

# Identification of Lactobacilli and Bifidobacteria

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Selective culture media and phenotypic tests enable lactobacilli to be differentiated from morphologically similar bacteria. The accurate identification of *Lactobacillus* species can be accomplished by reference to 16S rRNA gene sequences. Species-specific, PCR primers that target the 16S-23S rRNA spacer region are available for a limited number of *Lactobacillus* species. Molecular methods for the comprehensive identification of *Bifidobacterium* species are not yet available. Only DNA-DNA reassociation provides a reliable means of species identification for this genus at present. Bifidobacteria can be differentiated from morphologically similar bacteria by the use of genus-specific, PCR primers or oligonucleotide probes.

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## Introduction

Marked changes have occurred in bacterial classification since the application of molecular technologies to this task. The impetus for major change has resulted from the observation that 16S ribosomal RNA (rRNA) sequences can be used as evolutionary chronometers (40). Some regions of the 16S rRNA molecule are conserved throughout all bacterial species and can be used to align sequences obtained from different isolates. Alignment of these conserved regions permits comparison of the remaining regions (Figure 1, V 1 to V9) which are variable as to nucleotide base sequence between many species (6, 33, 36). From a practical point of view, the 16S rRNA gene sequences (rDNA) can be used in the reliable identification of many bacterial species through the derivation of specific oligonucleotide probes or polymerase chain reaction (PCR) based techniques (19, 36, 38, 39). Other regions of the genome also offer opportunities as targets for identification procedures (10). These molecular approaches allow *Lactobacillus* species to be reliably identified, but much developmental work remains to be accomplished in the case of the bifidobacteria.

## Identification by Phenotype

Traditionally, lactobacillus and bifidobacterial species have been identified on the basis of cell morphology, analysis of fermentation products and associated enzyme activities, and the ability to utilise various carbohydrate substrates. The application of these approaches in the classification and identification of Lactic Acid Bacteria has been the subject of several reviews and will not be covered in this article (11, 28, 32, 43). Suffice it to say that, in general, phenotypic methods suffer from a lack of reproducibility generated by conditions of culture related to different laboratories, and to the diversity of strains (biotypes) that comprise the recognised species (1, 11,

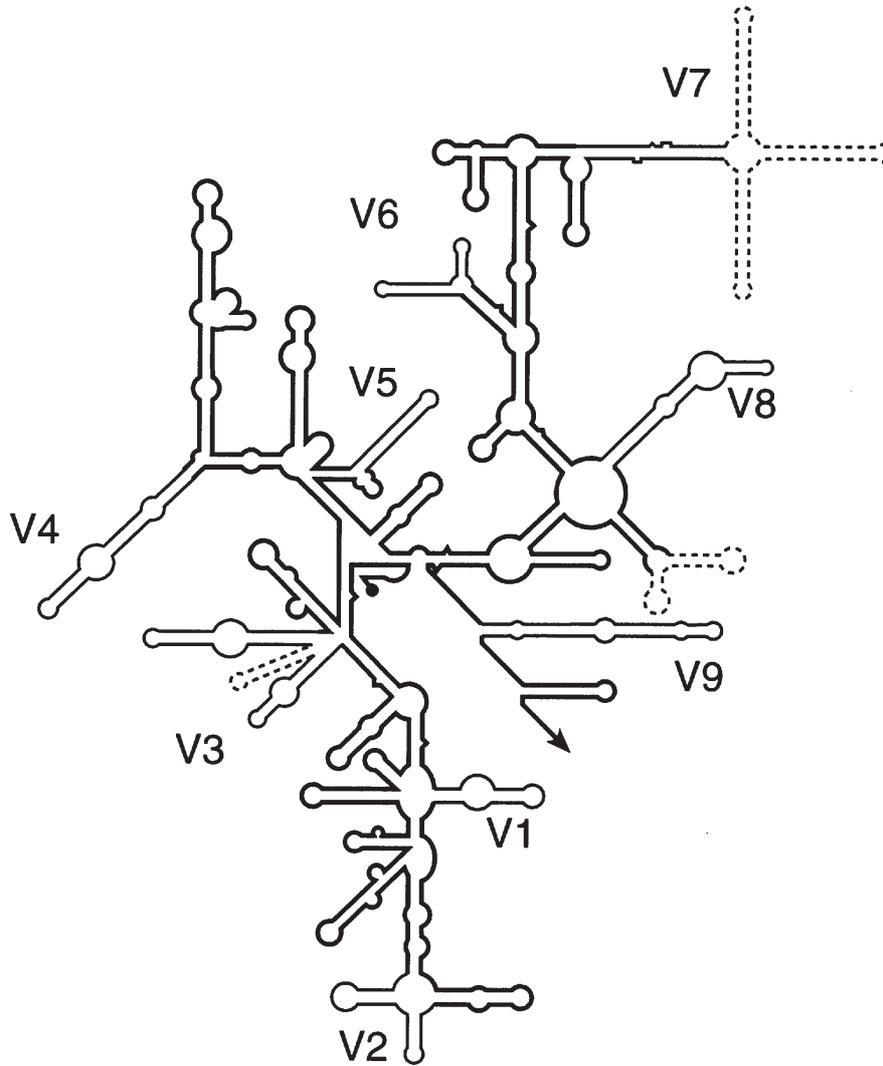


Figure 1. Secondary structure model for prokaryotic 16S rRNA. Regions where the nucleotide base sequence is most variable are labelled V1 to V9. After Neefs and colleagues (26).

32). Nucleic acids are universal in cellular biology, and the nucleotide base sequence of these molecules is not influenced by culture conditions. Analysis of nucleic acids thus provides a basis for identification methods that are reproducible from one laboratory to another. Genotypic approaches hold the most promise for the rapid and accurate identification of lactobacilli and bifidobacteria.

Table 1. *Lactobacillus* species

<i>L. acetotolerans</i>	<i>L. acidophilus</i>
<i>L. agilis</i>	<i>L. alimentarius</i>
<i>L. amylolyticus</i>	<i>L. amylophilus</i>
<i>L. amylovorus</i>	<i>L. animalis</i>
<i>L. aviarius</i> subsp. <i>araffinosus</i>	<i>L. aviarius</i> subsp. <i>aviarius</i>
<i>L. bifementans</i>	<i>L. brevis</i>
<i>L. buchneri</i>	<i>L. carnis</i>
<i>L. casei</i>	<i>L. catenaforme</i>
<i>L. cellobiosus</i>	<i>L. collinoides</i>
<i>L. coryniformis</i> subsp. <i>coryniformis</i>	<i>L. coryniformis</i> subsp. <i>torquens</i>
<i>L. crispatus</i>	<i>L. curvatus</i> subsp. <i>curvatus</i>
<i>L. curvatus</i> subsp. <i>melibiosus</i>	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i>	<i>L. delbrueckii</i> subsp. <i>lactis</i>
<i>L. farciminis</i>	<i>L. fermentum</i>
<i>L. fructivorans</i>	<i>L. fructosus</i>
<i>L. gallinarum</i>	<i>L. gasseri</i>
<i>L. graminis</i>	<i>L. hamsteri</i>
<i>L. helveticus</i>	<i>L. hilgardii</i>
<i>L. homohiochii</i>	<i>L. iners</i>
<i>L. intestinalis</i>	<i>L. jensenii</i>
<i>L. johnsonii</i>	<i>L. kandleri</i>
<i>L. kefiranoferiens</i>	<i>L. kefirgranum</i>
<i>L. kefiri</i>	<i>L. kunkeei</i>
<i>L. lindneri</i>	<i>L. malefermentans</i>
<i>L. mali</i>	<i>L. maltaromicus</i>
<i>L. manihotivorans</i>	<i>L. murinus</i>
<i>L. oris</i>	<i>L. panis</i>
<i>L. parabuchneri</i>	<i>L. paracasei</i> subsp. <i>paracasei</i>
<i>L. paracasei</i> subsp. <i>tolerans</i>	<i>L. parakefiri</i>
<i>L. paraplantarum</i>	<i>L. pentosus</i>
<i>L. plantarum</i>	<i>L. pontis</i>
<i>L. reuteri</i>	<i>L. rhamnosus</i>
<i>L. rogosae</i>	<i>L. ruminis</i>
<i>L. sakei</i> subsp. <i>carnosus</i>	<i>L. sakei</i> subsp. <i>sakei</i>
<i>L. salivarius</i> subsp. <i>salicinicus</i>	<i>L. salivarius</i> subsp. <i>salivarius</i>
<i>L. sanfranciscensis</i>	<i>L. sharpeae</i>
<i>L. suebicus</i>	<i>L. uli</i>
<i>L. vaccinostercus</i>	<i>L. vaginalis</i>
<i>L. vitulinus</i>	<i>L. zeae</i>

For further information, see reference 36.

## Lactobacilli

### *Genus Identification*

Members of the genus *Lactobacillus* can be selected on solid culture media that have an acidic pH (e.g. Rogosa SL agar). While many *Lactobacillus* strains used in the dairy industry can be cultured under microaerophilic, or even aerobic conditions, intestinal isolates proliferate best under anaerobic conditions. Lactobacilli are gram-positive, non-spore-forming rods (ranging from coccobacilli to long, slender bacilli). Confirmation that the isolates belong to the genus *Lactobacillus* can be accomplished by testing for the absence of catalase activity, and the presence of lactic acid as the major acid produced from the fermentation of glucose. Acetic, succinic and formic acids may be detected in minor quantities in some cultures (16). Assignment of lactobacillus isolates to homofermentative or heterofermentative categories (by simply observing, by means of a Durham tube, whether gas is produced from glucose) is also a useful phenotypic test. Genus-specific primers for PCR-based identification have not been described.

### Species Identification

The taxonomy of the lactobacilli has been subject to flux during recent years, but there are currently 70 species (Table 1). From the point of view of probiotics research, however, 19 species are currently of most interest (Table 2).

Table 2. *Lactobacillus* Species Detected in the Intestinal Tract and/or used in Probiotic Products

<i>L. acidophilus</i>	<i>L. crispatus</i>	<i>L. hamsteri</i>
<i>L. agilis</i>	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	<i>L. intestinalis</i>
<i>L. aviarius</i>	<i>L. gallinarum</i>	<i>L. plantarum</i>
<i>L. amylovorus</i>	<i>L. gasseri</i>	<i>L. rhamnosus</i>
<i>L. brevis</i>	<i>L. johnsonii</i>	<i>L. reuteri</i>
<i>L. casei</i>	<i>L. murinus</i>	<i>L. ruminis</i>
		<i>L. salivarius</i>

For further information, see references 9 and 11.

Comparison of 16S rDNA gene sequences from lactobacilli shows that the V1, V2 and V3 regions contain the species-specific information. Therefore, for identification purposes, amplification by PCR and sequencing of less than half of the gene (less than 750 bp) may be sufficient, but whole gene sequencing is still best for establishing phylogenetic relationships. 16S rDNA sequence homology of less than 97.0%, it has been observed, means that the bacteria being compared will usually have less than 60-70% DNA similarity over the whole chromosome (DNA-DNA reassociation; the “gold standard” for species delineation) and hence are unlikely to belong to the same species (34).

16S, 23S and 5S rRNA genes are arranged within an operon on the bacterial chromosome (Figure 2). Nucleotide base sequence information has become available for the region between the 16S and 23S genes: the 16S-23S spacer region. Many bacteria have multiple copies (alleles) of the rRNA operon per genome and the spacer region may vary in size within different operons. This relates to the number and type of tRNA genes (tRNA<sup>glu</sup>, tRNA<sup>ile</sup>, tRNA<sup>ala</sup>) located in some spacer regions. PCR primers that anneal to conserved regions of the 16S and 23S rRNA genes permit the amplification of the spacer regions (10). Often, “short” (absence of tRNA genes) and “long” (presence of one or two tRNA genes) spacer regions are detected, and these can be purified from agarose gels for subsequent sequencing. The nucleotide sequence of the spacer regions of several *Lactobacillus* species has been determined. These sequences show that the spacer region is hypervariable, and species-specific PCR primers, based on these regions, have been derived for the identification of lactobacilli of interest to the food industry (5, 25, 35).

Oligonucleotide probes based on the analysis of hypervariable domains in nucleic acid molecules have been derived for identification purposes (31). These include a number that are reported to be specific for *Lactobacillus* species (Table 3). It should be noted, however, that consistent, specific, results are sometimes difficult to achieve when using oligonucleotide probes: control of the temperature and ionic strength of the washing solution are critical for obtaining optimal results.

## Bifidobacteria

### Genus Identification

Several solid media have been devised for the selective cultivation of members of the genus *Bifidobacterium*. Antibiotics or acidic additives have been popular choices in conferring selective properties on the media (4). Few of these media are completely selective for bifidobacteria, however, although Beerens agar and Rogosa SL agar (incubated for three days) have proved useful in the author’s laboratory (23).

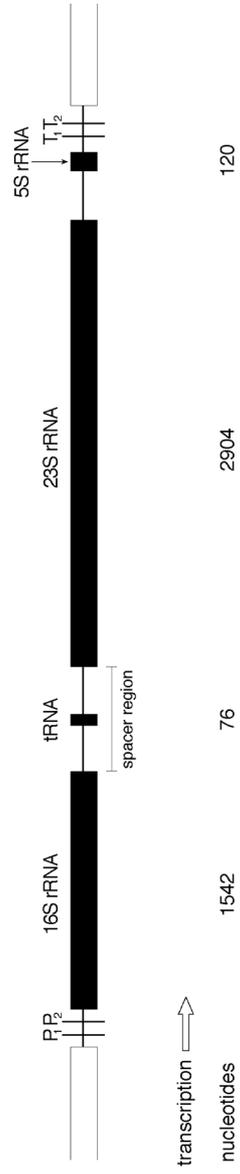


Figure 2. Generalised model of the rRNA operon of bacteria showing the 16S-23S spacer region. Some bacteria have two tRNA genes, others have either one or none. P<sub>1</sub>P<sub>2</sub> = promoters; T<sub>1</sub>T<sub>2</sub> = terminators. After Gurtler and Stanisch (10).

Table 3. Examples of rRNA-targeted Oligonucleotide Probes that Detect *Lactobacillus* Species

Species	Target	Reference
<i>L. acidophilus</i>	23S	28
<i>L. amylovorus</i>	16S	7
<i>L. brevis</i>	16S	37
<i>L. curvatus</i>	23S	13
<i>L. collinoides</i>	16S	7
<i>L. delbrueckii</i>	23S	14
<i>L. fermentum</i>	16S	37
<i>L. gasserii</i>	23S	28
<i>L. helveticus</i>	23S	14
<i>L. hilgardii</i>	16S	7
<i>L. johnsonii</i>	23S	28
<i>L. kefir</i>	16S	7
<i>L. kefiranoferiens</i>	23S	7
<i>L. paracasei</i>	23S	14
<i>L. parabuchneri</i>	23S	7
<i>L. pontis</i>	16S	37
<i>L. reuteri</i>	16S	7
<i>L. ruminis</i>	16S	12
<i>L. sake</i>	16S	13
<i>L. sanfrancisco</i>	16S	37

Bifidobacteria are gram-positive pleomorphic rods, ranging from uniform to branched, bifurcated Y and V forms, spatulate or club shaped. They are strictly anaerobic (although some strains can tolerate oxygen in the presence of carbon dioxide), non-motile and non-spore-forming. The branching nature of bifidobacteria is not only strain dependent but also depends on the medium used for cultivation. Comparison of cell morphology of bifidobacterial isolates grown on a standard medium can aid in identification (29). Cell morphology alone, however, must be supplemented with the results of biochemical tests to differentiate bifidobacteria from morphologically similar genera (*Lactobacillus*, *Actinomyces*, *Propionibacterium*, *Eubacterium*).

Bifidobacteria ferment hexoses by a fructose-6-phosphate phosphoketolase (F6PPK) shunt and are the only intestinal bacteria known to utilise this fermentation pathway. It is often termed the "bifidus pathway" (4, 29). F6PPK catalyses the splitting of fructose-6-phosphate to erythrose-4-phosphate and acetyl-phosphate. Demonstration of F6PPK activity in cellular extracts has been a useful method for differentiating bifidobacteria from morphologically similar bacteria (Figure 3).

Fermentation of glucose by the bifidus pathway produces acetic and lactic acids in a theoretical ratio of 3:2 (in reality, this is sometimes exceeded). Gas liquid chromatography of fermentation products thus provides another reliable means of differentiating bifidobacteria from other bacterial types (4, 29).

Biochemical tests for the identification of members of the genus *Bifidobacterium* are now largely superseded by the use of the genus-specific PCR primers described by Kok and colleagues (18) or Kaufman and colleagues (17). These primers amplify a 523 bp or 1.35 kbp region, respectively, of the 16S rRNA gene (Table 4). Genus-specific probes, Bif164 and Im3, have proved useful in the enumeration of total populations of bifidobacteria in faecal and food samples, respectively (17, 19).

### **Species Identification**

There are 32 species of bifidobacteria (Table 5). They can sometimes be differentiated, with great difficulty, using the results of fermentation tests together with observations of cell morphology and the electrophoretic mobilities of enzymes such as transaldolase (14 types) or 6-phosphogluconate dehydrogenase (19 types; 32).

<p><b>Reagents</b></p> <ol style="list-style-type: none"> <li>(1) 0.05 M sodium phosphate buffer (pH 6.5) containing 500 mg L-cysteine per litre.</li> <li>(2) Sodium fluoride (6 mg/ml) and potassium or sodium iodoacetic acid (10 mg/ml).</li> <li>(3) Hydroxylamine hydrochloride (13.9 gm/100 ml) adjusted to pH 6.5 with sodium hydroxide before use.</li> <li>(4) Trichloroacetic acid (15% weight/volume).</li> <li>(5) 4 M hydrochloric acid.</li> <li>(6) Ferric chloride (FeCl<sub>3</sub>·6H<sub>2</sub>O; 5% weight/volume in 0.1 M hydrochloric acid).</li> <li>(7) Fructose-6-phosphate (sodium salt; 70% purity; 80 mg/ml).</li> <li>(8) TPY broth (grams per litre; see reference 29): trypticase 10.0; phytone 5.0; glucose 5.0; yeast extract 2.5; tween 80 1.0 ml; cysteine hydrochloride 0.5; dipotassium monohydrogen phosphate 2.0; magnesium chloride 0.5; zinc sulphate 0.25; calcium chloride 0.15; ferric chloride 0.03; pH 6.5.</li> </ol> <p><b>Test</b></p> <p>Harvest cells by centrifugation from a 24-hour culture in TPY broth. Wash the bacterial cells in 10 ml of 0.05 M phosphate buffer. Suspend the cells in one ml of phosphate buffer in a microfuge tube.</p> <p>Centrifuge to deposit the cells, then remove supernatant and add 45 µl of mutanolysin solution (1mg/ml), 250 µl of lysozyme solution (6 mg/ml) and 650 µl of phosphate buffer. Suspend the cells. Disrupt the cells by sonication, keeping the preparations on ice.</p> <p>Transfer 50 µl aliquots of the sonicated material to three wells of a microtitre plate. Add 12 µl of the sodium fluoride-iodoacetic acid solution to each well. Then add 12 µl of fructose-6-phosphate to two of the wells (test) but not to the third well which serves as a negative control. Incubate the plate at 37 °C for 30 minutes. Stop the reaction by adding 75 µl of hydroxylamine solution to each well and leave at room temperature for 10 minutes. Then add 50 µl of trichloroacetic acid solution and 50 µl of 4 M hydrochloric acid. Add 50 µl of ferric chloride solution. A reddish-violet colour indicates fructose-6-phosphate phosphoketolase activity.</p>
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Figure 3. Fructose-6-phosphate phosphoketolase test for bifidobacteria. J. P. Burton, Department of Microbiology, University of Otago, after Scardovi (29).

The V1, V2 and V3 regions of the 16S rDNA sequences from 24 bifidobacterial species compared by McCartney (22) appeared to provide the best opportunity for the differentiation of species. Comparison of 16S rDNA sequences from 18 bifidobacterial species by Leblond-Bourget and colleagues (20), however, showed that they ranged in similarity from 92 to 99%. This high level of relatedness makes it impossible to differentiate between some bifidobacterial species on the basis of 16S rDNA sequence analysis (8).

Sequences (274 to 552 bp in length) obtained by PCR amplification of 16S-23S spacer regions of 18 bifidobacterial species gave similar phylogenetic information to that provided by 16S rDNA (20). The spacer region sequences were more variable, however, and Leblond-Bourget and colleagues have suggested that they provide a means of distinguishing between bifidobacterial strains (20). The spacer regions do not appear, therefore, to contain useful information that could be used in rapid tests to differentiate bifidobacterial species.

Only moderate progress has been made in the derivation of species-specific oligonucleotide probes for bifidobacteria (Table 4). Yamamoto and colleagues (42) derived probes 16 to 19 nucleotides in length that targeted 16S rRNA sequences. Probes specific for *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Bifidobacterium longum* were demonstrated using crude high-molecular-weight RNA preparations. The *B. bifidum* and *B. infantis* probes were not specific when tested using whole bacterial cells that had been fixed with formaldehyde. Mangin *et al.*, (21) reported that randomly-cloned fragments of chromosomal DNA could be used to differentiate *B. adolescentis*, *B. longum*, *B. bifidum* and *Bifidobacterium animalis*. Similarly, a randomly-cloned DNA fragment specific for *B. breve* has been reported by Ito and colleagues (15).

Table 4. Oligonucleotide Primers and Probes Described for use in the Identification or Detection of Bifidobacteria

Oligonucleotide	Specificity/Target	Application	Reference
Bif164	Genus/16S rRNA enumeration	Probe, detection,	19
Bif164/Bif662	Genus/16S rDNA identification	PCR, detection,	19
Im3	Genus/16S rRNA enumeration	Probe, detection,	17
Im3/Im26	Genus/16S rDNA identification	PCR, detection,	17
A6/17	<i>B. adolescentis</i> /unspecified	Identification	21
PAD	<i>B. adolescentis</i> /16S rRNA identification	Probe, detection,	42
an1	<i>B. animalis</i> /unspecified	Identification	21
bA7/2	<i>B. bifidum</i> /unspecified	Identification	21
BB22	<i>B. breve</i> /unspecified	Identification	15
PBR	<i>B. breve</i> /16S rRNA identification	Probe, detection,	42
PLO	<i>B. longum</i> /16S rRNA identification	Probe, detection,	42
L6/45	<i>B. longum</i> /phage integrase	Identification	21

It is clear, therefore, that rapid methods for the accurate and comprehensive identification of bifidobacterial species remain elusive. Considerable developmental work is required in this area since DNA-DNA reassociation is currently the only reliable method of *Bifidobacterium* species identification (1, 41).

#### Identification of Bacterial Species in Relation to Probiotic Research and Development

The prevalence of lactobacillus and bifidobacterial species in the intestinal tract of humans is not known accurately. Comprehensive studies of the species composing the human intestinal microflora have seldom been attempted, and those that have were conducted prior to the introduction of genotypic methods for the classification of bacteria (1). Probably the best comparisons available of *Lactobacillus* and *Bifidobacterium* species present in human faeces are those reported by Mitsuoka (24) and by Biavati and colleagues (2, 3). Mitsuoka listed *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* as the major species of the *Lactobacillus* microflora. *Lactobacillus johnsonii*, *Lactobacillus ruminis*, *Lactobacillus casei*, and *Lactobacillus brevis* were detected occasionally. Among the bifidobacteria, *Bifidobacterium longum* predominated in adult humans, while *Bifidobacterium bifidum* was detected occasionally. In contrast, *Bifidobacterium infantis* and *Bifidobacterium breve* were the most prevalent species in infant faeces, with *B. longum* and *B. bifidum* detected occasionally. The identity of the isolates was determined by phenotypic characterisation, and determination of the mole % G+C of genomic DNA in the case of the lactobacilli. Biavati *et al.*, (2),

Table 5. *Bifidobacterium* Species

<i>B. adolescentis</i> *	<i>B. angulatum</i> *
<i>B. animalis</i>	<i>B. asteroides</i>
<i>B. bifidum</i> *	<i>B. boum</i>
<i>B. breve</i> *	<i>B. catenulatum</i> *
<i>B. choerinum</i>	<i>B. coryneforme</i>
<i>B. cuniculi</i>	<i>B. dentium</i> *
<i>B. gallicum</i> *	<i>B. gallinarum</i>
<i>B. globosum</i> *	<i>B. indicum</i>
<i>B. infantis</i> *	<i>B. inopinatum</i>
<i>B. lactis</i>	<i>B. longum</i> *
<i>B. magnum</i>	<i>B. merycicum</i>
<i>B. minimum</i>	<i>B. pseudocatenulatum</i> *
<i>B. pseudolongum</i> subsp. <i>globosum</i>	<i>B. pseudolongum</i> subsp. <i>pseudolongum</i>
<i>B. pullorum</i>	<i>B. ruminantium</i>
<i>B. saeculare</i>	<i>B. subtile</i>
<i>B. suis</i>	<i>B. thermophilum</i>

\*Detected in human faeces. For further information, see reference 32.

using DNA-DNA reassociation as the primary method of identification, detected *B. infantis*, *B. breve*, and *B. longum* commonly in the faeces of human infants. *Bifidobacterium bifidum*, *B. pseudocatenulatum*, *B. dentium*, and *B. catenulatum* were isolated less frequently. In the case of samples from adults, *B. adolescentis*, *B. bifidum*, *B. catenulatum*, *B. longum*, and *B. pseudocatenulatum* were detected (3). Differentiation between species, in this latter study, was limited to the use of polyacrylamide gel electrophoresis of soluble cellular proteins.

Comprehensive studies utilising molecular approaches to identify the species commonly present in the intestine of humans inhabiting various geographic regions of the world should be initiated. Probiotics contain species of bifidobacteria and/or lactobacilli that are intended to generate an impact on the intestinal microflora (43, 44). It seems logical that the prevalence of the different species in various countries should be known, preferably before the consumption of probiotics becomes even more widespread. As part of the "environmental impact" report that should be completed for each probiotic that is consumed long term (44), an accurate assessment of the alterations, in terms of species, to the lactobacillus or bifidobacterial microfloras should be included. Some probiotics have been marketed in particular countries for many years. *Lactobacillus rhamnosus* GG, for example, has been marketed extensively to the general population as a component of milk and other food products in Finland. Has the *Lactobacillus* microflora of Finns altered dramatically since marketing of these products began? What changes have occurred in the proportions of *Lactobacillus* species detected in faeces since the introduction of probiotic products? What is the long term effect of any such change on the human population?

Accurate identification of strains of lactobacilli and bifidobacteria must also be accomplished so that consumers can be reliably informed of the content of probiotic products. Many probiotic yogurts are labelled as containing *Lactobacillus acidophilus*. The re-classification of the "acidophilus" group may mean that some products are currently mislabelled (11). Perhaps "Acidophilus" will be used in future more as a generic marketing term recognised by the consumer, but not necessarily indicative of the microbial content. From the point of view of health regulatory agencies, however, labelling of products should reflect exactly the species composition.

### Future Research

Major emphasis should be placed on the development of rapid and accurate methods for the identification of *Bifidobacterium* species. DNA sequences other than those of 16S rRNA genes will probably provide the basis of these methods. Comprehensive studies to detect the prevalence of *Lactobacillus* and *Bifidobacterium* species in the intestinal microflora of humans should be initiated once satisfactory identification methods are established. Companies that sell cultures to the dairy industry and other probiotic manufacturers must ensure that the strains in their culture collections are identified by genotypic procedures, and that they use current nomenclature.

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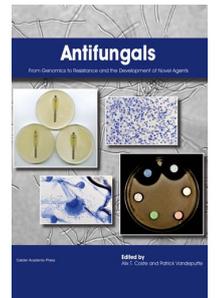
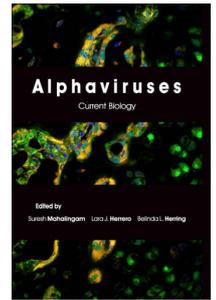
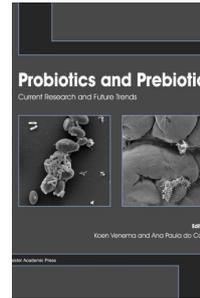
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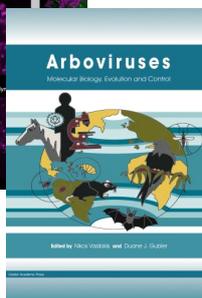
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