ELSEVIER

International Dairy Journal 12 (2002) 407–411

INTERNATIONAL DAIRY JOURNAL

www.elsevier.com/locate/idairyj

An application of PCR-DGGE analysis to profile the yeast populations in raw milk

Luca Cocolin[a], Daniele Aggio[a], Marisa Manzano[a], Carlo Cantoni[b], Giuseppe Comi[a,*]

[a] *Dipartimento Scienze degli Alimenti, Università degli studi di Udine, via Marangoni 97, 33100 Udine, Italy*

[b] *Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza degli Alimenti, Università degli studi di Milano, via Celoria 10, 20121 Milan, Italy*

Received 3 July 2001; accepted 19 December 2001

Abstract

Four different zones from the Friuli Venezia Giulia region, North East of Italy, were sampled for the study of the yeast bio-diversity in raw milk. Samples were analysed by traditional methods to isolate different yeast strains that were subjected to identification by sequencing the D1–D2 domains of the 26S rRNA gene. Twelve different species of yeast were identified, six of them belonging to the genera *Candida* and two to the genera *Kluyveromyces*. The identified strains were then used for the optimization of a method based on polymerase chain reaction and denaturing gradient gel electrophoresis that was used for a direct monitoring of the populations in the samples. Applying the method to the DNA extracted directly from the raw milk samples, new bands appeared in the gel underlining a different bio-diversity in respect to the traditional method. The approach described is a powerful and reliable tool to monitor directly yeast ecology in milk and milk products without the need of traditional isolation and it could be used to follow specific populations to prevent spoilage or to control contamination. © 2002 Published by Elsevier Science Ltd.

Keywords: Raw milk; PCR; DGGE; Yeast ecology

1. Introduction

The microbial spoilage of milk is generally associated with the growth of bacteria (Cousin, 1982; Bishop & White, 1986). Very little consideration has been given to the ability of yeasts to grow in milk (Fleet, 1990). However, a range of observations indicate an ability of yeasts to metabolize milk constituents. These observations include the occurrence and growth of yeasts in many cheeses, especially soft mould-ripened cheeses (Fleet, 1990; Roostita & Fleet, 1996), the spoilage of condensed milks and yogurts by yeasts (Fleet, 1990), isolated incidences of yeast spoilage of pasteurized milks (Craven & Macauley, 1992) and previous studies by Fleet and Mian (1987) showed the potential of yeast species to exhibit significant growth in UHT processed milk.

Although milk is the raw material of most dairy products, surprisingly, few studies have been conducted on the specific occurrence of yeasts in either raw or pasteurized milks. Often, information on yeasts is reported as an appendage to more detailed bacteriological studies. Generally the available information shows that yeasts occur in both raw and pasteurized milks, but at low and insignificant size of populations (Fleet, 1990). Populations less than 10^3 cells mL^{-1} are mostly reported but, occasionally, counts as high as 10^4 cells mL^{-1} can occur. Such yeasts rarely grow in milk during refrigerated storage and are quickly overgrown by psychrotrophic bacteria (Cousin, 1982; Bishop & White, 1986). However, yeast growth might occur in milk where bacterial growth has been inhibited by residual antibiotics. Also, yeasts might develop in milk as secondary flora, after bacterial growth and spoilage.

Recently, a variety of new methods have been developed to directly characterize the micro-organisms in particular habitats without the need for enrichment or isolation (Head, Saunders, & Pickup, 1998). Typically these strategies examine the total microbial DNA (or RNA) derived from mixed microbial populations to identify individual constituents (Hugenholtz & Pace, 1996). This approach eliminates the necessity for strain isolation, thereby negating the potential biases inherent to microbial enrichment. Moreover, studies which have

*Corresponding author. Tel.: +39-04-32-590745; fax: +39-04-32-590719.

E-mail address: giuseppe.comi@dsa.uniud.it (G. Comi).

0958-6946/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0958-6946(02)00023-7

employed such direct analysis have repeatedly demonstrated a tremendous variance between cultivated and naturally occurring species, thereby dramatically altering perception on the true microbial diversity present in various habitats (Hugenholtz, Goebel, & Pace, 1998).

In this paper, the application of denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified ribosomal RNA (rDNA) genes, to study the natural distribution of yeast species in raw milk from the North East regions of Italy, is described. Yeast DNA was isolated directly from the milk and used as a template for amplification of individual 26S rRNA gene fragments. These fragments were then separated by DGGE and the resultant bands isolated and sequenced to permit strain identification. In this report we demonstrate that PCR-DGGE is a potential alternative to traditional plating schemes for simple monitoring of yeast populations in raw milks from different geographic areas.

2. Materials and methods

2.1. Raw milk samples

Samples were collected directly from local laboratories of four different geographic areas of the North East of Italy, covering different zones of the Friuli Venezia Giulia region. A total of eight samples were collected from each zone during a 15 days period. Raw milk was kept at 4°C during sampling and delivery, and subjected immediately to analysis. Traditional isolation on agar plates and DNA extraction followed by amplification and DGGE were performed.

2.2. Traditional isolation and identification of the isolates

Milks were subjected to traditional microbiology analysis to determine the yeast content and to isolate strains that were subsequently identified. Raw milk and further decimal dilution were plated on malt extract agar (Oxoid, Milan, Italy) supplemented with tetracycline (1 mg mL^{-1}) after sterilization at 115°C for 15 min. Plates were incubated at 28°C for 48–72 h and after this period, yeast colonies were counted. Five colonies, with different morphology characteristics, were isolated and a total of about 120 strains were identified. The identification was performed by sequencing the 5′ end of the large subunit rDNA encompassing the D1 and D2 expansion domains using the previously described primers NL1 and NL4 (Kurtzman & Robnett, 1998). In particular, following DNA extraction (Cocolin, Bisson, & Mills, 2000), 2 μL of DNA were loaded in a 50 μL PCR reaction containing 10 mM Tris-HCl pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.2 mM each primer and 1.25 UI *Taq*-polymerase (Promega, Milan, Italy). Amplification was performed in a Mini-Cycler (Genenco, Florence, Italy) using an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 60 s, 56°C for 90 s and 72°C for 120 s, and ended by a final extension at 72°C for 7 min. After purification using a commercial kit (Roche diagnostics, Milan, Italy), the PCR products were sent to a commercial sequencing facility (MGW Biotech, Edersberg, Germany) for sequencing.

2.3. Total DNA isolation from milk samples

Two milliliters of milk samples were centrifuged at 14,000 × *g* for 10 min at 4°C, put at −80°C for 30 min to solidify the sample and the fat layer, separated by centrifugation, was removed from the tube. After thawing, the supernatant was discarded and the precipitated cells were re-suspended in 1 mL of 8 g L^{-1} NaCl solution and transferred to a microcentrifuge tube containing 0.3 g of 0.5 mm diameter glass beads. The mixture was centrifuged at 14,000 × *g* for 10 min at 4°C and the supernatant discarded. The cell/bead pellet was re-suspended in 300 μL of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA pH 8) and 300 μL of phenol–chloroform–isoamyl alcohol 25:24:1 (Sigma, Milan, Italy) were added. The cells were then homogenized in a bead beater (Mini-BeadBeater 8™, Biospec Products Inc., OK, USA) three times, each for 30 s at maximum speed at room temperature. 300 μL of TE (10 mM Tris, 1 mM EDTA pH 7.6) were added and the tubes were centrifuged at 12,000 × *g* for 10 min at 4°C. The aqueous phase was collected and the DNA was precipitated with 1 mL ice-cold absolute ethanol. After centrifugation at 14,000 × *g* for 10 min at 4°C, the pellet was dried under vacuum at room temperature and re-suspended in 50 μL of sterile distilled water containing 2 IU DNase-free RNase (Roche Diagnostics, Milan, Italy). The samples were then incubated at 37°C for 30 min before storage at −20°C.

2.4. PCR-DGGE protocol

The D1 region of the 26S rRNA gene was amplified by PCR using the primers NL1GC (5′-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GGC CAT ATC AAT AAG CGG AGG AAA AG-3′) (the GC clamp is underlined) and a reverse primer LS2 (5′-ATT CCC AAA CAA CTC GAC TC–3′) (Cocolin et al., 2000). PCR was performed in a final volume of 50 μL containing 10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.2 mM of the primers, 1.25 UI *Taq*-polymerase (Promega, Milan, Italy) and 2 μL of the extracted DNA. The amplification was carried out as follows: initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 60 s, 52°C for 45 s and

72°C for 60 s, and a final extension at 72°C for 7 min. Five microliters of the amplified mixture was analysed in a 2% 0.5X TBE agarose gel electrophoresis containing 0.5 mg mL^{-1} ethidium bromide, visualized under UV illumination and photographed using a DS34 camera system (Polaroid, MO, USA). For PCR products sequence specific separation, DGGE analysis with the Dcode™ Universal Mutation Detection System (BioRad, CA, USA) were then carried out. Electrophoresis were performed in a 0.8 mm polyacrylamide gel (8% w/v acrylamide: bisacrylamide 37.5:1) using a denaturing gradient from 30% to 50% of urea and formamide (100% corresponds to 7 M urea and 40% w/v formamide) increasing in the direction of the electrophoresis run. The electrophoresis was performed at a constant voltage of 130 V for 4.5 h at constant temperature of 60°C. After the run the gels were stained for 20 min in 1.25X TAE containing 1X SYBR Green (Molecular Probes, OR, USA) and photographed under UV illumination.

2.5. Sequencing of DGGE bands

Small pieces of DGGE bands were punched from the gel using a sterile pipette tip. The blocks were, then, transferred, in 50 μL sterile water and incubated overnight at 4°C to allow diffusion of the DNA. Two microliters of the DNA was used for the re-amplification and the PCR products, generated with the GC clamped primer, were checked by DGGE using the milk sample DNA as a control. Only products migrating as a single band and at the same position with respect to the control, were amplified with the primer without GC clamp, purified and sent for sequencing.

2.6. Sequence analysis and nucleotide accession numbers

Searches in the GenBank with the Blast program (Altschul, Gish, Miller, Myers, & Lipman, 1990) were performed to determine the closest known relatives of the partial 26S rDNA sequence obtained. The GenBank accession numbers for the sequences deposited are given in Table 1.

3. Results and discussion

Milk samples from the North East of Italy, Friuli Venezia Giulia region, were studied to assess the yeast bio-diversity using direct and indirect microbiological methods. Samples were subjected to plating analysis for the isolation of the yeasts present, which were then identified by sequencing the D1–D2 domains of the 26S rDNA. Moreover, isolates were used to optimize a PCR-DGGE protocol to analyse PCR products obtained from the DNA extracted directly from the milk samples. Yeasts were subjected to PCR amplification using two sets of primers: NL1/NL4 for the sequencing identification (Kurtzman & Robnett, 1998) and NL1GC/LS2 for the DGGE analysis (Cocolin et al., 2000). The results obtained from the yeast identification by sequencing approach are described in Table 1 and their related percentage of isolation are reported in Table 2. Six different species of *Candida*, including *C. catenulata, C. pararugosa, C. parapsilosis, C. zeylanoides, C. pseudointermedia* and *C. rugosa,* two species of *Kluyveromyces*, *K. marxianus* and *K. lactis*, *Saccharomyces cerevisiae*, *Pichia guilliermondii*, *Trichosporon mucoides* and *Cryptococcus curvatus* were identified.

Table 1
Sequences information from the 26S rDNA PCR product obtained from the yeast strains isolated from the milk samples

Strain number	Closest relative	% Identity[a]	Accession number
11.3	*Candida catenulata*	97.8	AF374607
15.1	*C. pararugosa*	99.8	AF374608
19.1	*C. parapsilosis*	97.8	AF374609
5.1	*C. zeylanoides*	99.6	AF374610
23.2	*C. pseudointermedia*	100	AF374611
17.3	*C. rugosa*	98.3	AF374612
28.2	*Kluyveromyces marxianus*	97.8	AF374613
6.3	*K. lactis*	98.5	AF374614
5.4	*Saccharomyces cerevisiae*	100	AF374615
7.3	*Pichia guilliermondii*	99.6	AF374616
7.2	*Trichosporon mucoides*	99.5	AF374617
4.4	*Cryptococcus curvatus*	99.0	AF374618

[a] Identical nucleotides percentage in the sequence obtained from the DGGE band and the sequence found in GenBank.

Table 2
Results of the yeast identification by sequencing approach and their isolation percentage

Yeast species	% of isolation
Candida spp.	
catenulata	8.6
pararugosa	18.8
parapsilosis	13.0
zeylanoides	8.6
pseudointermedia	4.3
rugosa	2.9
Total	56.2
Cryptococcus curvatus	10.6
Kluyveromyces spp.	
marxianus	20.3
lactis	1.4
Total	21.7
Pichia guilliermondii	4.3
Saccharomyces cerevisiae	2.9
Trichosporon mucoides	4.3

Candida spp. counted 56.2% of the total isolates representing the main yeasts found in the milk samples, followed by *Kluyveromyces* spp., in particular *K. marxianus* (20.3%), and *Cr. curvatus* (10.6%). *P. guilliermondii*, *S. cerevisiae* and *T. mucoides* were isolated less frequently. When the strains were subjected to PCR using the primers NL1GC/LS2 and analysed by DGGE, a good differentiation was obtained (Fig. 1). Specific migrations allowed an easy and rapid identification, at the species level, of the isolates. By simple DGGE analysis it was possible unequivocally to identify different species from the same genera, due to the different patterns obtained in the gel. All the strains belonging to the same species showed the same migration in the gel and no differences were obtained when results from the sequencing were compared to DGGE profiles. The DGGE protocol, optimized using the isolated strains, was then applied for the study of the yeast populations directly in milk without the need for traditional isolation. Four areas of the Friuli Venezia Giulia region were considered in the study representing geographic areas with different characteristics by the point of view of the territory. The results obtained are shown in Fig. 2 where the profiles of the zones studied are reported. Different patterns were detected, underlining the diversity of the population in the different zones considered, but the specific geographic distribution of the yeast strains cannot be demonstrated since the number of samples processed were not statistically significant. Some bands were present in all of the samples, while others were specific for some zones. For the identification of the yeast population represented in the gel, bands of interest were excised and after re-amplification, sent for sequencing. Analysing the results obtained, new yeast species were detected directly in raw milk. *Galactomyces* spp., *C. kefyr, S. bayanus/pastorianus* and *C. humulis* were represented in the gel by specific bands migrating in different position in respect of the standard strains used, but were not isolated by the plating method. However, not all the species isolated on malt agar were present as bands in the gel. *C. catenulata, C. parapsilosis, C. zeylanoides, Cr. curvatus, P. guilliermondii* and *T. mucoides* did not give a band in the gel underlining a population less than 10^4 cfu mL^{-1}, defined as the sensitivity of the PCR-DGGE protocol used (Cocolin et al., 2000). Comparing the two methods, differences in the bio-diversity can be detected which have to be analysed carefully considering the biases related with both traditional and PCR method. Traditional methods are enable to characterize micro-organisms for which selective enrichments and culturing are problematic or impossible, thereby eliminating entire populations from consideration, and PCR can amplify also dead cells that can be detected as specific bands in the DGGE gels, evidencing yeast populations not viable in the samples.

The method described in this paper opens the possibility for a fast and easy identification of yeast

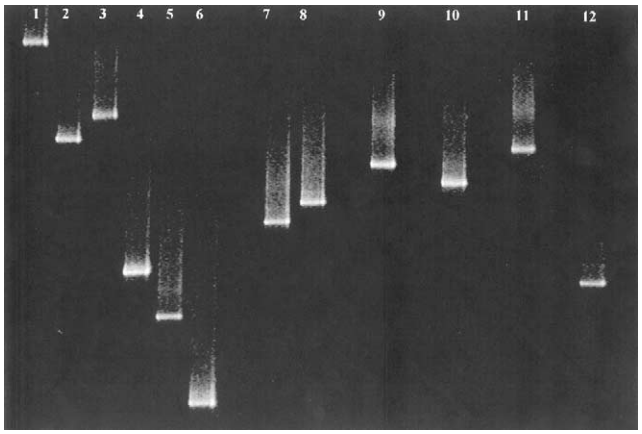

Fig. 1. DGGE profiles of the yeast strains isolated from milk: Lane 1, *Candida pararugosa*; lane 2, *C. parapsilosis*; lane 3, *C. zeylanoides*; lane 4, *C. pseudointermedia*; lane 5, *C. catenulata*; lane 6, *C. rugosa*; lane 7, *Kluyveromyces marxianus*; lane 8, *K. lactis*; lane 9, *Trichosporon mucoides*; lane 10, *Pichia guilliermondii*; lane 11, *Cryptococcus curvatus*; lane 12, *Saccharomyces cerevisiae*.

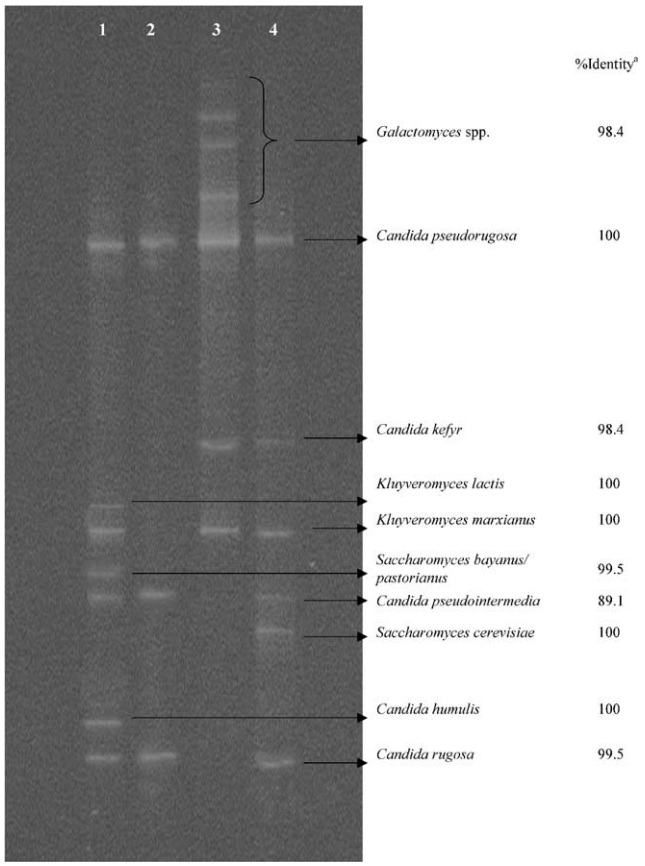

Fig. 2. DGGE profiles of the PCR products obtained from the DNA extracted directly from milk: Lane 1, zone 4; lane 2, zone 3; lane 3, zone 2; lane 4, zone 1. Arrows indicate the identified species after band sequencing.

strains isolated from milk and for the direct study of yeast populations spread in milks from different geographic areas, avoiding the potential biases of traditional methods. The simplicity of the results interpretation, significant characteristics of the PCR-DGGE method performed, must be taken into consideration if compared to the traditional methods or to the sequencing approach. Traditional identification is problematic and time consuming. Moreover the results often come from personal evaluation of the biochemistry characteristics. The sequencing approach can be a good substitute because of its rapidity, but sometimes, especially for species that are very close genetically, it is difficult to obtain fast and undoubted identification. DGGE profiles are simple to interpret and for this reason the identification is immediate. Moreover, the possibility to exploit DGGE to study yeast ecology directly in the sample, allows a better understanding of the bio-diversity in milk. This approach has been already used in the wine microbiology to follow the yeast dynamics during commercial wine fermentation (Cocolin, Heisey, & Mills, 2001). Here the method is applied to raw milk, obtaining results that open up the possibility for extension of the application to dairy products. Using the protocol, specific yeast strains during production can be followed or their presence can be monitored to control contamination or to avoid spoilage.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410.

Bishop, J. R., & White, C. H. (1986). Assessment of dairy product quality and potential shelf-life—a review. *Journal of Food Protection*, *49*, 739–753.

Cocolin, L., Bisson, L. F., & Mills, D. A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, *189*, 81–87.

Cocolin, L., Heisey, A., & Mills, D. A. (2001). Direct identification of the indigenous yeasts in commercial wine fermentation. *American Journal of Enology and Viticulture*, *52*, 49–53.

Cousin, M. A. (1982). Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. *Journal of Food Protection*, *45*, 172–207.

Craven, H. M., & Macauley, B. J. (1992). Microorganisms in pasteurised milk after refrigerated storage. 1. Identification of types. *Australian Journal of Dairy Technology*, *47*, 38–45.

Fleet, G. H. (1990). Yeasts in dairy products. *Journal of Applied Bacteriology*, *68*, 199–211.

Fleet, G. H., & Mian, M. A. (1987). The occurence and growth of yeasts in dairy products. *International Journal of Food Microbiology*, *4*, 145–155.

Head, I. M., Saunders, J. R., & Pickup, R. W. (1998). Microbial evolution, diversity and ecology: A decade of ribosomal rna analysis of uncultivated microorganisms. *Microbial Ecology*, *35*, 1–21.

Hugenholtz, P., Goebel, B. M., & Pace, N. R. (1998). Impact of culture-independent studies on emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology*, *180*, 4765–4774.

Hugenholtz, P., & Pace, N. R. (1996). Identifying microbial diversity in the natural environment: A molecular phylogenetic approach. *Trends in Biotechnology*, *14*, 90–97.

Kurtzman, C. P., & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek*, *73*(4), 331–371.

Roostita, R., & Fleet, G. H. (1996). Growth of yeasts in milk and associated changes to milk composition. *International Journal of Food Microbiology*, *31*, 205–219.