Enumeration of Yeasts in Dairy Products: A Comparison of Immunological and Genetic Techniques

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ABSTRACT

Enzyme-linked immunosorbent assay (ELISA) and PCR techniques have been developed for the detection of spoilage yeast species in dairy products. Polyclonal antibodies against live yeast cells (AY) were raised in rabbits by inoculation of a mixture of 10 yeast species frequently associated with dairy products spoilage. AY antibodies were used for the development of two ELISA formats (indirect and double-antibody sandwich ELISA) for the detection of yeast species in milk and yogurt. A PCR assay was also developed for yeast detection in dairy products, using primers designed to amplify a conserved 250-base-pair fragment of the 18S rRNA of the yeast species. The results obtained in this work show that ELISA techniques using polyclonal antibodies against viable yeast cells are of limited value for the detection and enumeration of spoilage yeast species in dairy products. On the contrary, PCR amplification of a conserved region of the 18S rRNA of the yeast species allows the homogeneous detection of all the yeast species tested and, combined with an overnight enrichment of samples, could be used for the detection of low levels of viable spoilage yeast species in dairy products.

Yeast species are known to be the most important contaminants in some types of dairy products. This is especially relevant in fermented products such as yogurt and sour milk, where yeast species are a major cause of spoilage because the low pH offers a selective environment for their growth (23). When yogurts are produced under good manufacturing practices, they should contain less than 10 yeast cells per g, and a shelf life of 3 or 4 weeks may be expected if they are properly refrigerated (5°C). However, yogurts that are contaminated with an initial load of 100 or more yeast cells per g will quickly spoil as the yeast cells multiply. Ingredients such as sugar and fruits added to yogurts provide additional sources of contamination, thus increasing the risk of yeast spoilage during storage (26). Spoilage becomes evident when the yeast population reaches $10^5$ to $10^6$ cells per g and is first seen as swelling of the yogurt package due to gas production by yeast fermentation (9). Also, the yogurt may acquire a yeasty, fermentative off-flavor and gassy appearance, and occasionally, yeast colonies are seen under the surface of the package lid (26).

Economic losses because of spoilage by yeast species have increased in European companies because of less severe preservation procedures (lower concentration of preservatives), packaging in modified atmospheres, or new formulations that occasionally permit the growth of yeast species. The control of this type of spoilage has become one of the main concerns of manufacturers of dairy products (9, 16).

To control spoilage, it is essential to use effective techniques for detecting and quantifying yeast populations in raw materials and finished products. The routine detection and enumeration of yeast species are based on the determination of the colony count (1, 29). Colony count techniques, although simple, convenient, and widely accepted, have a number of disadvantages; they are labor-intensive and, most importantly, plate counting is slow to provide data (3 to 5 days), which are available only for retrospective evaluating and are, therefore, of limited value in quality and process control (5). Several methods have been proposed to overcome the disadvantages inherent in traditional plate count methods. These include an ISO-GRID membrane filtration system (7), electrometric techniques (6), a direct epifluorescent filter technique, and ATP measurement by bioluminescence technology (8). They have been mainly applied for the rapid detection of yeast species in beer, wine, and carbonated beverages.

Immunoassays and PCR technology offer accurate and rapid alternatives to conventional methods for the detection of spoilage and pathogenic microorganisms in foods (10, 25). However, they have been applied mainly for the detection of pathogenic bacteria in food products. Immunoassays for the detection of yeast species are scarce (17–19, 30). Moreover, genetic techniques have been applied for characterization of yeast isolates (22, 27) but not for the detection of spoilage yeast species in foods.

The objective of this research was to compare immunological and PCR techniques for the rapid detection of spoilage yeast species in milk and yogurt.
MATERIALS AND METHODS

Microorganisms and culture conditions. The yeast strains Kluyveromyces marxianus (CECT 10584), Kluyveromyces lactis (CECT 1121), Debaryomyces Hansenii (CECT 10360), Pichia anomala (CECT 10314), Saccharomyces cerevisiae (CECT 1347), Rhodotorula mucilaginosa (CECT 10359), Yarrowia lipolytica (CECT 10358), Candida boidinii (CECT 10029), and Candida zeylanoides (CECT 10048) were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). Yeast species were grown in LM medium (10 g/liter glucose, 5 g/liter polypeptone, 3 g/liter yeast extract, and 3 g/liter malt extract) at 25°C in an orbital shaker at 125 rpm for 24 h.

The molds Rhizopus microsporus (CECT 2763), Aspergillus niger (CECT 20156), Penicillium roquefortii (CECT 290ST), Penicillium camemberti (CECT 2267), and Mucor racemosus (CECT 2670) were obtained from the Spanish Type Culture Collection and grown on LM agar at 25°C for 3 days.

Sabouraud dextrose agar (pH 5.6) (Oxoid Ltd., Basingstoke, UK) supplemented with chloramphenicol (0.4 g/liter) was used to enumerate yeast and mold species. Plates were incubated at 25°C for 72 h before colonies were counted.

Staphylococcus aureus, Escherichia coli, Salmonella Typimurium, Yersinia enterocolitica, Lactococcus lactis, Enterococcus faecium, and Lactobacillus bulgaricus were grown to the late log phase (approximately 10^8 cells/ml) of the yeast strains described above were mixed in a 1.5-mL microcentrifuge tube. The mixture was centrifuged at 10,000 × g for 3 min. The pellet was washed three times with 1 mL of sterile phosphate-buffered saline (PBS; 136 mM NaCl, 1.4 mM KH_2PO_4, 8.09 mM Na_2HPO_4·12H_2O, and 2.6 mM KCl, pH 7.2) and finally resuspended in 0.5 mL of the same buffer.

Preparation of antigenic extracts. Yeast species were grown to the late log phase (approximately 10^8 cells/ml) in LM medium. Then, a 0.1-mL culture of each of the 10 yeast strains described above were mixed in a 1.5-mL microcentrifuge tube. The mixture was centrifuged at 10,000 × g for 3 min. The pellet was washed three times with 1 mL of sterile phosphate-buffered saline (PBS; 136 mM NaCl, 1.4 mM KH_2PO_4, 8.09 mM Na_2HPO_4·12H_2O, and 2.6 mM KCl, pH 7.2) and finally resuspended in 0.5 mL of the same buffer.

Production of polyclonal antibodies. Polyclonal antibodies against yeast surface antigens (AY) were raised in New Zealand male white rabbits. Immunization started by intradermic injection of 0.5 mL of the antigenic extract emulsified in Freund's Complete Adjuvant (Sigma Chemical Co., St. Louis, Mo.) at multiple dorsal sites. Six booster doses made in Freund's Incomplete Adjuvant (Sigma) were applied alternatively by intradermic or intramuscular injection every 3 weeks. Two weeks after the last injection, the rabbits were bled, and the blood was allowed to clot for 1 h at 20°C. Serum was collected by centrifugation at 2,000 × g for 10 min. Immunoglobulins from the crude antisera were partially purified by ammonium sulfate precipitation (12). Samples (1 mL) of the partially purified antisera were stored at −20°C.

Biotinylated antibodies. Biotinamidocaproate N-hydroxysuccinimide ester (Sigma), dissolved at 1 mg/mL in dimethyldioctamide (Merck, Darmstadt, Germany), was added at a molar excess of 50 to the AY antibodies dissolved in PBS (1 mg/mL) and incubated for 2 h at 20°C (2). Unbound reagent was separated from the conjugated antibodies by dialysis overnight at 4°C against PBS. Samples of biotinylated AY antibodies (0.1 mL) were stored at −20°C until use in a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA).

Milk and yogurt samples. Reference samples of ultrahigh-temperature milk and skimmed yogurt were inoculated with individual pure yeast cultures to obtain yeast counts of 10^5 to 1 cell per ml. They were used in the ELISA techniques.

Extraction of yeast species from milk and yogurt samples. One-milliliter samples of milk or yogurt were thoroughly mixed in a microcentrifuge tube with 0.5 mL of a milk clearing solution (Promega Corp., Madison, Wis.). The tubes were then centrifuged at 12,000 × g for 5 min. After the centrifugation of milk samples, a cream pad was formed on top of a clear supernatant, and a cell pellet was formed at the bottom. However, for yogurt samples, a clear supernatant and a thick elastic pellet were formed. The supernatant and creamy pad were carefully removed, and the cell pellet containing the yeast species was resuspended in 1 mL of sterile PBS, centrifuged at 12,000 × g for 5 min, and finally resuspended in 1 mL of sterile PBS for ELISA experiments.

Indirect ELISA procedure. Flat-bottomed micro-ELISA plates (Costar, Cambridge, Mass.) were filled with 0.1 mL of the yeast species (recovered from milk, yogurt, or pure culture) diluted in PBS, pH 7.2, and incubated for 1 h at 37°C. The wells were washed five times with PBS and coated with 0.2 mL of 0.1% bovine serum albumin (Sigma) in PBS for 30 min at 37°C. The wells were then washed five times with PBS (containing 1% Tween 20), 0.1 mL of the AY antibodies diluted in PBSTM (PBST containing 1% skimmed milk powder) was added to the wells, and the plates were incubated on a plate shaker (LKB-Pharmacia, Uppsala, Sweden) for 1 h at 20°C. After another five washes with PBST, 0.1 mL of peroxidase-conjugated goat anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) diluted 1:1,000 in PBST was added to the wells, and the plates were incubated with shaking for 1 h at 20°C. Wells were washed five more times with distilled water before the addition of 0.05 mL of the substrate solution (3,3',5,5'-tetramethylbenzidine, Roche Diagnostics, Mannheim, Germany). After a 15-min incubation, the reaction was stopped by the addition of the 0.05 mL of 1 M H_2SO_4. The yellow color developed in a spectrophotometer (EM-reader MeF, Labsystems, Vantaa, Finland).

Sandwich ELISA procedure. Flat-bottomed micro-ELISA plates (Costar) were filled with 0.1 mL of the capture AY antibodies diluted 1:100 in carbonate coating buffer (sodium carbonate-bicarbonate buffer, pH 9.6) and incubated for 1 h at 37°C. The wells were washed five times with PBST and coated with 0.2 mL of 0.1% bovine serum albumin in PBS for 30 min at 37°C. After five washes with PBST, 0.1 mL of the milk, yogurt, or culture samples diluted in PBST was added to the wells, and the plates were incubated with shaking for 1 h at 20°C. After another washing with PBST, 0.1 mL of biotinylated AY antibodies, diluted 1:100 in PBST, was added to the wells, and the plates were incubated with shaking for 1 h at 20°C. After another washing with PBST, 0.1 mL of biotinylated AY antibodies, diluted 1:100 in PBST, was added to the wells, and the plates were incubated with shaking for 1 h at 20°C. After a final washing with distilled water, 0.05 mL of the substrate solution was added to each well, and the reaction was visualized as described for the indirect ELISA.

Enrichment of yeast species in milk and yogurt samples. Reference samples of ultrahigh-temperature milk and skimmed yogurt were inoculated with a pure K. marxianus culture to obtain yeast counts of 10^7 to 1 cell per ml. DNA was extracted from inoculated samples and from uninoculated reference samples immediately after inoculation and also after incubation at 25°C for 16 h with shaking at 125 rpm.

DNA extraction. Microorganisms were harvested from pure liquid cultures by centrifugation at 12,000 × g for 5 min. When contaminated milk or yogurt samples were to be analyzed, micro-
organisms were previously recovered using a milk clearing solution, as described above. The pellet containing the microorganisms was washed three times with sterile PBS before extraction of DNA. DNA from yeast, mold, and bacterial species was then extracted from the resulting pellet using a Wizard Genomic DNA Purification kit (Promega) following the manufacturer’s directions for isolation of genomic DNA from yeast species.

**PCR amplification.** Primers 18SBDIR (5’-GCGATAACGAA-CGAGACCTTAA-3’) and 18SDINV (5’-TTGAGCCATAATTGCAGTGTC-3’) (11) were designed for the amplification of a fragment of the 18S rRNA gene on the basis of a comparison of sequences available in the GenBank database for D. hansenii (accession number AB013568), C. zeylanoides (AB013509), K. marxianus (X89522), S. cerevisiae (J01353), Cryptococcus albidus (AB032616), P. anomala (D86914), R. mucilaginosa (AB021668), Y. lipolytica (AB018158), R. microsporus (AF113438), A. niger (D63697), Penicillium freii (AJ005446), M. racemosus (AB113430), Bos taurus (AF176811), and Sus scrofa (AF102857). This set of primers should produce amplicons of approximately 250 base pairs (bp) from yeast DNA. Sequence analysis was performed using the Wisconsin Package, version 9.0 (Genetics Computer Group, Madison, Wis.).

**PCR amplification reactions** were performed in a total volume of 50 μl containing 1 to 5 ng of template DNA from pure microbial cultures or 2 μl of genomic DNA extracted from milk or yogurt samples. The reaction mixture consisted of 2 mM MgCl₂, 10 pmol of each primer, 200 μM of each dNTP, and 2 U of Tth DNA polymerase (Biotools, Madrid, Spain) in a reaction buffer containing 75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM (NH₄)₂SO₄, and 0.001% BSA. PCR amplification was carried out in a Progene Thermal Cycler (Techne Ltd., Cambridge, UK), programmed to perform a denaturation step of 96°C for 5 min, and was followed by 35 cycles consisting of 45 s at 95°C for denaturation, 45 s at 60°C for primer annealing, and 45 s at 72°C for extension. The last extension step was 5 min longer.

PCR products were electrophoresed in a 1.5% low electroendosmosis D1 agarose gel (Hispanlab S.A., Torrejón, Spain) containing 1 μg/ml of ethidium bromide in Tris-acetate buffer (0.04 M Tris-acetate and 0.001 M EDTA, pH 8.0) for 45 min at 100 V. The resulting DNA fragments were visualized by UV transillumination and analyzed by the Geldoc 1000 UV Fluorescent Gel Documentation System-PC (Bio-Rad Laboratories, Hercules, Calif.).

**FIGURE 1.** Relationship between indirect ELISA results and colony counts for pure cultures of the yeast species K. marxianus (Km), K. lactis (Kl), D. hansenii (Dh), S. cerevisiae (Sc), P. anomala (Pa), C. boidinii (Cb), R. mucilaginosa (Rm), Y. lipolytica (Yl), and C. zeylanoides (Cz); the mold species M. racemosus (Mr) and P. roqueforti (Pr); and the bacterial species S. aureus (Sa) and E. coli (Ec). The AY antibody was diluted 1:2,000 (vol/vol), and the conjugate was diluted 1:1,000 (vol/vol).

**RESULTS**

Characterization of antiserum against yeast species by indirect ELISA. To obtain an immunoserum showing broad specificity against yeast species, a mixture containing live cells from selected strains of K. marxianus, K. lactis, D. hansenii, P. anomala, S. cerevisiae, Y. lipolytica, C. albidus, C. boidinii, and C. zeylanoides, all of them isolated from foods, was used in this work for immunization of rabbits.

An indirect ELISA was used to evaluate the antibody titer of the induced serum to recognize different yeast, mold, and bacterial species. Optimum conditions for the indirect ELISA were obtained using the AY antibodies diluted 1:2,000. The results obtained in the indirect ELISA for pure yeast cultures (Fig. 1) showed that the AY antibodies recognized K. marxianus, D. hansenii, Y. lipolytica, C. boidinii, C. zeylanoides, P. anomala, and S. cerevisiae, with a detection threshold of 10⁴ to 10⁸ CFU/ml, depending on the species. However, the AY antibodies did not react to R. mucilaginosa. The specificity of the antiserum was also evaluated against bacteria and molds. The antibodies did not recognize the bacterial species tested (E. coli and S. aureus), but they showed some degree of cross-reactivity to the mold species P. roqueforti and M. racemosus (Fig. 1).

Detection of yeast species in yogurt by an indirect ELISA. Yogurt samples were seeded with individual cul-
FIGURE 2. Relationship between indirect ELISA results and colony counts for yogurt samples inoculated with pure cultures of K. marxianus (Km), D. hansenii (Dh), C. zeylanoides (Cz), and R. mucilaginosa (Rm). The control sample was yogurt without inoculum (○). The AY antibody was diluted 1:100 (vol/vol), and the conjugate was diluted 1:1,000 (vol/vol).

FIGURE 3. Relationship between double-antibody sandwich ELISA results and colony counts for samples of ultrahigh-temperature milk inoculated with pure cultures of K. marxianus, D. hansenii, C. zeylanoides, and R. mucilaginosa at a range of concentrations from 10^8 to 10^2 CFU/ml. The AY antibodies did not recognize the yeast species seeded in yogurt samples (data not shown); thus, separation of the yeast cells from the yogurt matrix was required before analysis by the ELISA technique.

The addition of a milk clearing solution to yogurt samples seeded with yeast species facilitated the recovery of the microorganisms without yeast components that might interfere in the ELISA procedure. Figure 2 shows the indirect ELISA results obtained with the AY antibodies diluted 1:100 when yogurt samples seeded with D. hansenii, C. zeylanoides, K. marxianus, and R. mucilaginosa in the range 10^2 to 10^8 CFU/ml were treated with the milk clearing solution. A positive correlation was found between ELISA absorbance values and yeast concentrations, except for R. mucilaginosa. Indirect ELISA was capable of detecting more than 10^5 yeast per ml of yogurt. Nevertheless, absorbance values obtained from yogurt samples were remarkably lower than those of the same yeast numbers in pure culture.

Detection of yeast species by double-antibody sandwich ELISA. In an attempt to improve the immunological detection of spoilage yeast species, a sandwich ELISA format was tested, using AY antibodies to capture the yeast species from samples and biotinylated AY antibodies for detection. Nevertheless, differences in the immunoreactivity of the yeast species tested, seeded in ultrahigh-temperature milk, were even wider than those obtained in the indirect ELISA (Fig. 3). Highest absorbance values were achieved for the detection of C. boidinii, with a detection threshold of 10^6 CFU/ml, which was followed by K. marxianus and P. anomala, with a detection threshold of 10^5 CFU/ml. K. lactis and C. zeylanoides were also recognized by the AY antibodies in the sandwich ELISA, but lower absorbance values were obtained for these species with a detection threshold of 10^7 CFU/ml. The AY antibodies did not react to R. mucilaginosa, D. hansenii, Y. lipolytica, and S. cerevisiae when the sandwich ELISA assay was used.

PCR detection of yeast species. Published 18S rRNA sequences from yeast and mold species were searched for
regions of sequence conservation in yeast to devise oligonucleotides suitable for amplification. Primers 18SBDIR and 18SDINV span a DNA fragment of approximately 250 bp in the yeast species searched.

PCR was optimized for specificity by titrating the concentrations of individual reaction mix components using a known concentration of purified microbial DNA obtained from pure liquid cultures. The set of primers 18SBDIR and 18SDINV amplified the expected 250-bp fragment from purified genomic DNA from *D. hansenii*, *Y. lipolytica*, *S. cerevisiae*, *R. mucilaginosa*, *C. boidinii*, *P. anomala*, *C. zeylanoides*, *K. lactis*, *K. marxianus*, and *C. albicans* (Fig. 4A). Amplification was absent when purified genomic DNA from molds (*R. microsporus*, *A. niger*, *P. roqueforti*, *P. camemberti*, and *M. racemosus*), bacteria (*S. thermophilus*, *P. fluorescens*, *L. lactis*, *E. faecium*, *L. sake*, *E. coli*, *Salmonella Typhimurium*, and *Y. enterocolytica*), or animal species (*B. taurus*, *S. scrofa domesticus*, and *Gallus gallus*) were included as a template in the PCR reactions (Fig. 4B and 4C). The amplification band obtained from *K. marxianus* was purified and sequenced to confirm that the amplified products were the target 18S rRNA gene. The 251-bp sequence obtained was aligned and compared to the *K. marxianus* 18S rRNA sequence from GenBank (X89522), and it was confirmed that they were 98% identical.

Reference milk and yogurt samples were seeded with 1 to 10^7 CFU/ml of yeast cells (*K. marxianus*), and their DNA was obtained from the contaminated samples before and after incubation at 25°C with shaking for 16 h. Figure 5 shows the effect of yogurt sample enrichment on the sensitivity of the PCR technique for yeast species detection. A detection limit of approximately 10^5 CFU/ml was obtained before incubation of experimentally contaminated yogurt samples, while samples with initial counts of 10 CFU/ml tested positive after 16 h of enrichment. Similar results were obtained for yogurt and milk samples (data not shown).

**DISCUSSION**

Among the methods used for the rapid detection and counting of microorganisms in foods, immunochemical techniques are promising because of their sensitivity, rapidity, and suitability for simultaneous analysis of a large number of samples (3, 10). However, immunoassays for the detection of spoilage yeast species in foods are scarce (17–19, 30), and they are not commercially available for the food industry. Polyclonal antibodies were raised by Middelhoven and Notermans (18, 19) against thermostable immunogenic extracellular polysaccharides (EPSs) released to the growth medium by several yeast species. Differences were also found in the amount of EPS excreted by yeast species under different growth conditions and

FIGURE 5. Agarose gel electrophoresis (1.5%) of PCR products obtained from yogurt samples inoculated with pure cultures of K. marxianus CECT 10367 without incubation (A) and after incubation at 25°C for 16 h (B). K. marxianus was inoculated into yogurt samples in the following quantities: (1) control without inoculation, (2) 10⁷ CFU/ml, (3) 10⁶ CFU/ml, (4) 10⁵ CFU/ml, (5) 10⁴ CFU/ml, (6) 10³ CFU/ml, (7) 10² CFU/ml, and (8) 10 CFU/ml. P, 100-bp ladder; N, control without DNA; C, positive control with K. marxianus DNA.

growth phases. Thus, they concluded that immunoassays based on the detection of EPSs were not feasible for the detection and enumeration of yeast species in foods. Yoshida et al. (30) used a different approach to develop an immunoassay for the detection of spoilage yeast species in orange juice. They produced polyclonal antibodies against live cells of yeast species often associated with orange juice spoilage. Immunosera obtained against Candida intermedia and Candida parapsilosis showed cross-reactivity against other yeast species and were used to develop an indirect ELISA that detected the homologous yeast species in orange juice with a detection threshold of 10⁴ or 10⁵ CFU/ml. However, the assay was qualitative, and the authors did not state a detection threshold for yeast species different other than the detection limit of the species against which the immuno serum was obtained.

In the work developed herein, instead of selecting a single yeast species for immunization of rabbits, immunoserum was obtained against a mixture of 10 yeast species frequently isolated from spoiled dairy products. The aim was to obtain polyclonal antibodies that could recognize any yeast species in order to establish a correlation between the absorbance values obtained in the ELISA technique and the yeast counts.

Although many different yeast species may be isolated from dairy products, several authors conclude that dairy products, as a whole, present a unique ecological niche, selecting for the growth and occurrence of only a few main species. K. marxianus and D. hansenii, with their anamorphs Candida famata and Candida kefyr, followed by others like Y. lipolytica, Candida sp., R. mucilaginosus, Pichia guilliermondii, and S. cerevisiae, are the most frequently isolated yeast species in dairy products (9, 23, 24, 26, 28). Accordingly, we selected for immunization strains of K. marxianus, K. lactis, D. hansenii, P. anomala, S. cerevisiae, R. mucilaginosus, Y. lipolytica, C. albicans, C. bov dinii, and C. zeylanoides, all of which were isolated from foods. Nevertheless, when the AY antibodies were used in an indirect ELISA format (Figs. 1 and 2), the immunoserum reacted with different specificity against the yeast species analyzed. Moreover, although the sandwich ELISA is considered a more specific format than the indirect ELISA, it did not improve the results obtained (Fig. 3).

Immunological tests, as well as conventional colony counts, depend on phenotypic expression of cells to produce the target antigens to be detected by the available antibodies. Accordingly, they can be affected by growth conditions and environmental and chemical stresses. Genotypic characteristics of a cell are far more stable. Thus, there has been a push in recent years to champion genetic testing procedures as the confirmatory and definitive identification technique in diagnostic microbiology (25). PCR technology, based on amplification of selected fragments of target DNA, offers a very accurate and rapid alternative for the detection of spoilage and pathogenic microorganisms in food. Moreover, automated quantitative PCR assays have been recently commercialized for screening bacterial pathogens in food samples directly from an overnight enrichment of the target organisms (14, 20, 21).

Several PCR-based techniques have been proposed for the identification and typing of food-associated yeast and mold species (4, 15, 16, 22, 27). Nevertheless, they have not been advanced as alternatives to colony count procedures when rapid enumeration of food spoilage yeast species is desired. We explored in this work the feasibility of PCR for the detection and enumeration of yeast species in dairy products.

The detection of a specific microorganism or group of microorganisms by PCR is highly dependent on the speci-
ficiency of the primer set used. Although sequence analysis of the 18S rRNA has been used for differentiation and identification of yeast and mold species (4, 13), we have compared published 18S rRNA sequences from several yeast and mold species to design primers complementary to conserved regions in all the yeast species. Primers 18SBDIR and 18SDINV (11) amplified a fragment of approximately 250 bp in all yeast species analyzed with similar efficiency, while no amplification was achieved from the mold, bacterial, and animal species tested (Fig. 4).

The two main drawbacks frequently associated with PCR techniques in food microbiology are the presence of food compounds that can interfere with the PCR reaction and result in false negatives and the possibility of amplification of DNA from dead microorganisms (10). In this work, the inhibitor compounds from milk and yogurt were eliminated using a milk clearing solution to recover microorganisms from the samples before DNA extraction. Once DNA free of inhibitor compounds was available, PCR products were obtained from all yeast-contaminated samples, with a detection threshold of approximately 10⁵ CFU/ml (Fig. 5A).

An enrichment step was used in this work to make the assay useful for the qualitative detection of low numbers of viable spoilage yeast species in dairy products. Incubation of yogurt and milk samples at 25°C for 16 h before DNA extraction allowed amplification of the yeast-specific fragment from samples with initial counts of 10 CFU/ml. Sample enrichment not only allows improvement in the sensitivity of the assay, but also permits differentiation of viable and dead cells. This is very important for the food industry, because only viable cells will multiply and cause spoilage of dairy products during refrigerated storage.

In conclusion, the results obtained in this work show that ELISA techniques using polyclonal antibodies against viable yeast cells are of limited value for the detection and enumeration of spoilage yeast species in dairy products. These results are in agreement with those previously described in the literature using polyclonal antibodies against live yeast cells and also against thermostable EPSs. The main drawbacks of the immunological methods described to date for rapid enumeration of spoilage yeast species come from the different specificities of the antiserum for different yeast species. On the contrary, PCR amplification of a conserved region of the 18S rRNA of the yeast species allows the homogeneous detection of all the yeast species tested. The detection limit of the PCR assay after 16 h of incubation of the samples is approximately 10 viable yeast species per ml.

The rapid PCR technique developed in this work is able to qualitatively identify milk and yogurt samples with viable yeast counts higher than 10 CFU/ml in less than 48 h. The availability of such a technique represents a breakthrough for quality control in the dairy industry, because it will facilitate the identification and withdrawal of product batches that could be spoiled by yeast before their predicted shelf life date.

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