Taxonomy and important features of probiotic microorganisms in food and nutrition[1–4]

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ABSTRACT Lactic acid bacteria are among the most important probiotic microorganisms typically associated with the human gastrointestinal tract. Traditionally, lactic acid bacteria have been classified on the basis of phenotypic properties, eg, morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, and fermentation of various carbohydrates. Studies based on comparative 16S ribosomal RNA sequencing analysis, however, showed that some taxa generated on the basis of phenotypic features do not correspond with the suggested phylogenetic relations. Thus, some species are not readily distinguishable by phenotypic characteristics. This is especially true for the so-called *Lactobacillus acidophilus* group, the *Lactobacillus casei* and *Lactobacillus paracasei* group, and some bifidobacteria, strains of which have been introduced in many probiotic foods, eg, the novel yogurt-like commodities. Consequently, modern molecular techniques, including polymerase chain reaction-based and other genotyping methods, have become increasingly important for species identification or for the differentiation of probiotic strains. Probiotic strains are selected for potential application on the basis of particular physiologic and functional properties, some of which may be determined in vitro. The classification and identification of a probiotic strain may give a strong indication of its typical habitat and origin. The species, or even genus name, may also indicate the strain's safety and technical applicability for use in probiotic products. Molecular typing methods such as pulsed-field gel electrophoresis, repetitive polymerase chain reaction, and restriction fragment length polymorphism are extremely valuable for specific characterization and detection of such strains selected for application as probiotics. *Am J Clin Nutr* 2001;73(suppl):365S–73S.

KEY WORDS Probiotic strains, lactic acid bacteria, gastrointestinal tract, functional properties, molecular typing

INTRODUCTION

A beneficial association of microorganisms on the human host was probably first suggested by Döderlein (1), who proposed that vaginal bacteria produced lactic acid from sugars to prevent or inhibit the growth of pathogenic bacteria. Such lactic acid bacteria (LAB) were also found in association with fermented milk products and were advocated for their health benefits by Metchnikoff (2) in 1908. He considered the longevity of white persons to be related to their high intake of fermented milk products. However, in contrast with present-day interpretations, Metchnikoff suggested that gut microbes were detrimental rather than beneficial to human health, although he admitted that the substitution of gut microbes by yogurt bacteria may be beneficial. In this context, LAB and their major metabolite of sugar fermentation, ie, lactic acid, were especially promoted by Metchnikoff. Early taxonomic and gut (fecal) ecology studies on LAB were conducted by Moro (3) in 1900, and by Beijerinck (4) and Cahn (5) in 1901.

Originally defined as "…microorganisms promoting the growth of other microorganisms" (6), *probiotics*, according to present-day interpretation, refers to viable microorganisms that promote or support a beneficial balance of the autochthonous microbial population of the gastrointestinal tract (GT). Such microorganisms may not necessarily be constant inhabitants of the GT, but they should have a "…beneficial effect on the general and health status of man and animal" (7, 8). In recent years, probiotics have been defined more precisely as "…mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora" (9). In relation to food, probiotics are considered as "viable preparations in foods or dietary supplements to improve the health of humans and animals" (10). According to these definitions, an impressive number of microbial species and genera are considered as probiotics (**Table 1**). However, only those strains classified as LAB are considered of importance in regard to food and nutrition and thus are the strains that will be addressed in this article.

PHYSIOLOGIC PROPERTIES OF LACTIC ACID BACTERIA, THEIR HABITATS, AND MODERN TAXONOMY

LAB are gram-positive, nonsporing, catalase-negative organisms that are devoid of cytochromes and of nonaerobic habit but are aerotolerant, fastidious, acid-tolerant, and strictly fermentative; lactic acid is the major end product of sugar fermentation (12). However, exceptions from this general description do occur

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TABLE 1
Microorganisms considered as probiotics[1]

Lactobacillus species	*Bifidobacterium* species	Other lactic acid bacteria	Nonlactic acid bacteria
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*[2]	*Bacillus cereus var.* toyoi[2,3]
L. amylovorus	*B. animalis*	*Enterococcus faecium*	*Escherichia coli* strain nissle
L. casei	*B. bifidum*	*Lactococcus lactis*[4]	*Propionibacterium freudenreichii*[2,3]
L. crispatus	*B. breve*	*Leuconstoc mesenteroides*	*Saccharomyces cerevisiae*[3]
L. delbrueckii	*B. infantis*	*Pediococcus acidilactici*[4]	*Saccharomyces boulardii*[3]
subsp. *bulgaricus*[4]	*B. lactis*[5]	*Sporolactobacillus inulinus*[2]	
L. gallinarum[2]	*B. longum*	*Streptococcus thermophilus*[4]	
L. gasseri			
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

[1] Data from reference 11.
[2] Main application for animals.
[3] Applied mainly as pharmaceutical preparations.
[4] There is either little known about the probiotic properties or the microorganism is nonprobiotic.
[5] Probably synonymous with *B. animalis.*

because some species can form catalase or cytochromes on media containing hematin or related compounds (13–15). The production of a nonheme catalase, called pseudocatalase, by some lactobacilli can also cause some confusion in the identification LAB (16).

In an early approach, Orla-Jensen (17) subdivided LAB into the genera *Betabacterium, Thermobacterium, Streptobacterium, Streptococcus, Betacoccus, Tetracoccus,* and *Microbacterium* on the basis of their morphologic and phenotypic features (**Table 2**). Today, only the name *Streptococcus* is still valid, whereas *Enterococcus, Lactococcus*, and *Vagococcus* have been separated from the original genus *Streptococcus* (18, 19). With the exception of *Streptococcus thermophilus*, this genus represents mainly pathogenic streptococci, compared with the technically important *Lactococcus* sp., which are generally considered to be nonpathogenic and safe, and the *Enterococcus* sp., some strains of which may be involved in opportunistic infections, some strains that are considered to play some role in food fermentations, and some strains that act as commensals in the GT. Taxonomy of other LAB genera has also undergone considerable changes since the time of Orla-Jensen, resulting in the genera listed in Table 2. Such taxonomic knowledge of a strain may therefore give an indication of the strain's origin, habitat, and physiology, and have important consequences for the selection of novel strains for application in food fermentation or for use as a probiotic.

Although *Lactobcillus acidophilus,* which is one of the most important probiotic species, is phenotypically difficult to assess, its heterogeneity was recognized in the 1960s by Lerche and Reuter (20), who suggested 4 different biotypes of the species. DNA-DNA hybridization studies reported in 1980 (21, 22) confirmed this heterogeneity, suggesting the existence of 6 different homology

TABLE 2
Key to differentiating lactic acid bacteria and a comparison with current taxonomic classification[1]

Genus	Shape	Catalase	Nitrite reduction	Fermentation	Current genera
Betabacterium	Rod	−	−	Hetero-	*Lactobacillus*[2] *Weissella*[2]
Thermobacterium	Rod	−	−	Homo-	*Lactobacillus*[2]
Streptobacterium	Rod	−	−	Homo- and hetero-	*Lactobacillus*[2] *Carnobacterium*
Streptococcus	Coccus	−	−	Homo-	*Streptococcus* *Enterococcus* *Lactococcus*[2] *Vagococcus*
Betacoccus	Coccus	−	−	Hetero-	*Leuconostoc*[2] *Oenococcus*[2] *Weissella*[2]
Microbacterium	Rod	+	+	Homo-	*Brochothrix*
Tetracoccus	Coccus	+[3]	+	Homo-	*Pediococcus*[2] *Tetragenococcus*[2]

[1] Data from reference 17.
[2] Genera generally recognized as safe on the basis of scientific information and practical experience.
[3] Pediococci are generally catalase negative but some strains produce a pseudocatalase that results in false-positive reactions.

TABLE 3
Typical properties of species of the so-called acidophilus group[1]

Species	Habitat	mol% guanine plus cytosine in DNA	Biotypes[2]	DNA homology groups	
				Reference 22	Reference 21
Lactobacillus acidophilus	All	32–37	I, II	I a	A-1
Lactobacillus amylovorus	P, C	40	IV (III)	I b	A-3
Lactobacillus crispatus	H, G	35–38	III	I c	A-2
Lactobacillus gallinarum	G	33–36	—	I d	A-4
Lactobacillus gasseri	H, C	33–35	I	II a	B-1
Lactobacillus johnsonii	H, P, G	32–38	I, II	II b	B-2

[1] Data modified according to reference 75 and G Reuter, unpublished observations, 1997. H, humans; P, pigs; C, cattle; G, poultry.
[2] Data from reference 20.

groups (**Table 3**). As a consequence, only strains belonging to the homology group that showed a high degree of DNA relatedness with *L. acidophilus* remained in this species, whereas members of the other homology groups were classified as separate species, ie, *Lactobacillus amylovorus, Lactobacillus gallinarum, Lactobacillus crispatus*, *Lactobacillus gasseri,* and *Lactobacillus johnsonii*. Although these are regarded as a separate species, they are closely related and have been suggested as belonging to one phylogenetic group or branch (23, 24) (**Figure 1**). Exact identification of members of the *L. acidophilus* group may also give an indication to the origin or typical host of a species (Table 3).

Relevant *Bifidobacterium* sp. that act as probiotics (Table 1) are generally strict anaerobes and are difficult to cultivate in milk or other food substrates. The most important species may be distinguished to some degree by relatively simple phenotypic criteria, ie, the fermentation of the sugars and sugar alcohols. L-arabinose, D-xylose, D-mannose, salicin, D-mannitol, D-sorbitol, and D-melezitose may serve as key characteristics (25). Analysis of the cell wall peptidoglycan composition was found particularly suitable for the identification of *Bifidobacterium longum, Bifidobacterium infantis,* and *Bifidobacterium suis* (26). Using genomic methods, however, Bonaparte (26) could show that most strains isolated from probiotic dairy products in Germany belonged to *Bifidobacterium animalis*.

The phylogeny of bacteria must be based on the comparison of highly conserved molecules that are present in all microorganisms. Therefore, genes encoding ribosomal RNA (rRNA), comprising conserved and variable domains, are chosen for phylogenetic studies. Comparing the sequence of rRNA is currently considered to be the most powerful and accurate technique for determining the degree of phylogenetic relation of microorganisms (27). Initially, DNA-rRNA hybridizations or rRNA cataloging methods were used for this purpose (12, 28). Advances in molecular biological techniques enabled sequencing of long stretches of rRNA, first by the use of reverse transcriptase and later by direct polymerase chain reaction (PCR) sequencing of 16S or 23S rDNA molecules, which resulted in large sequence databases. On the basis of the available information on 16S/23S rRNA sequences, phylogenetic trees or dendrograms were created. All gram-positive bacteria cluster in 2 of the 17 eubacterial phyla, which coincide with their DNA base composition (23, 24). Practically all organisms used in probiotic foods or food supplements are representatives of the genera *Lactobacillus*, *Enterococcus,* or *Bifidobacterium*. The genus *Bifidobacterium* shares some phenotypic features with typical LAB but traditionally and also for practical purposes, bifidobacteria are still considered to form part of the LAB. Phylogenetically distinct, bifidobacteria exhibit a relatively high guanine plus cytosine (G + C) content of 55–67 mol% in the DNA and form part of the so-called Actinomycetes branch. The "true" LAB form part of the so-called *Clostridium* branch, which is characterized by a G + C content of <55 mol% in the DNA (23, 24). The phylogenetic relation of the different genera of "true" LAB are shown in **Figure 2** and are based on the comparison of 16S rRNA sequences. *Carnobacterium*, *Enterococcus, Vagococcus, Aerococcus, Tetragenococcus,* and the newly described genus *Lactosphaera* are related more closely to each other than to any other LAB. *Lactococcus* and *Streptococcus* appear to be relatively closely related, whereas *Lactobacillus* is phylogenetically diverse. 16S rRNA sequencing data showed that *Lactobacillus* and *Pediococcus* are phylogenetically intermixed as 5 species of a *Pediococcus* cluster with 32 homo- and heterofermentative *Lactobacillus* spp. in the so-called *Casei* and *Pediococcus* group (29). 16S rRNA sequence data of pediococci and lactobacilli clearly indicate that the taxa generated on the basis of phenotypic properties, such as cell morphology and fermentation type, do not correspond with the phylogenetic branching. As a consequence, certain species of LAB may have to be reclassified. The challenge for taxonomists is to find easily determinable characters that correlate with the phylogenetically based grouping. This has become an increasingly important issue with respect to species nomenclature and the typing and characterization of new probiotic strains.

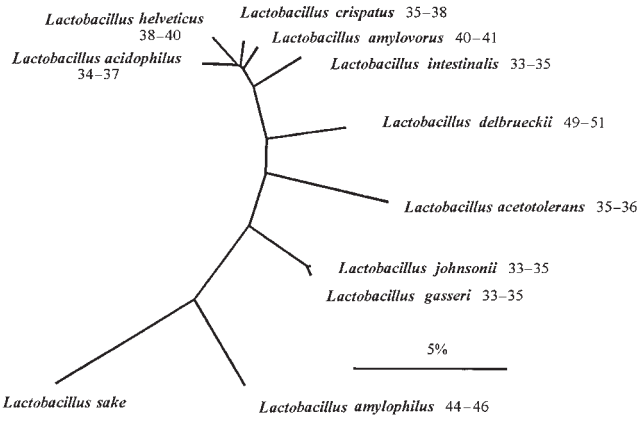

FIGURE 1. Relative phylogenetic relations of members of the *Lactobacillus acidophilus* group on the basis of comparative 16S rRNA sequence analysis (mol% guanine plus cytosine of the DNA). Adapted from references 23 and 24.

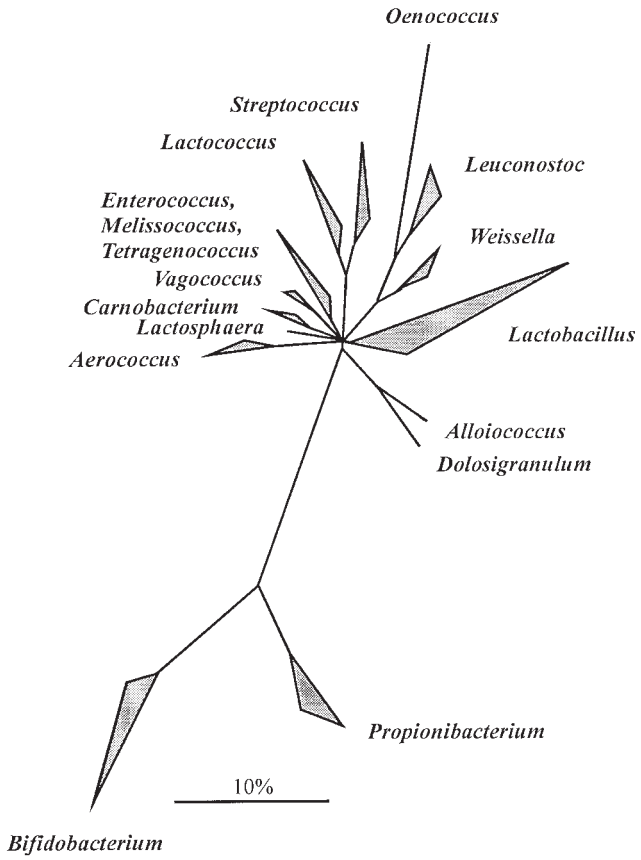

FIGURE 2. Consensus tree, based on comparative sequence analysis of 16S rRNA, showing major phylogenetic groups of lactic acid bacteria with low mol% guanine plus cytosine in the DNA and the nonrelated gram-positive genera *Bifidobacterium* and *Propionibacterium.* Adapted from references 23 and 24.

SELECTION OF PROBIOTIC STRAINS

LAB are associated with habitats that are rich in nutrients, such as various food products and plant materials. They can be found in soil, water, manure, sewage, and silage and can ferment or spoil food. Particular LAB are inhabitants of the human oral cavity, the intestinal tract, and the vagina, and may have a beneficial influence on these human ecosystems. They may therefore also be candidates for application as probiotics. Against this background, Reuter and Lerche (20, 30–32) comprehensively studied the lactobacilli typically associated with the human GT. On the basis of their precise and well-documented observations (**Table 4**), it can be assumed that homofermentative lactobacilli that are typical of the human host are represented by 3 groups: *1*) the *L. acidophilus* group, involving strains that are recognized today as *L. acidophilus*, *L. gasseri*, *L. crispatus*, and *L. johnsonii*; *2*) *Lactobacillus salivarius*; and *3*) the *Lactobacillus casei* group, involving strains of *paracasei* and *Lactobacillus rhamnosus*. In addition, Reuter and Lerche identified some heterofermentative lactobacilli as part of the normal microbial population of the human GT, which include mainly *Lactobacillus reuteri* and, to a lesser extent, *Lactobacillus fermentum, Lactobacillus oris*, and *Lactobacillus vaginalis*.

APPLICATION OF TYPICAL STRAINS IN COMMERCIAL PRODUCTS

Food products or supplements and pharmaceutical preparations containing viable probiotic strains are supplied on the market either as fermented food commodities or in lyophilized form. Among the yogurt-type products (33, 11), strains of *L. acidophilus* (including the related species *L. crispatus* and *L. johnsonii*), *L. casei* and *L. paracasei*, and *Bifidobacterium* spp. predominate (**Table 5**). Strains with probably the longest history of proved health benefits and safe use are the *L. casei* Shirota strain and some strains of the *L. acidophilus* group. The functional properties and safety of particular strains of *L. casei*, *L. rhamnosus*, *L. acidophilus*, and *L. johnsonii* have been extensively studied and well documented (**Table 6**).

Because of lactase deficiency, a large proportion of the world's adult population suffers from symptoms such as flatulence, abdominal pain, and diarrhea. Lactose-intolerant people may consume milk products without adverse symptoms if high concentrations of lactase are present in the product. Classical starter culture bacteria are well adapted to the milk substrate and may ferment lactose more effectively than do most probiotic strains. Their sensitivity to intestinal conditions, eg, high bile salt concentrations, seems to result in permeabilization and the release of intracellular lactase (34). With respect to probiotic strains, this hypothesis still seems to be controversial (35, 36). Strain-specific effects of probiotic lactic cultures on the human immune system and on diarrhea are well documented, eg, for counteracting rotavirus- or antibiotic-associated diarrhea, examples of which are the *Lactobacillus* GG strain of *L. rhamnosus* and the Shirota strain of *L. casei* (*L. paracasei*) (37, 38). To understand the underlying mechanisms, continued research is focusing on adhesive and immunomodulating properties of effective strains (39–43). Although published criteria for the selection of new strains are provisional, research data on particular LAB strains as immunomodulators and oral vaccine vectors contribute to the rapidly increasing knowledge in this area (44–46).

Research on immune stimulation and modulation coincides partly with the focus on cancer prevention by probiotic cultures. Antiproliferative effects and antigenotoxic and antimutagenic activities are documented; to some extent, these also seem to be

TABLE 4
Autochthonous lactobacilli associated with the human host[1]

Homofermentative[2]
Lactobacillus acidophilus
Lactobacillus acidophilus sensu stricto
Lactobacillus gasseri
Lactobacillus crispatus
Lactobacillus johnsonii
Lactobacillus salivarius
subsp. *salivarius*
subsp. *salicinius*
Lactobacillus casei
subsp. *casei*
subsp. *tolerans*
Lactobacillus rhamnosus
Heterofermentative[3]
*Lactobacillus reuteri (*formerly *L. fermentum II),* including *Lactobacillus oris* and *Lactobacillus vaginalis*

[1] Data from G Reuter, unpublished observations, 1997.
[2] Mainly lactic acid but no carbon dioxide from glucose.
[3] Lactic acid and other organic acids (acetic and formic acids) and carbon dioxide from glucose.

TABLE 5
Lactobacillus strains used in probiotic yogurts or yogurt-like products

Probiotic strain[1]	Type of product	Identification on the basis of DNA-homology analysis	Viable counts[2]
			log CFU/g yogurt
L. acidophilus LA-1	Yogurt	*L. johnsonii*	7.1–8.0
L. acidophilus LA-7	Yogurt	*L. acidophilus*	3.9–6.1
L. acidophilus L1	Yogurt drink	*L. crispatus*	—
L. acidophilus LA-H3	Dietetic yogurt	*L. acidophilus*	5.8–8.4
L. acidophilus	Yogurt	*L. crispatus*	6.8–8.2
L. acidophilus	Yogurt	*L. acidophilus*	5.5–6.8
L. casei Actimel	Yogurt drink	*L. paracasei*	7.4–8.4
L. casei Shirota	Probiotic drink	*L. paracasei*	7.9–8.9
L. casei GG	Yogurt drink	*L. rhamnosus*	8.0
L. casei LC-H2	Dietetic yogurt	*L. casei*	4.7–5.3
L. casei	Yogurt	*L. paracasei*	6.2–7.8
L. casei	Yogurt	*L. paracasei*	8.6–8.7

[1] As indicated by the manufacturer.
[2] CFU, colony-forming units.

species and strain specific (47–49). Fecal enzymes [eg, β-glucuronidase (EC 3.2.1.31) and azobenzene reductase (EC 1.6.6.7)] related to colon carcinogenesis were found to be reduced by particular strains, eg, *L.* GG (50), but more strain-specific studies are still required.

The potential of probiotic cultures to reduce serum cholesterol concentrations is still a matter of debate with respect to underlying mechanisms. This property, however, seems to be specific for strains with a high-bile-salt hydrolytic activity (51).

The steady increase in the range of probiotic food products, including nondairy products, (eg, fermented meats and vegetable and fruit juices), has opened new questions and challenges with regard to the typing and description of strains selected for application.

MOLECULAR TYPING OF PROBIOTIC STRAINS

DNA-based typing methods

Many different genotyping techniques may be applied to LAB as tools for either species identification or differentiation of strains to the clonal level. The major advantages of these DNA-based typing methods lie in their discriminatory power (52) and in their universal applicability. Closely related strains with similar phenotypic features may now reliably be distinguished by DNA-based techniques. Molecular typing methods applicable to probiotic LAB include plasmid profiling, restriction enzyme analysis (REA), pulse-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), and ribotyping.

Plasmid profiling

For LAB, plasmid profiling was formerly considered suitable for the typing of individual strains within a species. However, as a result of the instability of extrachromosomal DNA, methods that use chromosomal DNA, are superior to this technique and have become more popular (53).

Restriction enzyme analysis

REA involves the digestion of chromosomal DNA with restriction endonucleases. The fragments obtained are usually separated in an agarose gel with use of conventional electrophoresis. This results in a complex banding pattern with fragments sized between 1000 and 20000 bp. The complexity of the banding pattern makes visual evaluation difficult and necessitates the use of computer-aided multivariate analysis (54). The selection of an appropriate restriction enzyme or a set of enzymes is important for obtaining revealing patterns. REA was applied successfully to differentiate between strains of *L. acidophilus* (55), *L. casei* and *L. rhamnosus* (56), and *L. reuteri* (56, 57).

TABLE 6
Successful probiotic strains and their functional properties[1]

Property	*Lactobacillus casei* Shirota	*Lactobacillus rhamnosus* GG (ATCC 53103)	*Lactobacillus johnsonii* LA1	*Lactobacillus acidophilus* NFCB 1748
Origin	Human	Human	Human	—[2]
Safety	Verified	Verified	Verified	Verified
Acid stability	Good	Good	Good	Good
Bile stability	Resistant	Resistant	Resistant	Resistant
Colonization	–	+	+	–
Bacteriocin production	No	—	Yes	No
Adherence (Caco-2)	No	Yes	Yes	No
Adherence (mucosa)	—	Yes	Yes	Yes

[1] Data from reference 10.
[2] Data not available.

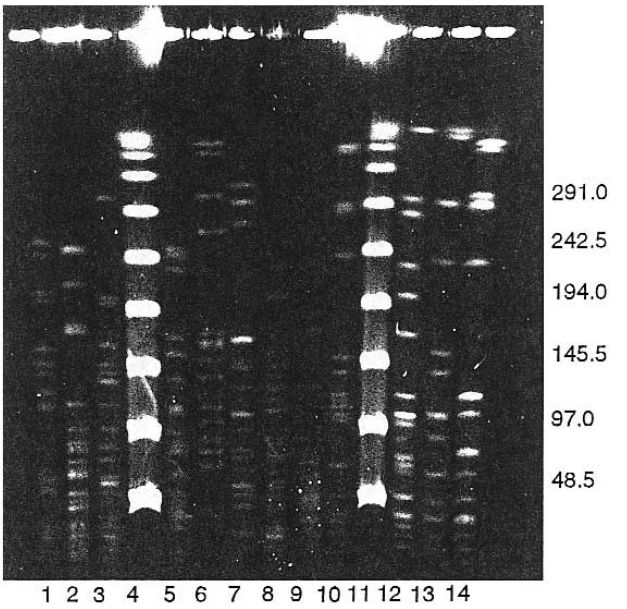

FIGURE 3. *SmaI* restriction endonuclease patterns obtained by pulse-field gel electrophoresis. Lanes 1–3: *Enterococcus faecium* strains; lanes 4 and 11: lambda concatemer as a size marker; lane 5: *E. faecium* LMG 11423^T; lane 6: *Enterococcus durans* LMG 10746^T; lane 7: *Enterococcus hirae* LMG 6399^T; lane 8: *Enterococcus casseliflavus* LMG 10745T; lane 9: *Enterococcus gallinarum* LMG 13129^T; lane 10: *Enterococcus faecalis* LMG 7937^T; lanes 12–14: isolates of *E. faecalis*.

Pulse-field gel electrophoresis

A modification of the genomic DNA restriction analysis became known as PFGE. This type of electrophoresis involves periodically changing the orientation of the electric field, thereby enabling the separation of high-molecular-weight fragments. PFGE allows the use of rare-cutting restriction endonucleases, which generates a low number of fragments, resulting in a banding pattern that is easy to interpret. This type of DNA fingerprint typically consists of 5 to 20 large well-resolved fragments ranging in size from 10 to 800 kb. It is a highly discriminatory and reproducible method and has been used to differentiate strains of important probiotic bacteria, such as bifidobacteria (58), *L. casei* (59), and *L. acidophilus* (55). It usually enables the differentiation between different clones of a particular species. Gordillo et al (60), for example, compared ribotyping with PFGE to differentiate strains of *Enterococcus faecalis*. With ribotyping, they could identify 7 patterns compared with 25 patterns with PFGE. Because of its high discriminatory power, PFGE enables intraspecies differentiation between probiotic and clinical strains of enterococci (61, 62). An example of the discriminatory power is shown in **Figure 3**. Three different strains of *E. faecalis* and *Enterococcus faecium,* together with 6 enterococcal-type strains, were subjected to PFGE. Each of the patterns generated was distinct from the others and clearly distinguished the strains that were analyzed. PFGE is now considered to be the standard for epidemiologic studies involving enterococci. A dendrogram based on *SmaI* PFGE patterns (**Figure 4**) suggested the applicability of PFGE for intraspecies differentiation of *Enterococcus* strains.

Ribotyping

With ribotyping, rRNA restriction patterns are created by hybridization with a 23S and 16S rRNA gene probe. Digestion of chromosomal DNA and agarose electrophoresis are followed by southern blotting, by which the DNA is transferred to a membrane for hybridization. In general, the fingerprint patterns are more stable and more easily interpretable than are those obtained by REA (54). Another advantage lies in the high reproducibility of this method and in the possibility of using a universal probe for all species because of the similarity of ribosomal genes (62, 63). Ribotyping has been used with some success to study the diversity of strains of *L. reuteri* and *L. fermentum* isolated from the mouse ileum (64) and to characterize strains of different *Lactobacillus* species (55, 65). However, ribotyping, shows high discriminatory power at the species level rather than on the strain level. For example, fresh isolates from the urogenital tract were reliably identified by ribotyping (66). The choice of a suitable restriction enzyme is important and different ribopatterns may be obtained by using different restriction enzymes. For differentiation of *Lactobacillus sake* strains, Björkroth and Korkeala (67) found EcoRI, HindIII, and ClaI to give the best results among 11 different restriction enzymes. An example of results obtained by ribotyping of different *E. faecalis* strains is shown in **Figure 5**.

Randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA is a simple and rapid method and is also based on the PCR. In the PCR reaction, short primers of random sequences are used under low-stringency annealing conditions, which results in the amplification of

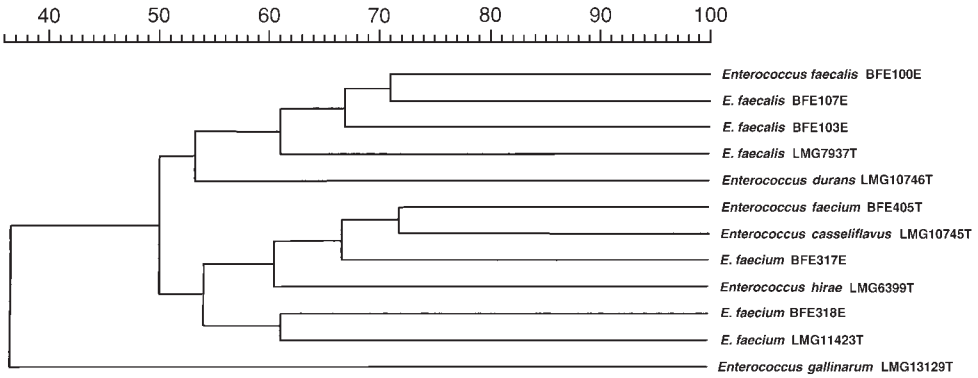

FIGURE 4. Similarities between the *SmaI* pulsed-field gel electrophoresis patterns. Dice coefficient correlation and unweighted pair group method with arithmatic mean clustering were used for the numerical analysis.

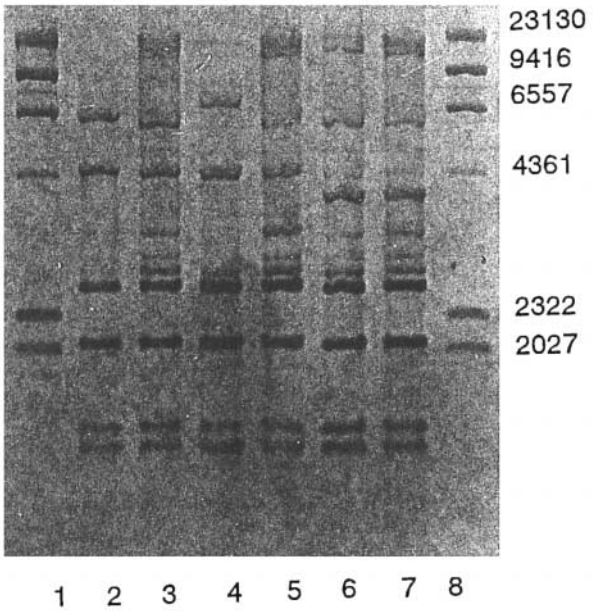

FIGURE 5. rRNA gene restriction patterns obtained by using *HIND*III restriction endonuclease. Lanes 1 and 8: lambda *HIND*III digest used as molecular weight marker; lanes 2–7: typical *HIND*III ribopatterns of *Enterococcus feacalis* isolates, showing differences between strains.

randomly sized DNA fragments. The reproducibility of RAPD patterns, however, is occasionally poor and the method needs to be performed under carefully controlled conditions. RAPD profiling has been successfully applied to distinguish between strains of *Bifidobacterium* (58) and between strains of the *L. acidophilus* group (68). A multiplex RAPD-PCR using a combination of two 10 mer primers in a single PCR reaction enabled differentiation of *Lactobacillus* strains from the GT of mice (69).

Oligonucleotide probes complementary to rRNA gene targets can be applied for in situ detection of bacteria in mixed populations, eg, species of potentially probiotic lactobacilli (62, 70–72), enterococci (72), and bifidobacteria (73). For example, genus-specific 16S rRNA hybridization probes were also developed for in situ detection of bifidobacteria in human feces (74), and chromosomal typing (ie, chromotyping) enabled differentiation among ribotype A strains of *L. rhamnosus* (66).

Other newly developed techniques for molecular typing, which include PCR-ribotyping, amplified DNA restriction analysis, rep-PCR, and restriction or amplified fragment length polymorphism, offer high sensitivity and discriminatory power for the identification and differentiation of probiotic microorganisms.

CONCLUSIONS

Realizing the complexity of the present market situation, it is clear that the identification of microorganisms at only the species level would no longer provide the transparency required by the consumer, by responsible scientists, and by industry and legislative bodies. It is a well-established fact that species, and even genus designation, may give a strong indication of typical habitats and the possible origin of an organism (Table 3). In addition, as indicated above, the generally accepted safety and technical applicability of a species, and especially of "new" strains, may be indicated strongly by the species or even the genus to which it belongs (Table 1). Furthermore, strains selected for their particular functional properties have to be clearly characterized below the species level, which is also in the interest of the manufacturer, especially after high investments in screening and selection procedures and in clinical studies. Molecular fingerprinting methods provide reliable and highly discriminatory solutions to these challenges.

In contrast with traditional starter cultures, eg, fermented milk products, long-term experience does not exist for most probiotic strains on the market. Legislative bodies and governmental control organs require exact indications of the properties and typing of strains applied in new products.

In Germany, species such as *L. rhamnosus*, *E. faecium*, and *E. faecalis* have been grouped into risk group 2, ie, potential pathogens. The investigators responsible for this classification, however, concede that strains of these species with a documented safe history may belong to a risk group 1 (ie, species constituting no risk). Such strains that have found application in food fermentations or certain probiotic products for a long time are considered as safe. The differentiation of these strains from those of a clinical, environmental, or animal origin poses a special challenge that may be solved by modern molecular typing techniques such as PFGE and PCR.

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