi, O., 2003. Kefir: a probiotic dairy-composition, nutritional and therapeutic aspects. Pak. J. Nutr. 2 (2), 54–59.
Ovreas, L., Forney, L., Daae, F.L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. 63 (9), 3367–3373.
Paraskevopoulou, A., Athanasiadis, A., Blekas, I.G., Koutinas, A.A., Kanellaki, M., Kiosseoglou, V., 2003. Influence of polysaccharide addition on stability of a cheese whey kefir–milk mixture. Food Hydrocolloids 17, 615–620.
Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W.M., Vaughan, E.E., 2002. Diversity, dynamics and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Appl. Environ. Microbiol. 68, 1882–1892.
Sanguinetti, C.J., Neto, E.D., Simpson, A.J.G., 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechnology 17 (5), 914–921.
Sekiguchi, H., Tomioka, N., Nakahara, T., Uchiyama, H., 2001. A single band does not always represent single bacterial strains in denaturing gel electrophoresis analyses. Biotechnol. Lett. 23, 1205–1208.
Silva, K.R., Rodrigues, S.A., Filho, L.X., Lima, A.S., 2009. Antimicrobial activity of broth fermented with kefir grains. Appl. Biochem. Biotechnol. 152, 316–325.
Smithers, G.W., 2008. Whey and whey proteins – From 'gutter-to-gold'. Int. Dairy J. 18, 695–704.
Tada, S., Katakura, Y., Ninomiya, K., Shioya, S., 2007. Fed-batch coculture of *Lactobacillus kefiranofaciens* with *Saccharomyces cerevisiae* for effective production of kefiran. J. Biosci. Bioeng. 103 (6), 557–562.
Van Beek, S., Priest, F.G., 2002. Evolution of the lactic acid bacterial community during malt whisky fermentation: a polyphasic study. Appl. Environ. Microbiol. 68, 297–305.
Wang, X., Haruta, S., Wang, P., Ishii, M., Igarashi, Y., Cui, Z., 2006. Diversity stable enrichment culture which is useful for silage inoculant and its succession in alfalfa silage. FEMS Microbiol. Ecol. 57, 106–115.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfang, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.