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REVIEWS

Application of Molecular Methods to Classification and Identification of Bacteria of the Genus *Bifidobacterium*

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Abstract—The molecular methods currently used in the classification and identification of bifidobacteria are reviewed. The sequencing of the 16S rRNA gene and some other genes considered to be phylogenetic markers is a universal and effective approach for taxonomic characterization of members of the genus *Bifidobacterium* and to reliable identification of new isolates. Various techniques of obtaining DNA fingerprints (PFGE, RAPD, rep-PCR) are widely used for solving particular problems in identifying bifidobacteria. Bacteria of the genus *Bifidobacterium* are important organisms in biotechnology and medicine. The research in the field of molecular systematics of bifidobacteria provides a basis not only for the solution of taxonomic problems, but also for monitoring of individual species in the environment and for more detailed study of the genetics and ecology of this group of microorganisms.

Key words: bifidobacteria, identification, classification, molecular methods.

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Bacteria of the genus *Bifidobacterium* occur in the oral cavity and intestinal tract of humans, warm-blooded animals, and insects, as well as in wastewater. Bifidobacteria are a component of natural microflora of human and animal intestines. They colonize gastrointestinal tracts of newborn children in the first few days after birth and amount to 99% of the intestinal microflora of a healthy infant. With age, the quantity of bifidobacteria decreases and they rank third after *Eubacterium* and *Bacteroides* [1, 2]. The important role of bifidobacteria as a component of normal intestinal microflora of humans and animals has resulted in a significant increase in scientific interest in this genus and has served to intensify research into the biology of these microorganisms [3, 4].

The use of bacteria of the genus *Bifidobacterium* as probiotics requires the development and application of prompt, accurate, and convenient methods for detection and identification of these microorganisms in foods and for studying the composition of natural populations of bifidobacteria in human and animal intestines. At present, there is a numbers of reviews on the molecular methods of identification and detection of probiotic microorganisms [5–8]; however, only one review is devoted to the application of these methods to bifidobacteria [9].

Our review pursues this issue and gives much more attention to specific features of using molecular methods for accurate generic- and species-level classification of bifidobacteria, which is the basis of their effective identification.

History of the Systematics of Bifidobacteria

Bifidobacteria were first isolated from the feces of an infant and described in 1900 under the name of *Bacillus bifidus communis* [10]. In 1924, Orla-Jensen, who was the first to use biochemical methods for characterizing bifidobacteria, referred this group of microorganisms to an independent genus, *Bifidobacterium*, which, in his opinion, formed a connecting link between lactic-acid and propionic acid bacteria [11].

Up to the middle of the 20th century, bifidobacteria had changed their generic affiliation several times. They were referred to the genera *Bacillus, Lactobacillus, Bacteroides*, and *Actinomyces* [12, 13]. Frequent reclassification was due to predominance of morphological or physiological–biochemical criteria for describing generic level taxa and to pronounced pleomorphism of bifidobacteria. Since the early 1940s up to the 1960s, bifidobacteria were most often considered

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within the genus *Lactobacillus* under the species name *L. bifidus* [12] because of the similar morphological and cultural characteristics of these genera. Bifidobacteria are gram-positive, nonmotile, non-spore-forming rods of variable shape and size. They require complex organic media for their cultivation. Members of the genus *Bifidobacterium* are traditionally referred to anaerobic microorganisms, although sensitivity to oxygen varies between different species and even strains. The optimal growth temperature for most bifidobacterial strains isolated from humans is 37–40°C, whereas the temperature optimum for bacteria isolated from the intestines of animals is a bit higher, 41–43°C. The optimal pH value varies within 6.5–7.0 [3, 12, 14–16].

Studies in 1965–1967 resulted in the discovery of a unique enzyme, fructose-6-phosphate phosphoketolase (P6PPK), and of the hexose–fructose-6-phosphate shunt, a catabolic pathway characteristic of bifidobacteria only [17, 18]. To date, P6PPK is one of the key criteria for differentiating the genus *Bifidobacterium* from other genera, because only the phylogenetically close genus *Gardnerella* is also characterized by the presence of this enzyme [19]. One more distinguishing feature of bifidobacteria has been revealed: the ability to ferment hexoses with the formation of lactic and acetic acids in a molar ratio of about 2 : 3 and without production of carbon dioxide [13]. At the same time, it has been shown that bifidobacteria considerably differ from members of the genus *Lactobacillus* in the G+C content of DNA [20].

The 1960s–1980s was a period of explosive growth in chemotaxonomic studies of microorganisms, which showed that many genera of gram-positive bacteria, actinomycetes in particular, are characterized by specific compositions of cell walls, phospholipids, fatty acids, and menaquinones [21]. Unfortunately, the data on chemotaxonomic characteristics of bifidobacteria are fragmentary, because their determination is not required for the description of new species of this genus. Nevertheless, the integrity of available data suggest that representatives of the genus *Bifidobacterium* are characterized by a type VIII cell wall (ornithine as a typical component), PI-type phospholipids (absence of nitrogen-containing phospholipids as a distinctive feature), and unbranched saturated and monounsaturated fatty acids with an even number of carbon atoms in the chain [21, 22]. The structure of peptidoglycan may vary insignificantly among strains of the genus *Bifidobacterium*, which is also typical of other genera of gram-positive bacteria; however, these variations are usually not used for intrageneric (species) differentiation due to the difficulties in their determination.

Thus, the independent generic status of bifidobacteria was beyond question by the end of the 1960s, and the genus *Bifidobacterium* Orla-Jensen 1924 with 11 comprising species was approved in 1974 in the 8th edition of Bergey's Manual [23].

At present, the genus *Bifidobacterium*, represented by 29 species, is referred to the family *Bifidobacteriaceae*, which also includes the monotypic genera *Aeriscardovia, Gardnerella, Parascardovia*, and *Scardovia* [24].

It has been thought for a long time that the genus *Bifidobacterium* is represented by only one species, *B. bifidum*, highly variable in many characteristics. Only in 1957 were five groups of uncertain taxonomic status distinguished within this genus based on the ability of bifidobacteria to ferment different carbohydrates [25]. These studies initiated the discovery of numerous biotypes of *Bifidobacterium*, which later became a basis for describing species and subspecies. In 1963, a classification scheme for bifidobacteria was developed that took into account not only sugar fermentation profiles but also serological properties of these microorganisms. Several species were proposed, of which *B. adolescentis, B. breve* and *B. longum* still persist [24, 26]. The species *B. animalis* was described in 1969; its strains were isolated from animals. Until that time, the genus *Bifidobacterium* included only strains isolated from humans. It was shown that strains of human and animal origin were clearly different in the optimal growth temperature and the pattern of carbohydrate fermentation [27].

Studies by Scardovi, who was the first to use the DNA–DNA hybridization method in the study of bifidobacteria, contributed particularly to the development of taxonomy of the genus *Bifidobacterium*. His research initiated serious attempts to substantiate the taxonomic position of this group of microorganisms on the basis of not only phenotypic but also genotypic characteristics. Taking into consideration the DNA–DNA hybridization data, Scardovi proposed six new species in addition to those described earlier [28]. A detailed scheme for classification and identification of bifidobacteria on the basis of phenotypic characteristics was presented in 1972 in the *Anaerobic Laboratory Manual* [12, 29].

The last of the species of the genus *Bifidobacterium* described to date is *B. psychraerophilum* [16]. The specific features of this microorganism are demonstrated by its species name: it can grow on the surface of solid media under aerobic conditions at 4°C. We can certainly expect the emergence of novel bifidobacterial cultures with atypical and even unusual properties. Molecular methods will play a key role in correct determination of their taxonomic status.

For a long time, identification of bifidobacteria was based on such phenotypic characteristics as cell morphology, the ability to grow at different temperature and pH values, fermentation of different carbohydrates and alcohols, nutritional requirements, etc. However, phenotypic identification of members of the genus *Bifidobacterium* is difficult due to the intraspecies variability of differentiating physiological–biochemical characteristics [30, 31]. In addition, many morphological, cultural, and physiological–biochemical properties of bifi-

dobacteria greatly depend on the composition of the medium, cultivation conditions, culture age, and some other factors [12, 14, 32]. The key characteristics for differentiation of bifidobacterial species are usually the ability to ferment L-arabinose, D-xylose, D-mannose, salicin, D-mannitol, D-sorbitol, and D-melezitose [33]. However, the ability of bifidobacteria to ferment particular carbohydrates is a characteristic rather of a strain than of a species and therefore precise identification of bifidobacterial species based solely on carbohydrate fermentation is highly complicated [5, 30, 31, 34–36]. At the same time, determination of the final products of glucose fermentation by gas–liquid chromatography is a reliable method for differentiation of bifidobacteria from other related genera [37]. Electrophoretic analysis of β-galactosidase isoenzymes makes it possible to differentiate the strains of *B. bifidum, B. breve, B. longum*, and *B. animalis* from other bifidobacterial species [38].

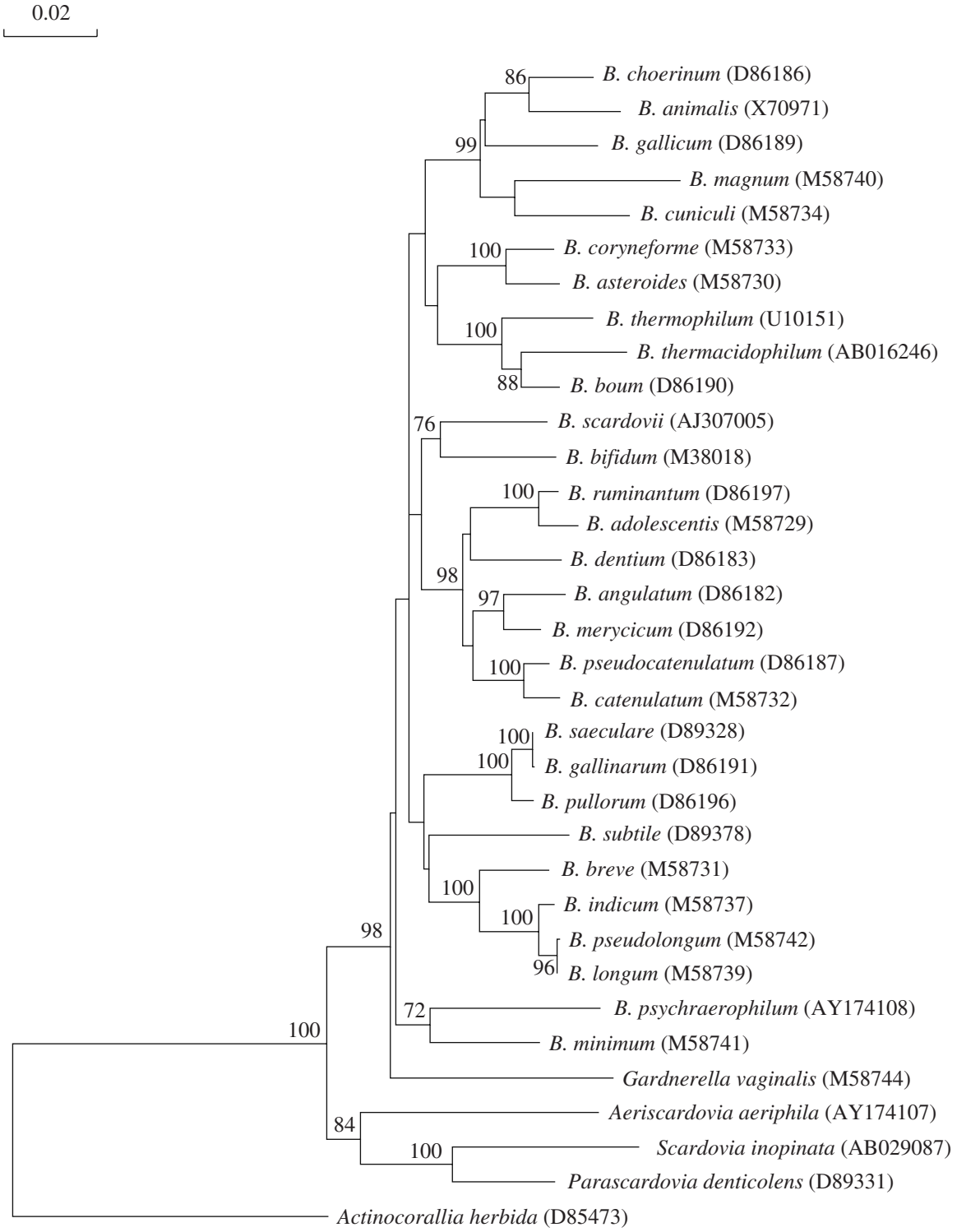
Molecular Methods in Classification of Bifidobacteria

The modern systematics of prokaryotic organisms is phylogenetic, since the hierarchic systems of classification in bacterial and archaeal domains, as well as substantiation of these domains, are primarily based on comparative analysis of nucleotide sequences of the 16S rRNA gene, coding for a molecule conservative in its structure and function [39]. The suprageneric taxonomic structure of bifidobacteria was proposed in 1997 [40] based on the description of clusters formed in the phylogenetic tree of actinobacterial 16S rRNA genes [41]. The genera *Bifidobacterium* and *Gardnerella*, which form a phylogenetic cluster, comprised a single family *Bifidobacteriaceae* of the order *Bifidobacteriales*, included in the class *Actinobacteria* (gram-positive bacteria with a high content of G+C pairs in DNA). The diagnosis of the order *Bifidobacteriales* and family *Bifidobacteriaceae* is based exclusively on signatures, i.e., positions in the 16S rRNA gene sequence that are different in composition from those in representatives of taxa of the same rank. Signatures of the order *Bifidobacteriales* and the family *Bifidobacteriaceae* were claimed to be 18 nucleotide positions located between positions 122 and 1326 of the 16S rRNA gene [40]. The topology of the phylogenetic tree and the composition of signatures differentiating suprageneric taxa may significantly change as a result of the description of new species and genera; however, the suprageneric taxonomic structure of the class *Actinobacteria* has not undergone any revision in the past decade. This "nonbiological" method of distinguishing most suprageneric taxa of bacteria and archaea is actively being discussed, by scientists but is not analyzed in this review.

The figure shows phylogenetic positions of all taxa currently included in the family *Bifidobacteriaceae*. The species *Aeriscardovia aeriphila* (formerly *Bifidobacterium aerophilum*), *Parascardovia denticolens* ("*Bifidobacterium denticolens*"), and S*cardovia inopinata* ("*Bifidobacterium inopinatum*") are separate phylogenetic branches with 16S rRNA similarity levels of about 90%. As a result of separate phylogenetic positions of *B. aerophilum*", "*B. denticolens"*, and "*B. inopinatum"* which follows from the analysis of nucleotide sequences of 16S rRNA genes and *hsp60* genes (coding for heat shock protein HSP60), as well as from the contents of G+C pairs in the DNAs, these species were removed from the genus *Bifidobacterium* and three new genera were described within the family *Bifidobacteriaceae* [16, 42]. At the same time, no significant (generic-level) phenotypic or ecophysiological differences have been revealed between representatives of these three new genera (*Aeriscardovia, Parascardovia, Scardovia*) and *Bifidobacterium.* The problem of congruence of genotypic and phenotypic criteria of taxa differentiation, attempts to solve which have been made in the framework of polyphasic taxonomy, remains extremely relevant for bifidobacteria. The genus *Bifidobacterium* contains several more subclusters with a 16S rRNA similarity level of about 90%; on the basis of their separate phylogenetic positions, they can also be considered potential new taxa of the generic level (see figure). The new taxa established based solely on phylogenetic markers are easily identified on the basis of the same markers, and this is probably the only indisputable argument in favor of the appropriateness of the proposed descriptions of these taxa.

One of the first molecular-genetic approaches that showed the phylogenetic distance of bifidobacteria from the group of lactic-acid bacteria, to which they had been traditionally referred, was determination of the G+C content in DNA. It was found that the G+C content in the DNA of bifidobacteria was 55–67 mol %, which allowed these microorganisms to be considered actinobacteria, whereas the group of lactic-acid bacteria proved to be close to the groups of clostridia and bacilli in their low G+C content (<55%) [43, 44].

The method of DNA–DNA hybridization is the "gold standard" in the differentiation of species-level taxa of bacteria and archaea. It is accepted that strains with the 10–60% level of DNA–DNA hybridization may belong to the same genus and those with the 70–100% level of DNA–DNA hybridization may be considered members of the same species. The 70% level of DNA–DNA hybridization corresponds to approximately 97% similarity in 16S rRNA gene sequences [45]. The method of DNA–DNA hybridization, used in the systematics of bifidobacteria for the first time by Scardovi in 1970, made a significant contribution to the establishment of the genus *Bifidobacterium* as an independent taxonomic unit and to the determination of its species composition [5, 34, 46]. One of the latest taxonomic revisions of the genus *Bifidobacterium* with the use of DNA–DNA hybridization data resulted in reclassification of the species "*B. infantis*" and "*B. suis*" as biotypes of *B. longum* [47]. The Subcommittee for Taxonomy of *Bifidobacterium, Lactobacillus*, and Related Organisms has agreed with reclassification of these



Phylogenetic tree based on the data of 16S rRNA gene analysis and showing positions of 29 species of the genus *Bifidobacterium* and of the monotypic genera *Aeriscardovia, Gardnerella, Parascardovia*, and *Scardovia* within the family *Bifidobacteriaceae.* The species names are followed, in parentheses, by the GenBank accession numbers of the used 16S rRNA gene sequences of the type strains. The tree also presents bootstrap analysis values showing the significance of the branching order. *Actinocorallia herbida* of the order *Bifidobacteriales* was used as an outgroup organism. The scale bar, corresponding to 2 nucleotide substitutions for 100 positions, is in the left upper corner of the figure.

species but is disposed to consider "*B. infantis*" and "*B. suis*" as subspecies but not biotypes of *B. longum* [48].

The study of the 16S rRNA gene sequences was a significant contribution to the understanding of the phylogeny of the genus *Bifidobacterium* and the establishment of its modern taxonomic position. Several years ago, studies of the type strains of bifidobacteria showed that the level of 16S rRNA gene sequence similarity between different *Bifidobacterium* species varies within 93–99% and they form a tight phylogenetic cluster, considerably distant from other genera [41, 49]. The data of the 16S rRNA gene analysis were supported by the results of phylogenetic studies of other, less conservative genes. The topologies of phylogenetic trees constructed on the basis of analysis of the genes *hsp60*, *recA* (coding for the RecA protein), *tuf* (coding for the elongation factor Tu), and the 16S rRNA gene are characterized by a high level of similarity [7, 50, 51].

It is probable that the accumulating data from the analyses of semiconservative phylogenetic markers correlating both with the results of 16S rRNA gene sequence analysis and with data from DNA–DNA hybridization will make it possible to turn to a new gold standard of distinguishing and describing taxa of the species level, namely, to sequencing of several particular genes. The analysis of 7 genes *clpC, dnaB, dnaG, dnaJ1, purF, rpoC, xfp* of the type strains of the genus *Bifidobacterium* performed in 2006 was one of the most interesting studies in this direction [52].

Molecular Methods in the Identification of Bifidobacteria

Molecular-genetic approaches have significantly accelerated the identification process and give more accurate and reliable results as compared with physiological–biochemical testing [5, 6, 31, 53]. A significant advantage of molecular-genetic approaches is their universality: the same methods can be applied to genomes or separate genes of different groups of microorganisms; similar methods are used for the characterization of both cultured (identification ex situ) and uncultured (identification in situ) organisms [54]. Only methods of identification of cultured bifidobacteria are considered in this review. These methods fall into two main groups: sequencing with the aim of identifying particular genes (more often, gene fragments) and obtaining of DNA fingerprints. The methods of obtaining DNA fingerprints are diverse, so that the most optimal of them can be selected for specific research purposes.

The phylogenetic hierarchic classification system for bacteria is based on the data from 16S rRNA gene analysis, and so we will begin considering the methods of identification of bifidobacteria with the approaches that employ differences in the nucleotide sequences of this gene. Correct affiliation of a bifidobacterium to a genus or even to a group of species can be based on sequencing of a 16S rRNA gene fragment approximately 500 nucleotides in length [55]. This is conditioned by the high density and good quality of 16S rRNA data in databases (GenBank, Ribosomal Database Project II, etc.), which contain the 16S rRNA gene sequences for the type strains of nearly all bacterial species described to date, including bifidobacterial. However, in many cases, even determination of the full sequence of this gene (approximately 1550 nucleotides) is insufficient for species affiliation of the tested culture, because the interspecies level of 16S rRNA gene similarity varies within 93–99% [5, 42, 53] and many bifidobacterial species are phylogenetically close to each other (see figure).

The data on the 16S rRNA gene sequences are used for development of taxon-specific primers (Table 1). Several genus-, group-, and species-specific primers have been found for bifidobacteria [36, 56–59], and primers Bflact2–Bflact5 are suitable for exact identification of *B. animalis* subsp. *lactis* [60]. Amplification with the genus-, group-, and species-specific primers in some cases may be performed concurrently in the same reaction mixture (the so-called multiplex PCR) [60].

Another approach employing the differences in the 16S rRNA gene sequences is Amplified Ribosomal DNA Restriction Analysis (ARDRA). The 16S rRNA gene is almost completely amplified with universal bacterial primers. The resulting amplicon is processed with a restriction endonuclease (usually, with a restriction endonuclease with recognition sites of 4–5 nucleotides), and the restriction products are separated by electrophoresis in agarose gel. This method has a lower resolution than sequencing of 16S rRNA gene fragments; however, ARDRA is an inexpensive and simple method for group and, in some cases, species identification of bifidobacteria [61, 62]. ARDRA with the enzymes *Bam*HI, *Sau*3AI, and *Taq*I makes it possible to differentiate the species *B. animalis, B. breve, B. bifidum,* and *B. adolescentis* [9].

Ribotyping method is also based on the analysis of 16S rRNA genes but employs not the differences in their nucleotide sequences, but different locations of these genes on a chromosome in different strains. Total DNA is treated by a restriction endonuclease; the restriction products are separated by electrophoresis in agarose gel and hybridized (Southern-hybridization) with the *rrnB* rRNA operon of *Escherichia coli*. The advantage of this method is the high degree of standardization and automation in obtaining fingerprints, since it is performed with a specially designed RiboPrinter device (Qualicon, United States), whereas its disadvantage is a still very limited database of the fingerprints of type and reference cultures. The ribotyping data available for bifidobacteria show the high resolution power of this method at the strain level but also the absence of correlation with the species affiliation of strains [63].

In the early 1990s it was proposed to identify bacteria by analyzing the nucleotide sequence of the intergenic 16S–23S rRNA region, or the so-called internal

Primers based on the 16S rRNA gene sequence for identification of suprageneric and intrageneric taxa of the genus *Bifidobacterium*

Taxon	Characteristics of primers	Reference
Class *Actinobacteria*	Several primers were designed; the most promising is the following pair: S-C-Act–235_a-S-20 (CGCGGCCTATCAGCTTGTTG) S-C-Act-878_a-A-19 (CCGTACTCCCCAGGCGGGG)	[56]
Order *Bifidobacteriales*	Not designed	
Family *Bifidobacteriaceae*	Not designed	
Genus *Bifidobacterium*	Several genus-specific primers were designed; the most promising is the following pair: Lm26 (GATTCTGGCTCAGGATGAACG) Lm3 (CGGGTGCTNCCCACTTTCATG)	[58]
Group of species	Group-specific primers were designed for the group of species close to *B. catenulatum* and the group of species close to *B. longum*	[35]
Species	Primers were designed for about 10 of the 29 species of the genus *Bifidobacterium.* For other species, design of species-specific primers based on the 16S rRNA gene is impossible due to its conservative nature	[59]
Subspecies	There is only one example of using primers based on the 16S rRNA gene for identification of subspecies *B. animalis* subsp. *lactis*: Bflact2 (GTGGAGACACGGTTTCCC) Bflact5 (CACACCACACAATCCAATAC). Reverse primer Bflact5 is located not on the 16S rRNA gene but on the 16S–23S intergenic region. Wide application of primers based on the 16S rRNA gene for intraspecies identification is impossible due to conservative nature of this gene.	[60]

transcribed spacer region (ITS region) [64]. This region is much more variable than the 16S or 23S rRNA genes, it can be entirely amplified with universal primers for the adjacent regions of the 16S and 23S rRNA genes, and its analysis has been successfully used for identification and differentiation of closely related bacteria from different taxa. The sequencing of the intergenic 16S–23S rRNA region is now one of the most accurate and reliable approaches for species identification of bifidobacteria [5, 41, 65].

Several researchers have shown that the data from the sequencing of some genes that are less conservative than the 16S rRNA gene also provide efficient species identification of bifidobacteria. The nucleotide sequence of a *recA* gene fragment approximately 300 nucleotides in length has proved to be sufficiently informative for identification of most species of the genus *Bifidobacterium* and allows differentiation of closely related organisms such as *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis* [66, 67]. Analogous results have been obtained from sequencing of the *hsp60* gene; over 50 strains of different *Bifidobacterium* species were exactly identified at the species and even intraspecies level [7, 34, 50]. Another gene promising for the species identification of bifidobacteria is that of transaldolase. Bifidobacteria have been shown to produce at least 14 different types of transaldolases varying in their electrophoretic mobility, amino acid sequence and, accordingly, nucleotide sequence of the encoding genes [68]. The nucleotide sequence of the transaldolase gene provides clear-cut differentiation of the species *B. catenulatum* and *B. pseudocatenulatum*, indistinguishable by the analysis of the 16S rRNA gene sequence; however, the species *B. catenulatum* and *B. angulatum*, which are clearly differentiated by the data of 16S rRNA gene analysis, are not distinguishable. Thus, the analysis of the transaldolase gene sequence can be used as an additional criterion for species identification of bifidobacteria [68]. However, despite successful application of the data from analysis of the nucleotide sequences of certain genes for species identification of bifidobacteria, the development of species-specific primers on the basis of these genes is difficult or impossible due to the absence of sufficiently long (15–20 nucleotides) conserved regions.

DNA fingerprints are a second group of methods successfully used for identification of bifidobacteria. The advantage of this technique is the possibility of analyzing a great number of isolates with relatively low financial and time expenditures [69]. Limitations of their use are associated with the low level of automation and interlaboratory standardization of protocols and, as a consequence, the absence of appropriate public databases allowing quick comparison of the fingerprints obtained. DNA fingerprints are obtained by restriction

analysis of DNA or by PCR (so-called PCR fingerprints).

One of the most sensitive and efficient methods of identification of bifidobacteria on the basis of DNA fingerprints is Pulsed-Field Gel Electrophoresis (PFGE). In short, a bacterial culture is lysed in agarose block, and high-molecular DNA is treated with a rare-cutting restriction endonuclease that recognizes a region of 6–8 bp. Then, the restriction products (DNA fragments of 10–80 thousand bp) are exposed to PFGE. The PFGE method is considered to be the gold standard of the typing of strains, since the fingerprints are well reproduced, reflect the specific features of genome as a whole, and are characteristic of particular strains. A complex study of a large sampling of bifidobacterial strains where PFGE was used along with 16S rRNA gene sequencing, DNA–DNA hybridization, electrophoresis of total cell proteins, and RAPD showed not only strain but also species specificity of PFGE fingerprints [70].

Randomly Amplified Polymorphic DNA (RAPD) is a method comparable to PFGE in its resolution power [71]. The most widespread of the several RAPD variants envisages PCR with only one primer with a random sequence of 9–11 nucleotides in length. The optimal primer cannot be constructed in silico and is chosen from a series of analogous primers by testing. Primers that give more amplification products and, accordingly, more bands on the fingerprints are usually chosen. Thus, in one of the studies on employment of RAPD for identification of bifidobacteria, only seven 10-nucleotide primers were selected out of the 80 tested [72]. The disadvantage of RAPD is poor reproducibility of fingerprints. RAPD needs strict standardization of PCR conditions, because the use of different polymerases or DNA/primer ratios or different annealing temperatures may lead to a discrepancy in the results obtained at different laboratories or in different time periods with the same samplings of strains.

PCR fingerprinting includes the group of rep-PCR (repetitive DNA element PCR) techniques: ERIC-PCR, REP-PCR, BOX-PCR, $(GTG)_5$-PCR, and some others. Their goal is to analyze repeated conservative sequences, 30–40 to about 150 nucleotides in length, which are present in numerous copies in the genomes of most gram-positive and gram-negative bacteria. Several studies have shown that the location of these sequences in microbial genomes is strain-specific.

One of these methods, ERIC-PCR, was tested with 89 strains representing 26 species of the genus *Bifidobacterium*. ERIC fingerprints were unique for each species but similar for closely related species, e.g., for *B. catenulatum* and *B. pseudocatenulatum* [73]. Comparison of several rep-PCR methods with the employment of 128 type, reference, and freshly isolated bifidobacterial cultures has shown that BOX-PCR with the BOXA1R primer is the most promising method for identification of bifidobacteria at the species, subspecies, and even strain levels [74].

DNA probes can also be used for generic and species identification of bifidobacteria [58, 75]; however, in the recent years, this approach has been used more and more rarely for identification of microbial cultures and more and more often for their detection directly in the environment [5, 8, 76].

In our opinion, already in the nearest future, the classification and identification of bifidobacteria, as well as of other groups of bacteria and archaea, will be based mainly on the data from the sequencing of several particular genes, obligatorily including the 16S rRNA gene. DNA fingerprints as a whole will gradually lose their significance for identification; however, such techniques as PFGE, which permit differentiation of strains at the infrasubspecies level, will long remain highly competitive with methods based on DNA sequencing.

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