APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2003, p. 220–226 Vol. 69, No. 1
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.1.220–226.2003
Copyright © 2003, American Society for Microbiology. All Rights Reserved.

Culture-Independent Analysis of Probiotic Products by Denaturing Gradient Gel Electrophoresis

R. Temmerman,[1]* I. Scheirlinck,[1] G. Huys,[1] and J. Swings[1,2]

Laboratory of Microbiology, Ghent University,[1] and BCCM/LMG Bacteria Collection,[2] B-9000 Ghent, Belgium

Received 31 May 2002/Accepted 8 October 2002

In order to obtain functional and safe probiotic products for human consumption, fast and reliable quality control of these products is crucial. Currently, analysis of most probiotics is still based on culture-dependent methods involving the use of specific isolation media and identification of a limited number of isolates, which makes this approach relatively insensitive, laborious, and time-consuming. In this study, a collection of 10 probiotic products, including four dairy products, one fruit drink, and five freeze-dried products, were subjected to microbial analysis by using a culture-independent approach, and the results were compared with the results of a conventional culture-dependent analysis. The culture-independent approach involved extraction of total bacterial DNA directly from the product, PCR amplification of the V3 region of the 16S ribosomal DNA, and separation of the amplicons on a denaturing gradient gel. Digital capturing and processing of denaturing gradient gel electrophoresis (DGGE) band patterns allowed direct identification of the amplicons at the species level. This whole culture-independent approach can be performed in less than 30 h. Compared with culture-dependent analysis, the DGGE approach was found to have a much higher sensitivity for detection of microbial strains in probiotic products in a fast, reliable, and reproducible manner. Unfortunately, as reported in previous studies in which the culture-dependent approach was used, a rather high percentage of probiotic products suffered from incorrect labeling and yielded low bacterial counts, which may decrease their probiotic potential.

According to the Food and Agriculture Organization, a probiotic is a live microorganism which, when administered in adequate amounts, confers a health benefit to the host. Due to the increasing interest in health during the past decade, there has been a proportional expansion of the probiotic product market (15). Although probiotics were originally based on fermented dairy products, at present numerous probiotic food supplements are also commercially available as tablets, powders, or capsules. Bringing a functional, safe, and correctly labeled probiotic product to the market requires careful monitoring of the whole production process (12). Previous analyses of probiotic products have demonstrated that the identity and number of recovered microbial species do not always correlate with the information stated on the product labels (3, 4, 6, 16, 17). These and other studies mainly relied on the use of culture media to isolate the bacteria present in the probiotic product, after which a selection of purified isolates were identified by using 16S ribosomal DNA (rDNA) sequencing (5, 17), restriction fragment length polymorphism analysis (18), or protein profiling (16).

However, because the cultivation-dependent approaches have proven limitations in terms of recovery rate and reproducibility, the set of recovered isolates may not always truly reflect the microbial composition of the product (1, 2, 11). Moreover, more comprehensive insight into the production process and the survival capacity of the introduced strains requires analysis of both viable and nonviable bacteria. In practice, the need to identify product isolates (mostly lactic acid bacteria) at least to the species level makes the cultivation-based procedure rather time-consuming.

In the present study, a cultivation-independent method to detect and identify bacteria in probiotic products in a fast and reliable manner was developed. Essentially, the protocol comprises three steps: (i) extraction of bacterial DNA from the probiotic product, (ii) PCR amplification of a specific part of the 16S rDNA gene, and (iii) electrophoresis of 16S rDNA amplicons by denaturing gradient gel electrophoresis (DGGE). At present, DGGE analysis is one of the most suitable and widely used methods for studying complex bacterial communities in various environments (8). Compared to highly complex ecosystems, such as an animal or human intestinal tract, a probiotic product can be considered a rather simple microbial community, and therefore the DGGE method should allow qualitative analysis of any probiotic sample. The DGGE-based approach presented in this paper can also be used as a culture-independent identification method. In less than 30 h, a probiotic product can be analyzed to verify the species composition stated on its label. In order to validate this DGGE approach, the same products were also screened by using conventional cultivation on selective isolation media, followed by identification of the recovered isolates by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) protein profiling.

MATERIALS AND METHODS

Probiotic products. Ten commercially available probiotic products were analyzed in this study, including five freeze-dried products, four dairy products, and one fruit drink (Table 1). Besides the type of product, the choice was also based on the number of different bacterial groups claimed on the product label. As shown in Table 1, the products investigated contained one to four bacterial species.

Bacterial strains. All products were examined by using a set of four isolation media under standardized cultivation conditions. For isolation of *Lactobacillus*

* Corresponding author. Mailing address: Laboratory of Microbiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium. Phone: 32-9-264 52 49. Fax: 32-9-264 50 92. E-mail: Robin.Temmerman@rug.ac.be.

Downloaded from http://aem.asm.org/ on January 27, 2021 by guest

Downloaded from http://aem.asm.org/ on January 27, 2021 by guest

TABLE 1. Overview of culture-dependent and culture-independent analyses of 10 probiotic products

Product	Producer (country)	Organism(s) stated on product label	Organism(s) detected by:	
			Culture-dependent analysis (SDS-PAGE of proteins)	Culture-independent analysis (PCR-DGGE)
Dairy products				
Actimel	Danone (France)	*Lactobacillus casei*, living yogurt cultures	*Lactobacillus casei*	*Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*
Activia	Danone (France)	*Bifidobacterium*, living yogurt cultures	*Bifidobacterium lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus* *Streptococcus thermophilus*, *Lactococcus lactis*	*Bifidobacterium lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactococcus lactis*
Vitamel	Campina (The Netherlands)	*Lactobacillus casei* GG, *Bifidobacterium bifidum*, *Lactobacillus acidophilus*	*Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Streptococcus thermophilus*	*Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Streptococcus thermophilus*
Yakult	Yakult (The Netherlands)	*Lactobacillus casei*	*Lactobacillus casei*	*Lactobacillus casei*
Fruit drink				
Provie	Skåne Mejerier (Sweden)	*Lactobacillus plantarum*	*Lactobacillus plantarum*	*Lactobacillus plantarum*
Freeze-dried products				
Aciforce	Biohorma (The Netherlands)	*Lactobacillus acidophilus*, *Lactococcus lactis*, *Enterococcus faecium*, *Bifidobacterium bifidum*	*Lactococcus lactis*, *Enterococcus faecium*	*Lactococcus lactis*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Bifidobacterium lactis*
Bacilac	THT (Belgium)	*Lactobacillus acidophilus*, *Lactobacillus rhamnosus*	*Lactobacillus helveticus*	*Lactobacillus helveticus*, *Lactobacillus rhamnosus*
Bactisubtil	Synthelabo (Belgium)	*Bacillus* sp. strain IP5832	*Bacillus cereus*	*Bacillus cereus*
Bififlor	Eko-Bio (The Netherlands)	*Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*	Yeast	*Lactobacillus acidophilus*, *Lactobacillus rhamnosus*
Proflora	Chefaro (Belgium)	*Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium*	*Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Bifidobacterium lactis*	*Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Bifidobacterium lactis*

and *Lactococcus* strains, De Man-Rogosa-Sharpe agar (MRSA) (catalog no. CM361; Oxoid, Basingstoke, United Kingdom) was used, whereas streptococci and enterococci were isolated on M17 medium (catalog no. CM785; Oxoid) and on kanamycin esculin azide agar base (catalog no. CM591; Oxoid), respectively. For isolation of bifidobacteria, *trans*-galactooligosaccharide medium was used; this medium contained 10 g of Trypticase soy broth (catalog no. 81-1768-0; Becton Dickinson, Sparks, Md.), 1 g of yeast extract (catalog no. L21; Oxoid), 3 g of KH_2PO_4 (catalog no. 1627; Vel, Leuven, Belgium), 4.8 g of K_2HPO_4 (catalog no. 1628; Vel), 3 g of $(NH_4)_2SO_4$ (catalog no. 1.01217.1000; Merck, Darmstadt, Germany), 0.2 g of $MgSO_4 \cdot 7H_2O$ (catalog no. 1433; Vel), 0.5 g of L-cysteine hydrochloride (catalog no. C4820; Sigma, Bornem, Belgium), 15 g of sodium propionate (catalog no. P1880; Sigma), 10 g of *trans*-galactooligosaccharides (Honsha, Tokyo, Japan), and 15 g of agar (catalog no. L11; Oxoid) dissolved in 1,000 ml of distilled water. Identification of the isolates was performed by using SDS-PAGE separation of extracted cellular proteins as described previously (16). In order to verify the reliability of the DNA extraction protocol for probiotic products and to verify the identification potential of DGGE, cell suspensions of type strains were made in order to simulate the species compositions of the products. These cell suspensions were prepared by harvesting half a loop of cells with a sterile iron loop from a freshly grown pure culture on MRSA (catalog no. CM 361; Oxoid) and homogeneously suspending the cells in 10 ml of peptone physiological solution (PPS) (0.1% [wt/vol] peptone [catalog no. L37; Oxoid], 0.85% [wt/vol] NaCl).

DNA extraction. The method used for extraction of total bacterial DNA was based on the method described by Pitcher and coworkers (10), with slight modifications depending on the type of starting material. For dairy products, 1 ml of product was centrifuged for 10 min at 13,000 rpm in a 5804R centrifuge (Eppendorf, Hamburg, Germany); then the supernatant was removed, and the pellet was resuspended in 1 ml of Tris-EDTA (TE) buffer. Because of the large fruit content in the fruit drink, 50 ml of the drink was centrifuged for 2 min at 1,000 rpm, after which 1 ml of the top liquid was removed and centrifuged for 10 min at 13,000 rpm. After removal of the supernatant, the remaining pellet was dissolved in 1 ml of TE buffer. In the case of the capsule-type products, the content of one capsule, corresponding to approximately 100 mg, was dissolved in 10 ml of sterile PPS and softly shaken until a homogeneous suspension was obtained. One milliliter of this suspension was transferred to an Eppendorf tube and centrifuged for 10 min at 13,000 rpm, after which the supernatant was removed and the remaining pellet was suspended in 1 ml of TE buffer. This procedure was also used for freeze-dried powders; 100 mg of each powder was weighed and suspended in 10 ml of PPS. For freeze-dried tablets, one tablet was crushed in a sterile mortar, and the powder obtained was dissolved in 10 ml of PPS and homogenized again. One milliliter of the suspension was centrifuged for 10 min at 13,000 rpm, and the pellet was dissolved in 1 ml of TE buffer. All cell suspensions in TE buffer were centrifuged for 5 min at 13,000 rpm. In each case the supernatant was removed, and 150 μl of a lysozyme solution (5 mg of lysozyme [catalog no. 28262; Serva, Heidelberg, Germany] in 150 μl of TE buffer) was added; this was followed by incubation at 37°C for 40 min. In the case of DNA extraction from pure cultures, only this lysozyme step was added to the protocol described by Pitcher and coworkers (10). The DNA obtained was dissolved in 200 μl of TE buffer overnight, after which an RNA-digesting step was performed by adding 35 μl of an RNase solution (10 mg of RNase [catalog no. 34390; Serva] in 1 ml of Milli-Q water). Finally, 8 μl of the DNA solution was mixed with 2 μl of loading dye (4 g of sucrose and 2.5 mg of bromophenol blue dissolved in 6 ml of TE buffer) and electrophoresed on a 1% (wt/vol) agarose gel in 1× TAE buffer (catalog no. 161-0773; Bio-Rad, Hercules, Calif.) for 30 min at 100 V to verify the DNA extraction. The quality of the DNA samples was verified by spectrophotometric measurements at 260, 280, and 234 nm.

PCR. PCR was performed with a *Taq* polymerase kit (Applied Biosystems, Foster City, Calif.). The primers used in this study were those described by Muyzer et al. (7), which amplify the V3 region of bacterial 16S rDNA. Forward primer F357-GC contained a GC clamp (5′-CGCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGGGG-3′) and had the following sequence: 5′-GC clamp-TACGGGAGGCAGCAG-3′. Reverse primer 518R had the following sequence: 5′-ATTACCGCGGCTGCTGG-3′. The PCR mixtures (50 μl) contained 6 μl of 10× PCR buffer containing 15 mM $MgCl_2$, 2.5 μl of bovine serum albumin, 2.5 μl of a deoxynucleoside triphosphate preparation (containing each deoxynucleoside triphosphate at a concentration of 2 mM), 2 μl of each primer (5 μM), 0.25 μl of *Taq* polymerase (5 U/μl), 33.75 μl of sterile Milli-Q water, and 1 μl of a 10-fold-diluted DNA solution. The following PCR program was used: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min; and final extension at 72°C for 7 min, followed by cooling to 4°C. The PCR was verified by mixing 8 μl of PCR product with 2 μl of loading dye and electrophoresing it on a 2% (wt/vol) agarose gel for 30 min at 100 V flanked by the EZ Load 100-bp molecular ruler (catalog no. 170-8352; Bio-Rad) (data not shown).

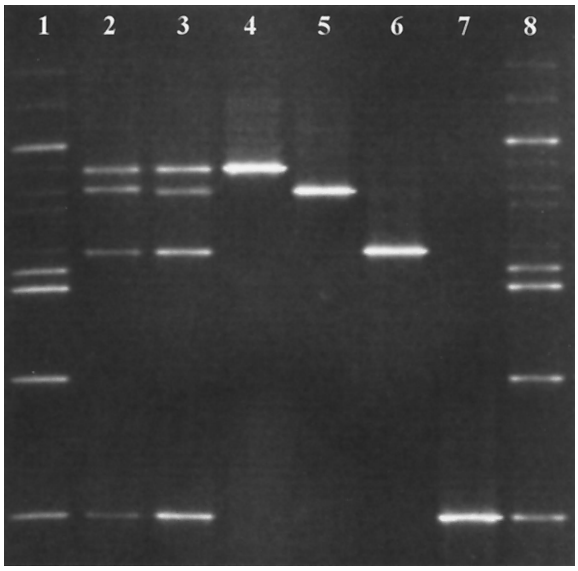

FIG. 1. Example of use of a 35 to 70% denaturant DGGE gel for product analysis. Lanes 1 and 8, reference pattern (V3 amplicons of *Enterococcus solitarius*, *Enterococcus flavescens*, *Bacillus cereus*, *Lactobacillus rhamnosus*, and *Bifidobacterium lactis*); lane 2, Aciforce amplicon; lane 3, amplicon from the cell suspension simulating Aciforce; lane 4, *Enterococcus faecium*; lane 5, *Lactobacillus acidophilus*; lane 6, *Lactococcus lactis*; lane 7, *Bifidobacterium lactis*.

DGGE analysis. PCR products were analyzed on DGGE gels by using a protocol based on the protocol of Muyzer and coworkers (7), with the following modifications. The polyacrylamide gels (160 by 160 by 1 mm) consisted of 8% (vol/vol) polyacrylamide (catalog no. EC-890; National Diagnostics, Atlanta, Ga.) in 1× TAE buffer. By diluting a 100% denaturing polyacrylamide solution (containing 7 M urea [catalog no. EC-605; National Diagnostics] and 40% formamide [catalog no. F-9037; Sigma, St. Louis, Mo.]) with a polyacrylamide solution containing no denaturing components, polyacrylamide solutions with the desired denaturing percentages were obtained. In this study two types of denaturing gradients were used, namely, a 35 to 70% gradient and a 40 to 55% gradient. The 24-ml gradient gels were cast by using a gradient former (catalog no. 165-4120; Bio-Rad) and a pump (catalog no. 731-8142; Bio-Rad) set at a constant speed of 5 ml/min. The denaturing gels were allowed to polymerize for 3 h, after which a 5-ml nondenaturing stacking gel containing a 16-well comb was poured on top. After 1 h of polymerization, PCR samples were loaded into the wells, and electrophoresis was performed for 16 h at 70 V in 1× TAE buffer at a constant temperature of 60°C by using the Dcode system (catalog no. 170-9081; Bio-Rad). The gels were stained with ethidium bromide (50 μl of ethidium bromide in 500 ml of TAE buffer) for 1 h; this was followed by visualization of DGGE band profiles under UV light. Digital capturing was performed by using a Foto/Analyst charge-coupled device camera (Fotodyne Inc., Hartland, Wis.) combined with the Iris Video Digitize software package (Inside Technology, Amersfoort, The Netherlands).

Processing of DGGE gels. For DGGE to be used as a direct identification method, a reference pattern consisting of six different type strain V3 amplicons was designed (Fig. 1). By including this reference pattern every six lanes on each DGGE gel, it became possible to digitally normalize the gel patterns by comparison with a standard pattern by using the BioNumerics (BN) software package, version 2.50 (Applied Maths, St.-Martens-Latem, Belgium). This normalization enabled comparison of DGGE gels, provided that they consisted of the same denaturing gradient. For each known probiotic species, the band position of the corresponding type strain was determined and stored in a BN database. The amplicons obtained from probiotic products were electrophoresed on a DGGE gel, and after normalization based on the standard reference pattern of the BN database, individual bands in the product band pattern could be identified. Amplicons of isolates (identified by SDS-PAGE) corresponding to the species claimed on the product label were electrophoresed next to the amplicon

Downloaded from http://aem.asm.org/ on January 27, 2021 by guest

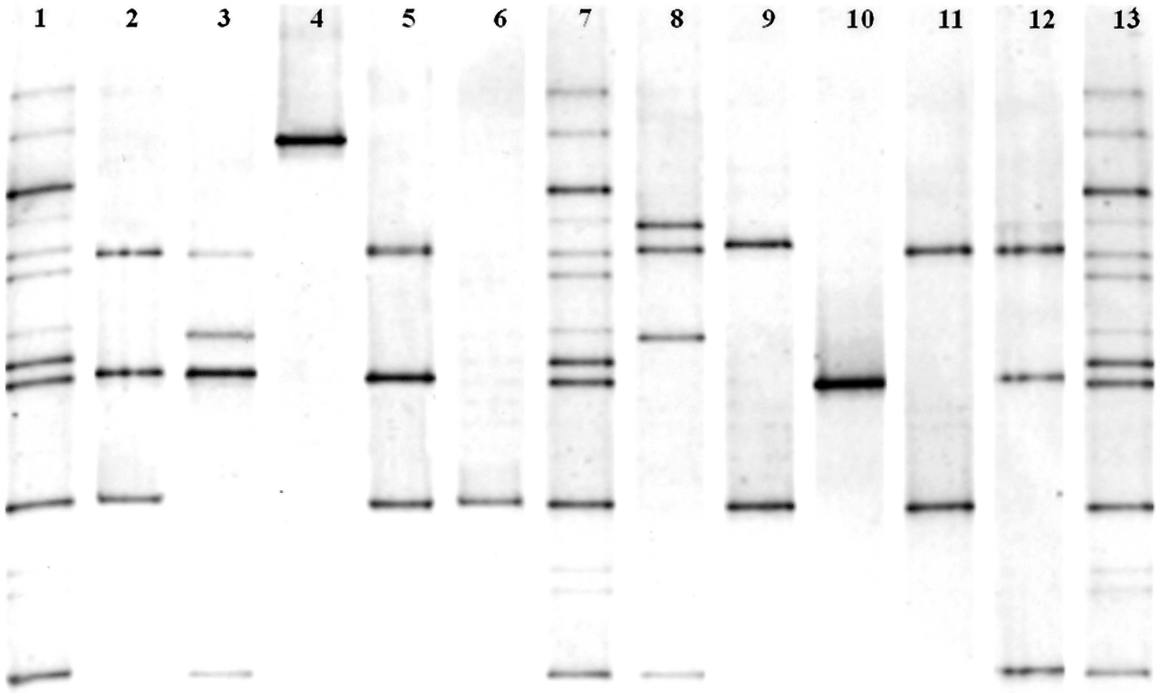

FIG. 2. Normalized 35 to 70% denaturant DGGE gel showing the V3 amplicons of 10 probiotic products. Lanes 1, 7, and 13, reference pattern; lane 2, Actimel; lane 3, Activia; lane 4, Provie; lane 5, Vitamel; lane 6, Yakult; lane 8, Aciforce; lane 9, Bacilac; lane 10, Bactisubtil; lane 11, Bififlor; lane 12, Proflora.

of the probiotic product itself as an additional visual confirmatory identification procedure (Fig. 1).

RESULTS

Culture-dependent analysis of probiotic products. Results of the isolation and identification of probiotic strains from the products tested are presented in Table 1. Isolation was performed with four selective isolation media, and this was followed by identification based on SDS-PAGE separation of whole-cell protein extracts and comparison of the species-specific patterns with a laboratory-based identification library, as described by Temmerman and coworkers (16). The colony counts on the media used were substantially lower in the case of the freeze-dried products; the yields were between 10^5 and 10^7 CFU/g of product, while the yields for the dairy products were between 10^7 and 10^9 CFU/ml. Furthermore, for six products (Actimel, Vitamel, Aciforce, Bacilac, Bififlor, and Proflora) not all species claimed on the labels could be isolated, and two products (Bacilac and Vitamel) contained a probiotic strain belonging to a species other than the species mentioned on the label (Table 1).

Culture-independent analysis of probiotic products. For cultivation-independent analysis, total bacterial DNA had to be extracted directly from the product. This was done reproducibly by adding lysozyme and a number of centrifugation steps to the original protocol described by Pitcher and coworkers (10). The PCR protocol described in this paper could reproducibly amplify the V3 region of the 16S rDNA of all samples tested. Sometimes, we found that adding 2 μl of DNA to the PCR mixture instead of 1 μl was necessary to enhance the intensity of some bands on the DGGE gels in order to aid visual interpretation of the results. For each of the 10 probiotic products, a 35 to 70% denaturant DGGE gel was used, on which the following PCR amplicons were loaded next to each other: the probiotic product, an artificial mixture of type strains simulating the species composition of the product, and individual type strains of the species claimed on the label (Fig. 1). Every six lanes, all gels contained the reference pattern. A gel on which the amplicons of all 10 product DNAs were loaded is shown in Fig. 2. Identification was performed after normalization of the gel with the standard reference pattern, followed by comparison of the band positions with those of identified type strains present in a newly built BN database. The identities were verified by electrophoresing the V3 amplicons of type strains or isolates originating from the culture-dependent analysis on a DGGE gel next to the probiotic product amplicons. In a few cases, two phylogenetically closely related species produced an amplicon that could not be clearly separated on a 35 to 70% denaturing gradient gel. Therefore, DGGE gels with a narrower 40 to 55% denaturing gradient were used to obtain greater band position resolution. As shown in Fig. 3, the amplicons representing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* may be confused with each other on a 35 to 70% denaturing gradient gel

Downloaded from http://aem.asm.org/ on January 27, 2021 by guest

(Fig. 3, top gel), but they can be clearly separated electrophoretically when a 40 to 55% denaturing gradient is used (Fig. 3, bottom gel).

The results of the culture-independent DGGE analysis of the 10 probiotic products, compared with the results of the culture-dependent analysis, are shown in Table 1. Two different scenarios were found. DGGE analysis of five products (Activia, Yakult, Provie, Bactisubtil, and Proflora) detected the same species that were detected with conventional isolation procedures. For the remaining five products (Actimel, Vitamel, Aciforce, Bacilac, and Bififlor), DGGE analysis was able to detect more claimed species than were recovered by isolation. For two products (Bacilac and Vitamel) species other than those mentioned on the label were isolated and identified, which was confirmed by DGGE analysis.

The detection limit of the DGGE method was also determined by preparing 10-fold serial dilutions of a pure culture of *Lactobacillus rhamnosus* LMG 18243 in PPS. After 100 μl of each dilution was plated on MRSA and incubated for 48 h at 37°C aerobically, DNA was extracted from the dilution, and PCR-DGGE analysis was performed. We found that this technique produced a clear band at dilutions corresponding to concentrations down to 10^4 CFU/ml. To determine the reproducibility of the technique, three different batches of each product were analyzed at different times. In all cases, identical results were obtained (data not shown).

DISCUSSION

Despite the expansion of the probiotic market (15) and the accompanying scientific research (9), a number of recent reports have clearly highlighted the poor quality of many probiotic products in terms of their contents and label information (3, 4, 6, 16, 17). In relation to safety and functionality, it is very important that these products are correctly labeled and contain well-documented probiotic strains (13). In this study, the culture-independent DGGE method was compared with a culture-dependent procedure for detection and identification of the strains in probiotic products. As demonstrated in a previous paper (16), we found that the numbers of bacteria isolated from the freeze-dried products were substantially lower than the numbers of bacteria isolated from the dairy products and the fruit drink. Furthermore, six products were not found to contain all the claimed species, as determined by the culture-dependent analysis. In addition to the fact that some of these products may have been mislabeled or had low production quality, the poor recovery results might to some extent be ascribed to the inherent selectivity of the isolation media used. Previous studies have stressed the need for culture-independent methods to circumvent the limitations of conventional cultivation (1, 2).

As part of such a culture-independent method, reliable DNA extraction and PCR analysis need to be performed. By preparing artificial mixtures of type strains to simulate the species compositions of the products analyzed, we were able to confirm the suitability of the DNA extraction and PCR method applied to the products in this study. So far, identification of DGGE bands has not been performed without additional steps, such as gel extraction and sequencing (2). By using a reference pattern included in each gel and the BN software, it

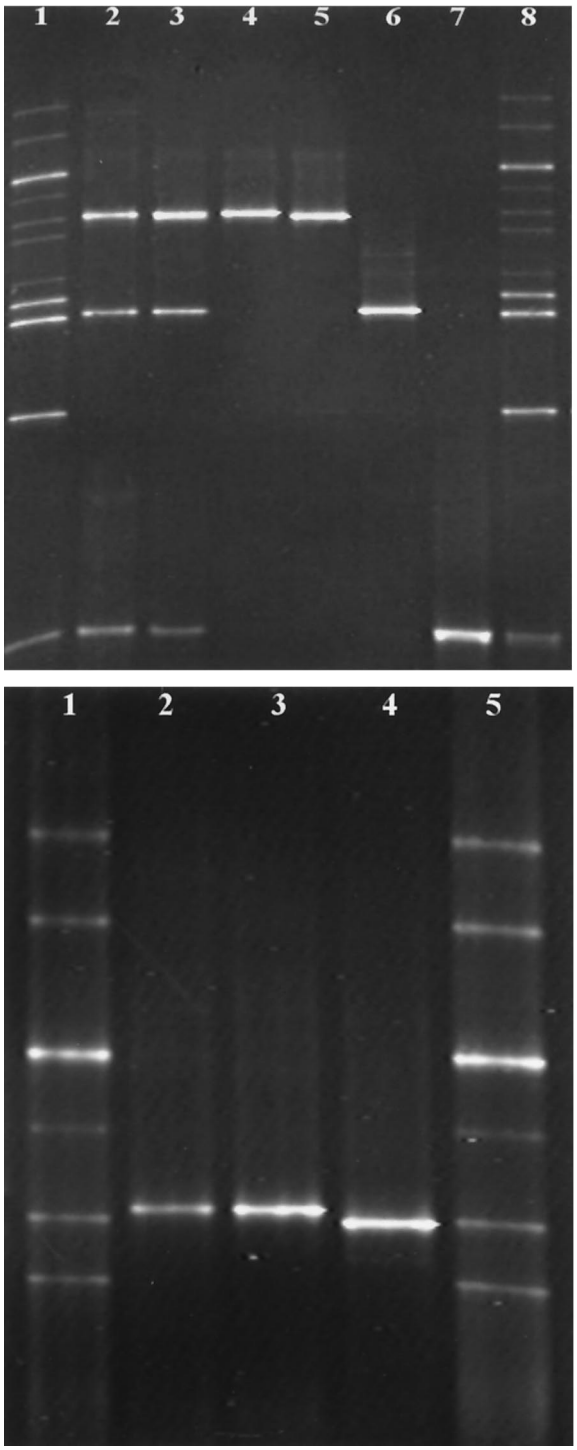

FIG. 3. (Top) Thirty-five to 70% denaturing DGGE gel showing analysis of Proflora. Lanes 1 and 8, reference pattern; lane 2, Proflora; lane 3, cell suspension simulating Proflora; lane 4, *Lactobacillus acidophilus*; lane 5, *Lactobacillus delbrueckii* subsp. *bulgaricus*; lane 6, *Streptococcus thermophilus*; lane 7, *Bifidobacterium lactis*. The difference in the positions of the *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* bands is not pronounced on this 35 to 70% denaturing gradient gel. (Bottom) Forty to 55% denaturing DGGE gel focusing on the difference in the positions of the *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* bands. Lanes 1 and 5, reference pattern; lane 2, Proflora; lane 3, *L. acidophilus*; lane 4, *L. delbrueckii* subsp. *bulgaricus*.

Downloaded from http://aem.asm.org/ on January 27, 2021 by guest

was possible to create a database containing all band positions for type strains representing probiotic species. Following digital normalization of the gels by comparison of the reference patterns with the standard pattern in the database, it was possible to assign an identity to each band in a band pattern representing a probiotic product. This identification based on DGGE could be confirmed by coelectrophoresing amplicons of pure cultures that were previously identified by protein profiling. Furthermore, multiple probiotic isolates belonging to one species produced bands whose positions coincided with the band positions of the type strain amplicon, indicating that band patterns are species specific. However, in the case of some phylogenetically closely related species (14), the differences in band positions between two species may sometimes be too small on a 35 to 70% denaturing gel to obtain clear-cut identification. This problem could be solved by using a narrower denaturing gradient, which increased the differences in the band positions. Alternatively, the use of other primers might result in amplicons which are readily separated from each other on DGGE gels, thereby making it possible to identify species that produce overlapping bands with the V3 primers. However, every change in the gradient, primer set, or electrophoresis conditions makes it necessary to build a new database corresponding to the new parameters. This implies that the use of DGGE as a direct identification method will be successful only with rather simple microbial ecosystems, such as probiotic products. With the rising complexity of a microbial ecosystem, it becomes necessary to change more parameters, making the method increasingly time-consuming. Thus, Ercolini and coworkers (2) studied the potential of DGGE to analyze natural whey cultures for cheese production, but they found that it was necessary to sequence the bands in the DGGE profile. In contrast, probiotic products can be considered ecosystems that developed from well-controlled fermentations with low taxonomic diversity. In the present study, analysis of type strains representing known probiotic species never resulted in two species with identical band positions. Moreover, of all the species investigated, only *Lactobacillus reuteri* produced multiple bands on a DGGE gel (data not shown). This species was not included in any of the products tested, but a previous study (16) showed that only 2 of 55 probiotic products contained this species.

When the results of the culture-dependent and culture-independent analyses of probiotic products are compared, it can be concluded that DGGE has a much higher potential for detection and identification. Whereas conventional isolation revealed that 6 of 10 products did not contain the species claimed on their labels, DGGE analysis was able to detect additional species in 5 of these 6 products. Nevertheless, four products (Vitamel, Bacilac, Bififlor, and Proflora) were considered to have incorrect labels after analysis by both approaches. This indicates that the previously reported poor product quality of probiotics (3, 4, 6, 16, 17) cannot be attributed solely to shortcomings of cultivation-based methods. Evaluation of three different batches of all products indicated that DGGE analysis is very reproducible, since in all cases the same bacterial species were detected. This was not the case for the culture-dependent approach, where one product produced a species previously not detected in another batch of the product. Mainly detection of bifidobacteria impairs the reproducibility of the culture-dependent approach, because of the lack of suitable selective isolation media (11). A potential drawback of the DGGE approach is that no information concerning the level of bacterial viability in probiotic products is obtained, implying that culture-dependent analysis may still add valuable information. Also, the detection limit, 10^4 CFU/ml as determined in this study, may result in the failure to detect species that are present at lower levels. In this regard, it can be seriously questioned whether organisms present at such low levels can exert any significant probiotic effect. In the near future, the linkage of real-time PCR to the DGGE method may result in a very powerful tool for both qualitative and quantitative analyses of all kinds of (bacterial) fermentation products. As in a previous study (16), we found that for a substantial percentage of probiotic products there are incorrect labels and low counts. Numerous studies (9) have demonstrated different probiotic effects exerted by different bacteria, but how can a consumer select the product containing the most suitable strain for his or her symptoms if the product labels are incorrect or the strains are absent? Nevertheless, this study clearly demonstrates that DGGE is a fast, reliable, and reproducible culture-independent approach for analysis of probiotic products and that it has greater detection and identification potential than conventional culture-dependent analysis.

ACKNOWLEDGMENTS

This research was supported financially by a Ph.D. grant from the Flemish Institute for the Promotion of Innovation by Science and Technology (IWT-Vlaanderen, Brussels, Belgium). G.H. is a postdoctoral fellow of the Fund for Scientific Research-Flanders (Belgium) (F.W.O.-Vlaanderen).

REFERENCES

1. **Ampe, F., N. Ben Omar, C. Moizan, C. Wacher, and J. P. Guyot.** 1999. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. Appl. Environ. Microbiol. **65:**5464–5473.
2. **Ercolini, D., G. Moschetti, G. Blaiotta, and S. Coppola.** 2001. The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water buffalo mozzarella cheese production: bias of culture-dependent and culture-independent analyses. Syst. Appl. Microbiol. **24:**610–617.
3. **Hamilton-Miller, J. M. T., S. Shah, and C. T. Smith.** 1996. 'Probiotic' remedies are not what they seem. Br. Med. J. **312:**55–56.
4. **Hamilton-Miller, J. M. T., S. Shah, and J. T. Winkler.** 1999. Public health issues arising from microbiological and labelling quality of foods and supplements containing probiotic microorganisms. Public Health Nutr. **2:**223–229.
5. **Holzapfel, W. H., P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger.** 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr. **73:**365S-373S.
6. **Hughes, V. L., and S. L. Hillier.** 1990. Microbiologic characteristics of *Lactobacillus* products used for colonization of the vagina. Obstet. Gynecol. **75:**244–248.
7. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
8. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek. **73:**127–141.
9. **Naidu, A. S., W. R. Bidlack, and R. A. Clemens.** 1999. Probiotic spectra of lactic acid bacteria (LAB). Crit. Rev. Food Sci. Nutr. **38:**13–126.
10. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
11. **Roy, D.** 2001. Media for the isolation and enumeration of bifidobacteria in dairy products. Int. J. Food Microbiol. **69:**167–182.

Downloaded from http://aem.asm.org/ on January 27, 2021 by guest

12. **Saarela, M., G. Mogensen, R. Fondén, J. Mättö, and T. Matilla-Sandholm.** 2000. Probiotic bacteria: safety, functional and technological properties. J. Biotechnol. **84:**197–215.
13. **Sanders, M. E., and J. Huis in't Veld.** 1999. Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. Antonie Leeuwenhoek **76:**293–315.
14. **Schleifer, K. H., and W. Ludwig.** 1995. Phylogeny of the genus *Lactobacillus* and related genera. Syst. Appl. Microbiol. **18:**461–467.
15. **Stanton, C., G. Gardiner, H. Meehan, K. Collins, G. Fitzgerald, P. B. Lynch, and R. P. Ross.** 2001. Market potential for probiotics. Am. J. Clin. Nutr. **73:**476S-483S.
16. **Temmerman, R., B. Pot, G. Huys, and J. Swings.** 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int. J. Food Microbiol. **81:**1–10.
17. **Yeung, P. S. M., M. E. Sanders, C. L. Kitts, R. Cano, and P. S. Tong.** 2002. Species-specific identification of commercial probiotic strains. J. Dairy Sci. **85:**1039–1051.
18. **Zhong, W., K. Millsap, H. Bialkowska-Hobrzanska, and G. Reid.** 1998. Differentiation of *Lactobacillus* species by molecular typing. Appl. Environ. Microbiol. **64:**2418–2423.

Downloaded from http://aem.asm.org/ on January 27, 2021 by guest