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Review

Review of molecular methods for identification, characterization and detection of bifidobacteria

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Abstract – Final identification of bifidobacteria based on phenotypic patterns (carbohydrate fermentation and enzymatic activity) can be difficult. The distinction between different species or strains of bifidobacteria such as *Bifidobacterium longum* and *Bifidobacterium infantis* or *Bifidobacterium animalis* and *Bifidobacterium lactis* is not reliable with the phenotypic identification. DNA-DNA hybridization, ribotyping, hybridization with a specific probe and sequence analysis of 16S rDNA were the first molecular methods used to identify bifidobacteria isolated from commercial products and the gastrointestinal tract. Now, a new group of molecular methods exist for the genus, species and strains identification. The PCR, multiplex PCR, amplified ribosomal DNA restriction analysis (ARDRA), sequencing of specific genes (*rec* A*, ldh, hsp* 60 and pyruvate kinase) and denaturing gradient gel electrophoresis (DGGE) are used for the detection, characterization and genus or species identification. Other molecular methods such as pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD) and the rep-PCR are used for typing strains of bifidobacteria. Real-time PCR or Q-PCR will be soon an interesting tool for the detection, identification and quantification of bifidobacteria in different samples and commercial products. With new molecular techniques it is easier now to have reliable identification, typing and quantification of bacteria such as *Bifidobacterium* spp.

human bifidobacteria / *Bifidobacterium* / molecular methods / identification / characterization / detection

Résumé – Revue des méthodes de biologie moléculaire pour la détection, l'identification et la caractérisation des bifidobactéries d'origine humaine et commerciale. Une identification finale basée uniquement sur des tests phénotypiques (fermentation des sucres et activité enzymatique) peut être difficile et la distinction entre certaines espèces comme *Bifidobacterium longum* et *Bifidobacterium infantis* ou *Bifidobacterium animalis* et *Bifidobacterium lactis* est pratiquement impossible. L'hybridation ADN-ADN, les profils polymorphiques de restriction (RFLP), l'hybridation avec des sondes spécifiques et le séquençage de l'ADNr16S étaient les premières méthodes moléculaires utilisées pour identifier les bifidobactéries. La PCR, la PCR-multiplex, l'analyse de profils de restriction des ADNr16S (ARDRA), le séquençage de gènes spécifique (*rec* A, *ldh*, *hsp* 60 et pyruvate kinase) et l'électrophorèse sur gel avec gradient dénaturant (DGGE) sont maintenant utilisés pour la détection, la caractérisation et l'identification au genre et à l'espèce. D'autres méthodes moléculaires comme l'électrophorèse sur gel en champ pulsé (PFGE), l'amplification aléatoire de l'ADN polymorphe (RAPD), et la rep-PCR peuvent être utilisées pour caractériser à la souche les bifidobactéries. La PCR en temps réel sera prochainement un outil intéressant et performant pour la détection,

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l'identification et la quantification des bifidobactéries dans différents types d'échantillons ou de produits commerciaux. Avec les nouvelles techniques moléculaires il est maintenant plus facile d'avoir une identification, une caractérisation et une quantification fiable des souches de *Bifidobacterium*.

bifidobactéries d'origine humaine / *Bifidobacterium* / méthodes moléculaires / détection / identification / caractérisation

1. INTRODUCTION

Bifidobacterium spp. constitute an important class of organisms in the intestinal microflora of healthy children and adults. Among the thirty bifidobacteria species, eleven have been isolated from human until now (Tab. I). There is an evolution in the intestinal microflora, the microorganisms including *Bifidobacterium* species change with the people's age [3, 29]. The most frequent *Bifidobacterium* species isolated in infants fecal sample are *B. bifidum, B. longum, B. infantis* and *B. breve*. In adults these species are *B. adolescentis, B. longum, B. bifidum* and *B. catenulatum/B. pseudocatenulatum* [25, 26, 45].

It is known that *Bifidobacterium* can have some beneficial effects on human health. These principal effects are: (1) stimulation of immune response, (2) reduction of growth of many potential pathogens and putrefactive bacteria, (3) prevention of constipation, diarrhea and other intestinal infections, (4) improvement of lactose-tolerance and (5) *Bifidobacterium* spp. could be used to restore the intestinal microflora after an antibiotic treatment [24, 29, 33].

The probiotic effect of *Bifidobacterium* explains the popularity of these bacteria in different commercial products. *Bifidobacterium* species frequently used in probiotic products are *B. longum, B. breve, B. bifidum, B. infantis* and *B. lactis* [26]. Yogurt, sour cream, cheese desserts, ice cream and powdered milk are some dairy products that sometimes contain *Bifidobacterium* [13]. The majority of strains isolated from dairy products were identified as *B. animalis* and were closely related ATCC 27536 [38]. Finally, some strains of *B. breve, B. infantis* and *B. longum* are included in VSL-3 [2], this product is used in the treatment of inflammatory bowel diseases.

Table I. Bifidobacterial species isolated in human and species used in commercial products.

Species isolated in human	Species used in commercial products
B. adolescentis	*B. animalis* [b]
B. angulatum	*B. bifidum*
B. bifidum	*B. breve*
B. breve	*B. infantis* [a]
B. catenulatum	*B. lactis* [b]
B. dentium	*B. longum* [a]
B. gallicum	
B. infantis [a]	
B. longum [a]	
B. pseudocatenulatum	
B. scardovii	

[a] Unification of *Bifidobacterium infantis* and *Bifidobacterium suis* as *Bifidobacterium longum*, this latter species can be divided into three biovars, namely the Infantis biovar, the Longum biovar and the Suis biovar [38].
[b] Reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* subsp. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp. nov. [23].

A right identification of these microorganisms is important for the producers of probiotic products. They have needed to select some bacteria which are usually present in human intestine and these bacteria must be also non-pathogenic and not have adverse effects of any sort. In the majority of laboratories, the phenotypic tests are the principal tools for identification of bacteria. Carbohydrate fermentation, cell wall analysis and detection of specific enzymes are generally used. But, these identification methods require a large amount of time and do not always give clear results [10, 14, 26]. For example, the distinction between some species currently used in commercial products such as *B. infantis* and *B. longum,* or *B. animalis* and *B. lactis* is difficult when phenotypic tests are only used [23, 37, 38]. To help microbiologists, researchers and producers to do the right identification and characterization of bacteria including *Bifidobacterium* some molecular methods have been developed. A review of these methods will be described in this paper.

2. THE FIRST MOLECULAR METHODS USED

The first molecular identification methods developed were DNA-DNA hybridization, sequence analysis of 16S rDNA, hybridization with a specific probe and RFLP analysis or ribotyping. In contrast to physiological and biochemical characteristics, the molecular identification is based on the constitutive composition of nucleic acids rather than on the products of their expression. These molecular methods are often used in association with the conventional microbiological identification. With DNA-DNA hybridization, percent homology between the test strain and the reference strain can be determined in using membrane filter for the DNA fixation and radioisotopes for detection [4, 41], or using microplate and photobiotin [6, 40, 52]. When the percentage of homology is higher than 70% between the isolated and the reference strain, and the phenotypic criteria agree with the species definition, these strains can be grouped together in the same species [14, 16, 51]. Homology between *B. longum* and *B. infantis* is often near 70% [14] and with *B. animalis* and *B. lactis* this homology is higher than 80% between the type strain of *B. lactis* and *B. animalis* strains [26]. These results confirm the similarity between *B. longum* and *B. infantis* and the confusion with *B. lactis* species.

The 16S rRNA gene is considered to be universally present in bacteria and shows a high degree of sequence conservation. The sequence homology analysis of this gene demonstrates some interesting results for the phylogenetic analysis of the genus *Bifidobacterium* [8, 21]. However, sequence similarities are very high with some species groups: *B. catenulatum* and *B. pseudocatenulatum* group (similarity 99.5%), *B. longum* and *B. infantis* group (similarity 99.1%), *B. lactis* and *B. animalis* group (similarity 99%) [28, 30]. Recently, the unification of *B. infantis, B. longum* and *B. suis* divided in three biotypes and the separation of *B. lactis* from *B. animalis* at the subspecies level have been proposed [23, 39, 46].

For the hybridization with a specific probe, the nucleic acid is fixed on a solid support, nitrocellulose or nylon membrane, by dot blot or colony hybridization [15, 17]. The probe can be a single oligonucleotide or cloned and characterized DNA fragment, labeled with biotin or digoxigenin to produce a colorimetric reaction [17] or radiolabeled [15, 53]. With the radiolabeled probes the amount of hybrid formed is determined by autoradiography. Fluorescence in-situ hybridization (FISH) on a microscopic slide was also used to detect and to determine the population of *Bifidobacterium* spp. in different samples [12, 20]. Some genus-specific and species-specific probes for *B. adolescentis, B. pseudocatenulatum, B. bifidum, B. breve, B. animalis*, and *B. longum* were proposed [3, 15, 53].

Ribotyping or restriction fragment length polymorphism (RFLP) of genes coding for rRNA uses a labeled probe containing 16S or 23S or both 16 and 23 ribosomal cDNA. Before hybridization the DNA is digested with *Bam*HI, *Eco*RV, *Pvu*II or *Nar*I [22, 27] and transferred on a membrane by Southern blot. The hybridization patterns or ribotypes produced by hybridization of probe to different fragments of DNA digested allow to do the differentiation between the following

species *B. adolescentis, B. animalis, B. bifidum, B. breve, B. infantis* and *B. longum* [27].

3. THE NEW GENERATION OF MOLECULAR METHODS

The PCR discovery, the new cloning, sequencing and fluorescence detection technology and the accessibility of a large quantity of information on the web helped the development of new molecular tools. The second generation of molecular methods for genus and species detection and identification are polymerase chain reaction (PCR), multiplex-PCR, sequencing of specific genes, amplified ribosomal DNA restriction analysis and denaturing gradient gel electrophoresis (DGGE).

3.1. Polymerase chain reaction (PCR)

The PCR is a fast, accurate, sensitive and easy operating technique. The amplification of bifidobacterial DNA directly extracted from a colony or a faecal sample, without prior culture, can be done [18, 31]. PCR inhibitors from faecal sample could be removed with some washing steps with buffers before breaking the bacterial cells. Instead of faecal samples, dairy products and bacterial culture can be used [26]. Bile salts and complex polysaccharides can also inhibit or reduce the amplification efficiency [2]. Another advantage of this technique for *Bifidobacterium* detection is that the PCR does not require anaerobic conditions compared to the classical culture method [26]. The amplification of a specific DNA fragments or a complete gene can be done with the PCR. The specificity of this technique is directly associated with the primers selection and the primers annealing temperature. Some primer pairs based on 16S rDNA sequences or on 16S to 23S internal transcribed spacer sequences, are selected for the detection of *Bifidobacterium* genus and for the principal human species (*B. bifidum, B. breve, B. infantis, B. longum*, *B. adolescentis, B. angulatum, B. catenulatum* group, *B. dentium,* and *B. gallicum*) [24, 25, 38, 43, 50] and strains used in commercial products (*B. lactis* and *B. animalis*) [35, 47].

3.2. Multiplex PCR

Multiplex PCR is similar to the conventional PCR. Many primer pairs are used in the same reaction at the same time, to the detection of many bacterial genus or different species. Others advantages of this technique are the reduction of the number of PCR reactions and the reduction of the time spent. With this method it's very important to keep in mind the following recommendations: (1) annealing primer temperature for all primers are needed to be in the same range; (2) primers used could not give any cross-reaction under multiplex conditions; (3) different PCR product sizes are necessary to be able to do the distinction between the different species or bacteria; and (4) this technique needed a larger amount of Taq polymerase than is used in conventional PCR [26]. Different multiplex have been designed for simultaneously detection of (1) *B. bifidum*, *B. breve*, *B. infantis*, *B. longum*, *B. adolescentis* [5], (2) *B. bifidum, B. breve, B. infantis*, (3) *B. angulatum, B. catenulatum/B. pseudocatenulatum* continuum, *B. dentium*, *B. longum* and (4) *B. adolescentis, B. scardovii, B. gallicum* [31]. The multiplex 1 was designed with specific primers based on 16S to 23S internal spacer sequences [5]. Some cross-reaction of *B. suis* with both *B. infantis* and *B. longum* primers based on 16S rDNA used in multiplex 2 and 3 have been observed [31].

3.3. Sequences of specific genes

Sequences of specific genes can be used for identification and characterization of *Bifidobacterium*. It's known that the DNA sequences of protein-coding genes are more effective than 16S rRNA gene sequencing for the characterization of *Bifidobacterium* species [16, 19, 37]. Some genes other than the 16S rRNA are used for the differentiation of bifidobacteria. These genes are: L-lactate deshydrogenase gene (*ldh*), *recA* gene, 60 $kg \cdot mol^{-1}$ heat shock protein (HSP60) gene, Pyruvate kinase (PK) gene [16, 19, 37, 44]. Before sequencing, a part

of gene is selected and amplified by PCR. The PCR product is sequenced, analyzed and compared with other sequences. With a short region of the *rec*A (231 bp) and *ldh* (312 bp) gene it is possible to distinguish between the principal human species *B. bifidum, B. infantis, B. longum, B. adolescentis, B. breve* and *B. animalis* strains frequently used in commercial products [19, 37]. Also, the analysis of a region of the *ldh* gene showed that nucleotide sequence of *B. lactis* DSM 10140 and *B. animalis* ATCC 27536 were identical but different between *B. lactis* DSM 10140 and *B. animalis* ATCC 25527^T [37]. Partial pyruvate kinase gene (300 bp) allows differentiating *B. infantis* from *B. longum* and also *B. animalis* from *B. lactis* [44]. Finally, analysis of partial *hsp*60 gene (538 bp) sequences is very effective for the differentiation between all human *Bifidobacterium* species. Sequence similarity is 93% between *B. catenulatum* and *B. pseudocatenulatum*, 98% between *B. longum*, *B. infantis* and *B. suis* and 98% between *B. an*imalis and *B. lactis* [16]. The *hsp*60 gene is a powerful tool for the phylogenetic study of *Bifidobacterium* species, this gene has a power higher than the 16S rRNA gene. So, the differentiation of the principal human bifidobacteria species after sequencing and alignment of a short sequence of a specific gene is possible. And the differentiation between *B. lactis* and *B. animalis* can be done with *hsp*60 and pyruvate kinase gene [16, 44].

3.4. Amplified ribosomal DNA restriction analysis (ARDRA)

Bacterial culture and DNA isolation are needed with the amplified ribosomal DNA restriction analysis (ARDRA) technique. The DNA is used to the PCR amplification of the totality or only a region of the 16S rRNA gene. This amplification is followed by a restriction digestion of the PCR products. The selection of restriction enzymes is important to have a clear distinction in ARDRA pattern to differentiate the larger amount of species. The digestion products are visualised under UV-light after agarose gel electrophoresis and ethidium bromide staining. Analysis and comparison of more than one restriction profile can be necessary to have a differentiation between some close species. A first study by Roy and Sirois [37], demonstrate the differentiation between *B. animalis, B. longum, B. infantis, B. breve, B. bifidum* and *B. adolescentis* after an amplification of 914 bp region of the 16S rRNA gene and restriction with *Bam*HI, *Sau*3AI and *Taq*I. The restriction pattern from *B. lactis* strains was identical to that of the *B. animalis* strains. *B. longum* was closely related to *B. infantis* but differentiation between these two species was obtained with the *Sau*3A1 restriction enzyme. Some similar results were obtained by Ventura et al. [48] after the restriction of the complete 16S rRNA gene with *Bam*HI and *Sau*3AI. However, this team demonstrated the possibility to also differentiate *B. catenulatum* and *B. pseudocatenulatum* with these patterns. Another study by Venema and Maathuis [45] showed the differentiation between all the *Bifidobacterium* species found in human alimentary tract, including *B. lactis* and *B. animalis*. This differentiation was done with a combination of six restriction patterns from a 511 to 525 bp 16S ribosomal gene fragment with the following enzymes *Taq*I, *Sau*3AI, *Rsa*I, *Alu*I, *Sau*96I and *Nci*I. ARDRA system is a good and reproducible molecular identification tool for human *Bifidobacterium* species. With this method the differentiation between *B. infantis* and *B. longum* or between *B. catenulatum* and *B. pseudocatenulatum* is possible [37, 45, 48]. *Nci*I enzyme is used to do the distinction between *B. animalis* and *B. lactis* [45].

3.5. Denaturing gradient gel electrophoresis (DGGE)

Denaturating gradient gel electrophoresis is based on the discontinuous phenomenon of strand dissociation, allows the resolution of DNA fragments differing by as little as a single nucleotide substitution. The principal difficulties with the DGGE are to choose the right running time and gel conditions to achieve the optimal separation. For DGGE analysis of PCR products a GC clamp is attached to the 5' end to either of the primers. But, the efficiency of the separation

can be different if the GC clamp is attached to the forward or the reverse primer [40]. Also, some closely related species like *B. infantis* and *B. longum* or *B. catenulatum* and *B. pseudocatenulatum* could not be separated from each other by this approach and the primer Bif164-f and Bif662-r specific for a 16S rDNA region [40]. The PCR amplification of the V2-V3 regions of the 16S rDNA or transaldolase DNA fragment followed by DGGE allow the separation of *B. animalis, B. lactis, B. bifidum, B. infantis, B. longum, B. adolescentis, B. pseudocatenulatum* and *B. breve* [7, 36]. In the PCR-DGGE approach identification of fragment can be confirmed by subsequent cloning and sequencing of the PCR products. This method is popular to evaluate the bacterial composition in fecal samples [37, 40] and can be an interesting and rapid method for the screening of the bifidobacterial composition of probiotic products [7].

4. MOLECULAR METHODS FOR SPECIES OR STRAINS IDENTIFICATION AND CHARACTERIZATION

These tools are very interesting to identify and characterize *Bifidobacterium* strains in commercial products and in the intestinal tract. These typing methods are principally: Random Amplified Polymorphic DNA (RAPD), Pulsed-Field Gel Electrophoresis (PFGE) and rep-PCR. Strains differentiation is possible with these techniques, but, it is impossible to be sure that some strains are the same only based on identical pattern, if the history of strains is unknown.

4.1. Random Amplified Polymorphic DNA (RAPD)

The Random Amplified Polymorphic DNA (RAPD) is frequently used to characterize probiotic bacteria. With this technique, the entire genome extracted from a bacterial culture is used to generate the DNA profile after PCR amplification with random primers [9, 39, 49]. These primers are often 10 nucleotides and only one is used in each reaction. The different size amplicons were visualised under UV-light after agarose gel electrophoresis and ethidium bromide staining. RAPD profiles can be combined and analysed with appropriate software. The advantage of RAPD is that it allows to distinguish strains within species that cannot be differentiated by ARDRA. Vincent et al. [49] used OPA-02, OPA-18, OPL-07, OPL-16 and OPM-05 primers to group in for different clusters *B. breve, B. adolescentis, B. bifidum* and *B. animalis*. Strains of *B. infantis* were grouped into a subcluster of *B. longum*. The similar results were obtained by Sakata et al. [39] with *B. infantis*, *B. longum* and *B. suis*. The comparison of RAPD profiles can also be used to recover a specific strain in different samples. Variation between species and strains could be identified with the number and size of DNA fragments. Sometimes the reproducibility of this technique is low and the utilization of control conditions and meticulous handling is needed. Finally, RAPD profiles may be altered by the presence of transposable elements or plasmids [49].

4.2. Pulsed-Field Gel Electrophoresis (PFGE)

A good reproducibility and efficient differentiation between *Bifidobacterium* strains is observed with this electrophoresis method. Compared to the others molecular techniques, the pulsed-field gel electrophoresis (PFGE) require a large amount of time and genomic DNA extraction in agarose block is necessary. This extraction allows to keep the DNA intact before the digestion. Rare cutting restriction enzymes could be selected to obtain a DNA profile containing 10 to 20 fragments. Periodical change in the electrical field orientation during 18 to 24 h migration allows the separation of large DNA fragments. With *Bifidobacterium* strains *Xba*I, *Spe*I, *Dra*I and *Ase*I are four enzymes generating selective patterns [1, 11, 32, 35, 38]. PFGE profiles were obtained with some commercial [11, 38] and reference strains of *B. animalis*, *B. infantis*, *B. longum*, *B. bifidum, B. adolescentis, B. angulatum, B. catenulatum, B. pseudocatenulatum, B. lactis* and *B. breve* [1, 32, 35, 38]. These profiles are generally different

between the different strains. However, *B. animalis* ATCC 25527 and ATCC 27674 have the same PFGE pattern after digestion with *Xba*I and *Spe*I [38], *B. animalis* ATCC 27536 and *B. lactis* DSM 10140 have also the same profile. The majority of *Bifidobacterium* strains isolated from European commercial preparations cannot be distinguished from *B. animalis* ATCC 27536, previously isolated from chicken feces [38]. PFGE can also be used to estimate the genome size. Some big variations were observed in genome size of some strains belonging to the same species. For example with *B. bifidum* the estimated size after digestion with *Xba*I were between 1.6 to 2.2 Mb [38]. Estimated *Bifidobacterium* genome size ranged between 1.1 to 2.2 Mb [1, 32, 38].

4.3. rep-PCR

Another method to obtain genomic DNA fingerprinting of bifidobacteria is the rep-PCR. This PCR uses primers to match short consensus repetitive sequences. Three different primers can be used namely BOX (originally described in *Streptococcus pneumoniae*). ERIC (originally described in *Salmonella typhimurium*) and REP (originally described in *Escherichia coli*) [10]. Differences in band sizes represent polymorphisms in the distance between the repetitive elements of different strains. Presently some study using BOX and ERIC primers for the characterization and differentiation of *Bifidobacterium* strains have been published [10, 42, 46]. With ERIC primer, it is possible to distinguish five different species of bifidobacteria *(B. pseudocatenulatum, B. infantis, B. longum, B. animalis* and *B. indicum*) [42]. This technique is also applicable to differentiate different strains in a same species. Furthermore, ERIC-PCR demonstrated that some strains of *B. lactis* and *B. animalis* (ATCC 27536, ATCC 27673 and ATCC 27674) were not comparable to any other *B. animalis* strains [46]. These observations confirm the results obtained by Roy et al. [37] with the sequences of *ldh* gene and the PFGE patterns. The differentiation of *B. breve, B. bifidum, B. longum, B. infantis* and *B. adolescentis* can also be done with BOX-PCR [10].

5. FUTURE PERSPECTIVES

Real-time PCR or Quantitative-PCR is effective for the detection and quantification of microorganisms in the same reaction. It is more sensitive than the conventional PCR and the amplification of PCR product is detected at each cycle. With the Real-Time PCR the fluorescence is used for the detection of PCR product. SYBR Green and fluorescing probes like *Taqman* probe are presently the most popular tools for the detection in real-time PCR. The fluorescing probes are more specific than the SYBR Green and with these probes it is possible to perform multiplex reaction. However, some specific conditions, like G-C percent, melting temperature, probe length, etc., must be respected for the construction of probes. In the reaction with SYBR Green the primers are mixed with the dye, while the DNA is extended by DNA polymerase, the SYBR Green is incorporated between the double-stranded DNA and the amplicon is detected. The fluorescence is a thousand times higher when the SYBR Green is fixed to the DNA compared to the free molecules in PCR reaction solution. With the *Taqman* probe, there is at each extremity of the probe a fluorochrome and a suppressor (quencher). In this PCR reaction, the primers and the probe fix the target and during the DNA extension the Taq DNA polymerase hydrolyse the probe, the fluorochrome (reporter) is separated to the suppressor or the quencher and there is light emission. Molecular Beacons probes and scorpion primer will be the next detection tools in real time PCR [34].

Presently the Real-Time PCR targeting the transaldolase gene can be used to detect and enumerate bifidobacteria in fecal samples [37]. Comparison of bifidobacterial count obtained by culture and real-time PCR showed a good correlation [26, 36]. This technique could be used to detect different *Bifidobacterium* species with some probes based on *hsp*60 gene and could also be used in multiplex to the detection of some *Bifidobacterium* species simultaneously in the same product.

Amplified restriction fragment polymorphism (AFLP) could be also an interesting technique for the characterization of

bifidobacteria. This technique is based on the selective amplification of restriction fragments from a total digest of genomic DNA. AFLP combines two strategies generally used in DNA fingerprinting: (1) the hybridization-based fingerprinting involving the cutting of genomic DNA with restriction endonuclease and (2) the PCR-based fingerprinting involving the amplification of particular DNA sequences using specific or arbitrary primers. The AFLP is highly sensitive and considered, along with the PFGE, as the most discriminating genotypic technique [23].

6. CONCLUSION

There are many molecular methods for the identification, characterization and detection of *Bifidobacterium* and many of these techniques are based on the 16S ribosomal gene. PCR and ARDRA are two easy and sensitive methods for the detection and identification of *Bifidobacterium* genus and species. The most accurate method for differentiation at the strain level is the PFGE, but it is impossible to be sure that some strains are the same only with an identical pattern. The distinction between some closely related *Bifidobacterium* species like *B. infantis* and *B. longum*, *B. catenulatum* and *B. pseudocatenulatum* or *B. lactis* and *B. animalis* is possible with some molecular tools. But, the unification of *B. infantis* *B. longum* and *B. suis* divided in three biotypes and the separation of *B. lactis* from *B. animalis* at the subspecies level are proposed [23, 39, 46]. And finally, in the future Real-Time PCR will be very popular for the detection, identification and quantification of *Bifidobacterium* spp.

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