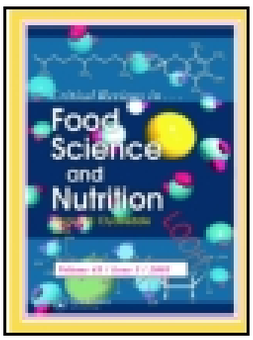

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# Contemporary nucleic acid-based molecular techniques for detection, identification, and characterization of *Bifidobacterium*

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**Abstract**

Bifidobacteria are one of the most important bacterial groups found in the gastrointestinal tract of humans. Medical and food industry researchers have focused on bifidobacteria because of their health-promoting properties. Researchers have historically relied on classic phenotypic approaches (culture and biochemical tests) for detection and identification of bifidobacteria. Those approaches still have values for the identification and detection of some bifidobacterial species, but they are often labor-intensive and time-consuming and can be problematic in differentiating closely related species. Rapid, accurate and reliable methods for detection, identification and characterization of bifidobacteria in a mixed bacterial population have become a major challenge. The advent of nucleic acid-based molecular techniques has significantly

 

advanced isolation and detection of bifidobacteria. Diverse nucleic acid-based molecular techniques have been employed, including hybridization, target amplification and fingerprinting. Certain techniques enable the detection, characterization and identification at genus-, species- and strains-level, whereas others allow typing of species or strains of bifidobacteria. In this review, an overview of methodological principle, technique complexity and application of various nucleic acid-based molecular techniques for detection, identification and characterization of bifidobacteria is presented. Advantages and limitations of each technique are discussed, and significant findings based on particular techniques are also highlighted.

**Keywords**

*Bifidobacterium*; nucleic acid; molecular techniques; hybridization; amplification; molecular fingerprinting;

## 1. Introduction

The genus *Bifidobacterium* belongs to phylum *Actinobacteria*, in which the species have irregular morphologies and appear as curved, rod-shaped, or branched (Madigan et al., 2003). Bifidobacteria are Gram positive, non-motile, and non spore-forming anaerobes, with few species capable of adapting in atmospheres containing 10% $CO_2$ (Perry and Staley, 1997). Several species from this genus constitute one of the major groups of probiotic bacteria that provide health-promoting functions to host when sufficient amount of viable cells are ingested (Fontana et al., 2013). With deeper study in the microflora of human gastrointestinal tract, probiotic lactic acid bacteria (LAB) and *Bifidobacterium* have gained considerable interests in food, pharmaceutical industries, and medical science (Turroni et al., 2013). Probiotic LAB and *Bifidobacterium* can survive inside the human gastrointestinal tract, and balance the intestinal microflora (Guarner and Malagelada, 2003; Ritchie and Romanuk, 2012). Several studies have shown the potential health benefits of *Bifidobacterium* to humans, such as treatmentment of constipation (Miller and Ouwehand, 2013), treatment of irritable bowel syndrome (Ortiz-Lucas et al., 2013), eradication of *Helicobacter pylori* infection (Wang et al., 2013), reducing incidence of antibiotic-associated diarrhea (Hempel et al., 2012), treatment and prevention of pouchitis and infectious diarrhea caused by *Clostridium difficile*(Ritchie and Romanuk, 2012), prevention of traveler's diarrhea (McFarland, 2007; Takahashi et al., 2007), providing beneficial effects on anthropometric parameters in preterm infants (Szajewska et al., 2010), and maintaining

remission of ulcerative colitis (Sang et al., 2010). Other health benefits include the reduction of serum cholesterol levels, improvement in lactose tolerance, anticarcinogenic activity, and anti-mutagenic properties (Cronin et al., 2011).

Strains of *Bifidobacterium* are supplemented to food and pharmaceutical products because of the beneficial effects of these microorganisms. These products have been sold worldwide as freeze-dried preparation, or in microencapsulated form. About $\geqslant 10^6$cfu/g of *Bifidobacterium* cells should be present in a product to produce beneficial effects (Marteau et al., 1992). However, some species of the genus *Bifidobacterium* do not produce probiotic effects because only specific strains of this genus have probiotic potential. For example, the genome sequence of *Bifidobacterium dentium* Bd1 reveals that this strain is an opportunistic pathogen associated with tooth demineralization and decay (Ventura et al., 2009). The probiotic effects produced from a specific strain does not hold true for other strains, even within the same species of *Bifidobacterium* (Williams, 2010). The strains of *Bifidobacterium* selected for supplementation to food or pharmaceutical products have to be originally isolated from humans. Strains isolated from animals or other sources cannot adapt in the human gastrointestinal tract, thereby not yielding probiotic effects. Non-human bifidobacterial isolate strains are potential pathogens to human hosts. Identification, classification, and characterization of *Bifidobacterium* are necessary steps to select potential probiotic strains. Prior to selection of these strains, knowledge of the bifidobacterial diversity and the distribution in human gastrointestinal tract are required.

Phenotypic and genotypic approaches are implemented into the detection, identification, and characterization of *Bifidobacterium*, as well as the microbial ecology of the human gut.

Phenotypic approaches of bifidobacterial identification, classification, and characterization are based on bifidobacterial cellular and colonial morphology, growth requirement for selective culture media, types of fermentation and fermentation products, as well as serological analysis and macromolecule analyses (cell-wall protein, fatty acid methyl ester, and fructose-6-phosphate phosphoketolase) (Fontana et al., 2013; McCartney, 2002; McCartney et al., 1996; Satokari et al., 2003; Tannock, 1999). Phenotypic approaches are often labor-intensive and time-consuming. Phenotypic analysis of *Bifidobacterium* lacks reliable and fully clear results because not all strains of *Bifidobacterium* are cultivable and these approaches have limited reproducibility due to lack of well-recognized protocol. More importantly, phenotype variation does not necessarily distinguish strains of *Bifidobacterium*, even for genetically closely species i.e., poor discriminatory power. Thus, these drawbacks of phenotypic approaches impel a shift towards genotypic approaches to elucidate bifidobacterial diversity.

The detection, identification and characterization of *Bifidobacterium* have benefited from the application of molecular methods, mainly nucleic acid-based genotypic approaches. These sensitive, rapid, and automated methods are adopted as complementary or alternative strategies to phenotypic approaches (Satokari et al., 2003). In contrast to the latter, genotypic approaches possess greater discriminatory power and produce better reproducible results. These approaches

are also employed in identifying and differentiating *Bifidobacterium* at species or strain levels among complex microflora, tracking or monitoring dynamic changes of bifidobacterial diversity and distribution in large-scale epidemiological analyses qualitatively or quantitatively, and allowing comparative study of phylogenetically related strains of *Bifidobacterium* (Donelli et al., 2013; McCartney, 2002; McCartney et al., 1996; Mohania et al., 2008; Satokari et al., 2003; Scott et al., 2002; Tannock, 2001). The nucleic acid-based genotypic approaches can be categorized into three techniques according to the methodologies: hybridization-based techniques, target amplification techniques, and fingerprinting techniques. In this review, the developed and applied nucleic acid-based genotypic approaches for detection, identification, and characterization of *Bifidobacterium* are described and detailed by exploring the technical principles of techniques. Strengths and limitations of each technique are also discussed.



## 2. Nucleic acid-based techniques

### 2.1 Hybridization techniques

Hybridization techniques have offered choices for the identification and detection of *Bifidobacterium* prior to the popular use of polymerase chain reaction (PCR). Hybridization techniques is on the basis of the ability of one single-stranded nucleic acid binds to another complementary single-stranded nucleic acid to form a hybrid, enabling the detection of specific nucleic acid sequences (Mohania et al., 2008). The sequences of interest is often identified with a

labeled nucleic acid probe.Hybridization can be carried out in forms of DNA-DNA, DNA-RNA, or RNA-RNA combinations.

### 2.1.1 Dot-blot hybridization

Five *Bifidobacterium* species (*B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum*; Table 1) often isolated from human feces were differentiated by dot-blot hybridization (DBH) (Yamamoto et al., 1992). In DBH, synthesized DNA probes corresponding to unique sequences within the 16S rRNA of five different *Bifidobacterium* strains were labeled with P. Crude RNAs with high-molecular weights were extracted and fixed to a positively charged nylon membrane with a microfiltration apparatus after denaturation. The radioactive DNA probes were hybridized with the RNAs, and the amount of hybrid formed was analyzed by autoradiography. These probes were specifically hybridized with the RNA extracted from target strains and used to hybridize with whole bifidobacterial cells fixed to the nylon membrane to test the specificity. The PBI probe and PIN probes were not specific for *B. bifidum* and *B. infantis*, respectively (Yamamoto et al., 1992). Mangin et al. (1995) developed four species-specific DNA probes isolated from recombinant DNA libraries of *B. longum*, *B. adolescentis*, *B. bifidum* and *B. animalis*, which were used in DBH to identify the strains of these species from closely relative ones. Malinen et al. (2003) developed DBH with rDNA-targeted oligoprobes to detect two *Bifidobacterium* species and four species of fecal bacteria. The array can specifically detect the

bacteria, but with low sensitivity (Malinen et al., 2003). DBH was also used to compare the bacterial groups within the human cecal and fecal microbiota (Marteau et al., 2001).

### 2.1.2 Colony Hybridization

Kaufmann et al. (1997) used colony hybridization to identify and quantify viable *Bifidobacterium* species isolated from food. The *Bifidobacterium* genus-specific probe labeled with digoxigenin (DIG) at the 3' end was developed from the V9 variable region of the bifidobacterial 16S rRNA sequence. This probe was specifically hybridized with *Bifidobacterium* other than non- *Bifidobacterium* species (Kaufmann et al., 1997). Bifidobacterial colonies were initially transferred to a nylon membrane, and cells on the membrane were lysed to allow the fixation of liberated DNA. Following prehybridization and hybridization processes, the hybrids appeared as dots on the membrane and detected by a commercial DIG luminescent detection kit. The detection of *Bifidobacterium* species by colony hybridization with species-specific probes was developed as microbial source tracking indicators (Balleste and Blanch, 2011; Lynch et al., 2002). This method was also used for detecting probiotic *B. lactis* LAFTI B94 in human fecal samples from a feeding trial (Su et al., 2005).

### 2.1.3 Microarray-based techniques

Microarray-based technology is a powerful molecular tool capable of large-scale high-throughput screening that enables simultaneous identification and characterization of

multiple genes of interest. A microarray is a collection of nucleic acid sequences (commonly DNA) which are hybrized with nucleic acid probes to generate data for either quantitative or qualitative analysis (Miller and Tang, 2009). Microarrays can be classified into solid and liquid microarrays according to the types of surface probe supporting platform. In a basic microarray platform, the probe molecules are bound to the microarray either by immobilization (solid microarrays) or suspension in solution (liquid microarrays). The target molecules, which are the fluorescently labelled sequences of interest then hybridize to the probe molecules, resulting in an elevation of fluorescent signals against to a background level that can be measured by fluorescent scanner (Miller and Tang, 2009). The technique was used by Wang et al. (2002a) to detect human intestinal bacteria in fecal samples. Three oligoprobes specific for each bacterial species (with a total of 60 oligogoprobes) were developed based on 16S rDNA sequences to detect these bacteria. Nine oligoprobes were specific to *B. longum*, *B. adolescentis*, and *B. infantis* (three oligoprobes for each *Bifidobacterium* species). PCR amplification of the rDNAs of predominant bacterial species in the human GIT was performed prior to hybridization. All designed oligoprobes were bound to small glass slides, and the PCR products were tested simultaneously against these probes. The microarray results have shown that the amount of PCR products bound to the oligoprobes determines the fluorescent intensity (Wang et al., 2002a). However, this method is costly because it requires expensive microarrayers and laser scanners (Wang et al., 2002b). Wang et al. (2002b) developed a membrane-array using the previously

designed oligoprobes with the principle analogous to microarray, except that the oligoprobes were fixed on the nitrocellulose membrane. A *Bifidobacterium* mixed-species microarray (BMS-microarray) was developed by Boesten et al. (2009) for high resolution discrimination between type strains and isolates belonging to nine intestinal *Bifidobacterium* species. In this microarray, the genomic DNAs (gDNAs) of six *Bifidobacterium* species were hybridized to a microarray carrying three sub-arrays (labeled as SA1, SA2 and SA3). Sub-array SA1 and SA2 comprise single-species gDNA libraries of *B. pseudolongum* and *B. longum*, respectively, whereas SA3 comprises a mixed-species gDNA library of *B. adolescentis, B. bifidum*, *B. animalis*, and *B. catenulatum*. The hybridization of the gDNAs of these species to the BMS-microarray manifested a distinct hierarchical clustering and allowed the separation of all intestinal *Bifidobacterium* species (Boesten et al., 2009). A total of 18 *Bifidobacterium* strains were used to test the applicability of the BMS-microarray as a taxonomic tool. This method can characterize *Bifidobacterium* at species- and strain- levels (Boesten et al., 2009). A validated phylogenetic microarray platform (named HTF-Microbi.Array) combined with real-time PCR was developed by Centanni et al. (2013) to characterize the *Bifidobacterium*-dominated gut microbiota of breast-fed infants. This microarray was based on the ligase detection reaction-universal array (LDR-UA), in which the reaction relied on the intolerant to base pair mismatching properties of the ligase (Centanni et al., 2013). In this study, bacterial 16S rRNA was amplified with universal primer set 27F/1492R. The PCR products were subjected to LDR

reaction with bacterial group-specific fluorescent labeled probes (without the *Bifidobacterium*-specific probe) following the series treatment. The LDR products were hybridized on universal arrays and have produced fluorescence signals detected and identified according to their location within the array (Candela et al., 2010; Centanni et al., 2013). The relative abundance of each bacterial group (without the bifidobacterial group) was calculated according to the fluorescence intensities. Real-time PCR was performed to quantify both *Bifidobacterium* and total bacteria, allowing the relative abundance of bifidobacterial and non-bifidobacterial populations to be obtained (bifidobacterial population subtracted from total bacterial population). Furthermore, the relative abundance of each bacterial group was adjusted proportional to the value corresponding to the relative abundance of the non-bifidobacterial population obtained from real-time PCR. All studied infants showed a *Bifidobacterium*-dominated microbiota with relative abundance ranging from 9% to 58% (Centanni et al., 2013). The combination of HTF-Microbi.Array with real-time PCR for the quantification of bifidobacterial population has advantages that can overcome the PCR-dependent bias observed when the HTF-Microbi.Array was used alone (Centanni et al., 2013).

### 2.1.4 Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) methods were developed to analyze *Bifidobacterium* species in human feces (Langendijk et al., 1995; Matsuki et al., 2004; Takada et al., 2004). FISH

allows the direct detection and quantification of whole bacterial cells in various samples (McCartney, 2002). Designed genus- or species-specific oligoprobes are labeled with fluorescents in FISH. These probes permeate into bacterial cells and the rRNA molecules capture the probe. Hybridization signals produced by the hybrids are detected and measured by fluorescence microscope or flow cytometry (Satokari et al., 2003). Langendijk et al. (1995) developed and tested three 16S rRNA hybridization probes (Bif164, Bif662 and Bif1278) for FISH. Bif164 yielded the highest fluorescence level among the three oligoprobes, and the fluorescent signal produced by this oligoprobe can be distinguished readily from that of the negative controls (Langendijk et al., 1995). *Bifidobacterium* genus- and species-specific 16S rRNA-targeted oligoprobes were validated and used in FISH for *Bifidobacterium* species (Matsuki et al., 2004). Multi-color FISH methods have been developed by Takada et al. (2004) to analyze seven of these species in human feces. A total of eight genus- and 16S rRNA-targeted oligoprobes were developed and labeled with different fluorescent dyes or a combination of those (Takada et al., 2004).



**2.2 Target amplification techniques**

Low sensitivity is often resulted when detection solely by hubridization due to insufficient amount of nucleic acid sequences (Wolk et al., 2001). The invention of *in vitro*, enzyme-facilitated nucleic acid amplification techniques have greatly improved the detection sensitivity. Target amplification, probe amplification, and signal amplification are the basic

strategies of nucleic acid amplification tests (NAATs) (Morel, 2011). Target amplification technique is led by PCR-based technique, which is the most developed and popular used technique among the available NAATs.

### 2.2.1 PCR

PCR is a commonly used target amplification technique that applies a thermocycling process and adopts 5'- and 3'- specific oligonucleotide primers complementing the region of interest to amplify DNA through a thermostable *Taq* DNA polymerase (Saiki et al., 1988). This technique allows a fragment of DNA to be amplified to up to $10^7$ fragments after cycling processes of denaturation, annealing, and elongating in a thermal cyclyer. This rapid, accurate, and sensitive technique overcomes the weaknesses of traditional phenotypic identification methods in bifidobacterial identification. PCR enables the amplification of bifidobacterial DNA directly extracted from various sources, such as feces, colonies, dairy products, and pharmaceutical products (Ward and Roy, 2005). Cultural identification of *Bifidobacterium* requires anaerobic conditions and a series of biochemical test, in which the protocols are often labor-intensive and time consuming (Tannock, 1999). Identification of *Bifidobacterium* by PCR is not restricted by cultural conditions, thereby yielding reproducible results in different laboratories. The accuracy and sensitivity of this process are accompanied by designing and careful primer selection. Group-specific, species-specific, and strain-specific primers for *Bifidobacterium* were continually developed., and Table 2 lists the published PCR primers for *Bifidobacterium* over the

past 17 years. The growing number of developed bifidobacterial specific PCR primers in the past two decades were resulted from the increased availability of information on bifidobacterial genome sequences. These primers were designed, either for direct detection of *Bifidobacterium* in various sample, or for phylogenetic analysis of *Bifidobacterium* species.

Most published bifidobacterial specific primers are designed to target the 16S rRNA gene of *Bifidobacterium*, which are highly conserved and present in multiple copies per bifidobacterial chromosome (Bourget et al., 1993; Frothingham et al., 1993; Leblond-Bourget et al., 1996). Real-time PCR primers and probes in published papers (in chronological order) are shown in Table 3. The 16S, 23S, and 5S rRNA genes encode the structural rRNA molecules. The 16S rRNA (16S rDNA) gene is universally found in bacterial chromosomes and is the most conserved among the three rRNA genes; this gene has also been recognized as "gold standard" for bacterial identification and classification (Kolbert and Persing, 1999; Pace et al., 1986). The variable regions (V1 to V9) in this gene contain specific information that provide sources for genus-specific, group-specific, species-specific, and strain-specific PCR primers (Donelli et al., 2013; Fontana et al., 2013; Satokari et al., 2003; Tannock, 1999; Ward and Roy, 2005). Several bifidobacterial specific primers were derived from the 16S–23S rRNA intergenic spacer region (ISR) (Brigidi et al., 2000; Tsai et al., 2008; Ventura et al., 2001b; Ventura and Zink, 2002). The ISR can be used for designing primers because it has more variations in sequence and length among the prokaryotes (Fontana et al., 2013; Leblond-Bourget et al., 1996). The 16S–23S ISR is

also less conserved than the 16S rRNA gene, and therefore bifidobacterial specific primers are derived from this region for the differentiation of *Bifidobacterium* at strain level (Ventura and Zink, 2002). No bifidobacterial specific primers have been derived based on the 23S rRNA gene because of its greater size (ca. 2,900 bp) (Kwon et al., 2005; Tannock, 1999). The first reported study employing the PCR method in bifidobacterial analysis was conducted by Roy et al. (1996), who developed species-specific primers to identify strains of *B. breve, B.infantis,* and *B. longum*. *B. longum*-specific primers reliably identified commercial strains of *B. longum*; however, this primer set cross-reacted with *B. magnum, B. thermophilum,* and *B. suis*. Primers specific for *B. breve* and *B. infantis* identified most test type strains with no cross-reactions (Roy et al., 1996). In the same year, species-specific primers were developed by Wang et al. (1996) to identify *B. adolescentis* ATCC15703 and *B. longum* ATCC15707. Primer sets BIA and BIL were designed from 16S rRNA gene sequences. Primer set BIL was sensitive enough to detect as few as two *B. longum* cells with pure culture, whereas primer set BIA detected 10,000 *B. adolescentis* cells (Wang et al., 1996). Several genus-specific primer sets targeted to the 16S rRNA gene have been published (Kaufmann et al., 1997; Kok et al., 1996; Matsuki et al., 2002). Primer sets Bif164/Bif662 and Bif16S3/Bif16S4 were developed to detect *Bifidobacterium* in infant feces (Kok et al., 1996). lm26/lm3 were used to directly detect Bifidobacteria in food without DNA isolation (Kaufmann et al., 1997). Primer set g-Bifid-F/g-Bifid-R was used to detect and identify the predominant bacteria in human feces (Matsuki et al., 2002). Primers targeting bifidobacterial

protein genes other than the ribosomal genes for PCR based analysis were also developed (Baffoni et al., 2013; Delcenserie et al., 2005; Drisko et al., 2005; Kullen et al., 1997; Requena et al., 2002; Roy and Sirois, 2000; Sheu et al., 2009; Sheu et al., 2010; Ventura et al., 2006; Ventura et al., 2003a). The highly conserved *recA* gene is universally present in both Gram positive and negative bacteria, and is encoded for multifunctional *recA* protein (Miller and Kokjohn, 1990).Bifidobacterial specific primers targeting the *recA* gene have been developed to reveal bifidobacterial intrageneric phylogeny (Kullen et al., 1997; Masco et al., 2007; Ventura and Zink, 2003). *ldh* gene encodes the key lactic acid fermentation enzyme fructose-1,6-biphosphate-dependent L-lactate dehydrogenase (LDH), and the LDH active site and several other domains have high degree of sequence conservation in prokaryotes and eukaryotes (Griffin et al., 1992). Primer set LDH has been developed for the sequence analysis of bifidobacterial *ldh* gene (Roy and Sirois, 2000). Transaldolase has an important function in central carbon metabolism, and is grouped into five subfamilies (Samland et al., 2012). Bifidobacteria were able to produce at least 14 isoenzymes of transaldolase, which can be electrophored and serologically differentiated (Requena et al., 2002). The transaldolase-encoding gene varies among *Bifidobacterium* species, and bifidobacterial specific primers targeting the conserved region of transaldolase gene have been developed to identify and detect *Bifidobacterium* species (Requena et al., 2002). The *tuf* gene encodes the elongation factor TU (EF-Tu) and presents as a single gene in bifidobacterial genomes (Ventura et al., 2003a).

Multiple alignment of *tuf* gene sequences have revealed that the conserved region of the gene sequence were differentiable among *Bifidobacterium* species; genus- and species-specific primers targeting the conserved region have been developed for bifidobacterial identification, detection, and enumeration (Sheu et al., 2009; Sheu et al., 2010; Ventura et al., 2003a). Primer set Bifbif-F/Bifbif-R failed to detect *B. bifidum*; however, a new primer set was designed to amplify a 513- bp fragment of β-galactosidase gene for *B. bifidum* identification(accession no. AJ224435) (Drisko et al., 2005). Surprisingly, the amplified product was not *B. bifidum* after sequencing and sequence homology analysis. This product was from *B. infantis* or *Bifidobacterium* species (Drisko et al., 2005). The heat-shock protein (*hsp* 60) gene encodes this highly conserved protein (a chaperonin) and is species-specific (Baffoni et al., 2013; Delcenserie et al., 2005; Jian et al., 2001).Bifidobacterial specific primer sets targeting the partial sequence of *hsp* 60 gene have been developed to identify, detect, and phylogenetically analyze *Bifidobacterium* species (Baffoni et al., 2013; Delcenserie et al., 2005; Jian et al., 2001).

In contrast to phenotypic identification methods, most PCR methods were unable to differentiate viable and nonviable bifidobacterial cells because bacterial chromosomal DNAs were extracted and amplified with either live or dead cells (Josephson et al., 1993; Ventura et al., 2001b). Chemical treatment to bifidobacterial cells prior to PCR- based method allows the differentiation of viable and dead cells. A study developed a PCR-based method with ethidium monoazide (EMA) to quantitatively detect viable *Bifidobacterium* in probiotic yogurt (Meng et al., 2010).

Fujimoto et al. (2011) developed the same method with propidium monoazide (PMA) to differentiate live *B. breve* strain Yakult from dead cells in human fecal samples. Quantitative detection of viable *B. bifidum* BF-1 cell in human feces using PCR-based method with PMA was also developed (Fujimoto and Watanabe, 2013). Both EMA and PMA are DNA intercalating agents that penetrate the dead cell membranes, but cannot penetrate the intact membranes of viable cells (Fujimoto et al., 2011; Fujimoto and Watanabe, 2013; Sun et al., 2010). These substances have been useful in combination with PCR-based method to selectively detect and quantify viable bifidobacterial cells (Fujimoto et al., 2011; Fujimoto and Watanabe, 2013; Sun et al., 2010). However, EMA is unsuitable to treat *B. breve* strain Yakult prior to the quantification of viable cells (Fujimoto et al., 2011). This finding suggests that EMA is toxic to some viable cells (Nocker et al., 2006; Pan and Breidt, 2007).

The feasibility of highly species-specific primers targeted to bifidobacterial 16S rRNA genes are limited by the high similarities of these sequences (Youn et al., 2008). Leblond-Bourget et al. (1996) compared the 16S rRNA gene sequences of 18 *Bifidobacterium* species, and found that the similarity of these sequences ranged from 92% to 99%. The phylogenetic analysis conducted by Miyake et al. (1998) also revealed that the similarity of the same gene sequences ranged from 93% to 99% among *Bifidobacterium* species. The high similarity imposes difficulty in the differentiation between these species. Moreover, the relatively small size of 16S rRNA gene (ca. 1,500bp) hampers the primer design for the identification and detection of *Bifidobacterium*

(Fontana et al., 2013; Tannock, 1999), which reflects on some designed species-specific primers based on the 16S rRNA gene that are in fact not species-specific. *B. adolescentis*-specific primer set BiADO and *B. longum*-specific primer BiLONg cross-reacted with *B. ruminantium* and *B. suis*, respectively (Matsuki et al., 1998). The *B. longum*-specific primer set BiLON-1/BiLON-2 cross-reacted with *B. susi* (Matsuki et al., 1999). The *B. animalis*-specific primer set Ban F2/Pbi R1 cross-reacted with *B. lactis* (Roy and Sirois, 2000). A work of the specificity and reproducibility of PCR conditions for identifying *Bifidobacterium* species showed that nonspecific reactions were found in many developed primer sets based on the 16S rRNA gene (Youn et al., 2008). The specificity, the yield of PCR products, and the fidelity are influenced by PCR protocol conditions including the concentration of dNTPS, $MgCl_2$ DNA templates, primers, and thermostable DNA polymerase, temperatures of denaturation, annealing, and strand elongation (Cha and Thilly, 1993; Sachse, 2004; Youn et al., 2008). Cross-reaction with other species was observed when origin PCR protocol conditions were duplicated (Youn et al., 2008). Stringent PCR protocol conditions must be optimized upon performing PCR to identify *Bifidobacterium* species. This method is also influenced by the presence of various inhibitors in the samples (Schrader et al., 2012). PCR identification of *Bifidobacterium* species is mostly employed in fecal samples and dairy products. Fecal sample contains complex polysaccharides, bile salts, lipids and urate, whereas dairy products contain plasmin and calcium ions (Schrader et al., 2012). These inhibitors interfere at different steps of the PCR protocol, resulting in reduced

sensitivity and amplification efficiency. The Removal of inhibitors during DNA preparation is required prior to PCR analysis. Several methods including additional extraction steps, selection of resistant polymerase, and inactivation of proteases by hot NaOH extraction have been developed and evaluated (Abu Al-Soud and Radstrom, 2000; Chaturvedi et al., 2008; Rossen et al., 1992).

### 2.2.2 Multiplex PCR

Multiplex mPCR was first developed based on the conventional PCR (Chamberlain et al., 1988). Unlike conventional PCR, two or more primer pairs are added to the same reaction, thereby enabling the simultaneous amplification of multiple sequences. The principle, reagents and protocol of mPCR are similar to that of conventional PCR. This method has been proven useful in the identification and detection of bacteria that cause infectious diseases (Markoulatos et al., 2002). Given that multiple target sequences are amplified in a single PCR, labor costs and time are reduced, and the accuracy and reliability are enhanced (Dong et al., 2000; McCartney, 2002). A total of eight studies have been conducted to employ mPCR to simultaneously identify and detect *Bifidobacterium* in complex microbial ecologies including mixed culture, human feces, commercially available probiotic products, and municipal sewage (Bonjoch et al., 2004; Dong et al., 2000; Germond et al., 2002; Kwon et al., 2005; Mullie et al., 2003; Sul et al., 2007; Ventura et al., 2001b). All mPCRs in these studies have been evaluated for their specificity upon comparison with their corresponding simplex PCRs. Cross-reactions were observed in two

studies, in which mPCR mixtures contained only species-specific primer pairs (Dong et al., 2000; Mullie et al., 2003). No cross-reactions were observed in the study where mPCR mixtures contained *Lactobacillus* species-specific and *Bifidobacterium* species-specific primer pairs (Sheu et al., 2009; Sul et al., 2007). Dong et al. (2000) observed that *B. infantis*-specific primer PIN710f cross-reacted with *B. minnimum,* and suspected that nonspecific reactions were also observed between *G. vaginalis* and *B. bifidum*-specific primer PBI245f, *B. breve*-specific primer PBR442f, and *B. adolescentis*-specific primer PAD805f. Mullie et al. (2003) observed that *B. suis* cross-reacted with both *B. infantis* and *B. longum* primers. No cross-reactions observed for studies that included both genus-specific and species-specific primer sets in mPCR protocol (Bonjoch et al., 2004; Germond et al., 2002; Kwon et al., 2005; Sul et al., 2007; Ventura et al., 2001b). Nested- PCR was performed and coupled with mPCR in two previous studies (Bonjoch et al., 2004; Ventura et al., 2001b). mPCR coupled with nested-PCR improved the sensitivity of detection upon the performance of the second round of amplification. However, this method also increases the risk of obtaining false-positive results as contaminations may be encountered (Markoulatos et al., 2002). As more complex reaction systems exist than conventional PCR, mPCR requires the optimization of the reaction conditions to specifically identify and detect *Bifidobacterium*. The amount of primers, the concentration of dNTP and $MgCl_2$, the dNTP/$MgCl_2$ balance, the concentration of PCR buffer, and the amount of template DNA and DNA polymerase are important for mPCR (Markoulatos et al., 2002). Moreover, primer dimer

formation and preferential amplification of a specific targets are often observed in mPCR as two or more primers are used. Preferential amplification of a specific target is driven by PCR drift and selection (Markoulatos et al., 2002). These mechanisms mainly cause different sensitivities and specificities for each amplification target. Primer- dimers are formed when insufficient DNA templates and excess primers exist; the yield of PCR products is reduced when excess DNA templates and insufficient primers exist (Markoulatos et al., 2002). The primer-to-template ratio must be optimally adjusted. Nonspecific reactions are undesired in mPCRs, and hot start DNA polymerases (hot Start PCR) are chosen to eliminate these reactions and primer dimer formation (Chou et al., 1992). Active enzyme is released at the initial thermocycling step of hot Start PCR to prevent the formation of primer dimers, mispriming, and primer annealing during pre-PCR processes (Chou et al., 1992; Erlich et al., 1991). Annealing primer temperature is an important parameter in mPCR primer design and selection (Henegariu et al., 1997; Markoulatos et al., 2002; Ward and Roy, 2005). Primer pairs in mPCRs are ideally within the same range of annealing temperature, GC content, and length; hence, the range of primer length, GC content, and annealing temperature should be 18 bp to 28 bp, 45% to 60%, and 56$^{o}$C to 60$^{o}$C, respectively (Henegariu et al., 1997; Markoulatos et al., 2002; Ward and Roy, 2005). The designed mPCR primer pairs should produce products in different sizes and exhibit either heterologous intraspecies or interspecies (Henegariu et al., 1997; Markoulatos et al., 2002; Ward and Roy, 2005).

### 2.2.3 Real-time PCR

PCR aids in bifidobacterial studies; however, conventional PCR yields results of presence or absence of *Bifidobacterium* in samples, and this method cannot generate quantitative results, i.e., bifidobacterial nucleic acid content is unknown. The revolutionary invention of real-time quantitative PCR (RQ-PCR) enables the detection and quantitative analysis of *Bifidobacterium* in the same reaction. A sudden increase in bifidobacterial studies using real-time PCR has occurred. Table 3 shows the newly established real-time PCR methods for these studies. Primers targeting the bifidobacterial rRNA and protein genes were developed for bifidobacterial identification and quantification. Multiple copies of 16S rRNA sequences present in bifidobacterial chromosome may influence the outcome of real-time PCR, in which the quantification of *Bifidobacterium* in sample tends to be overestimated (Masco et al., 2007; Requena et al., 2002; Solano-Aguilar et al., 2008). Protein genes targeting primers or probes were developed as alternatives to 16S rRNA gene-based real-time PCR analysis. Protein genes including transaldolase, *rec*A, *gro*ES, *gro*EL, *hsp*60, *tuf*, and *xfp* are present as single copies in bifidobacterial chromosomes (Cleusix et al., 2010; Junick and Blaut, 2012; Masco et al., 2007; Requena et al., 2002; Sheu et al., 2010; Solano-Aguilar et al., 2008). This method have become an important research technique in molecular biology because of its strong specificity, high sensitivity, good repeatability, rapid and accurate generated result, and closed reaction vessel (Bustin et al., 2005; Mackay, 2004). This method was first introduced by Higuchi et al. (1993).

Real-time amplification quantification target DNA is achieved by continous real-time monitoring of the appearance and amount of the fluorescence signals; this is analyzed upon comparison with internal or external standards with known amounts of DNA (Heid et al., 1996; Mohania et al., 2008). Real-time PCR operates in a closed vessel, thereby minimizing the risk of contamination. Analysis of *Bifidobacterium* using conventional PCR often requires post-PCR process where contaminations are encountered. For example, PCR products are often separated in agarose gels for analysis, and cross-contamination may occur through aerosols released from opening and closing the PCR tubes or incautious PCR operation (Heid et al., 1996). Real-time PCR detects the amplification of PCR products using fluorescent dyes and fluorescing probes at each thermocylcing steps. Hence, this method is more sensitive than conventional PCR, and false positive results are reduced (Mohania et al., 2008; Ward and Roy, 2005). A comparative study of real-time PCR and DBH in the quantification of selected fecal bacteria has shown this method was easier and faster to perform and has higher sensitivity (Malinen et al., 2003).

Fluorescent dye SYBR Green I and specifically designed fluorescing *Taqman* probes are popular reporter molecules in bifidobacterial studies using real-time PCR. SYBR Green I combines with double-strand DNA and produces fluorescent signals under the excitation light source. The strength of this signal represents the amounts of double-strand DNA. This substance further combines with the minor groove of double-strand DNA. No fluorescent signals are produced by free SYBR Green I, and fluorescent signals are produced dramatically upon combination with



 

double-strand DNA. The combinations of SYBR Green I and PCR products increase with the increase of PCR products, enabling the quantification of any target gene (Ward and Roy, 2005; Yin et al., 2001). The use of SYBR Green I is convenient, which is mixed with the primers and other PCR reagents in the same tube as in real-time PCR (Requena et al., 2002). This substance can be used to monitor the amplification of any DNA sequence and is not primers-specific. However, SYBR Green I also combines with nonspecific double-strand DNA such as primer dimers, thereby producing false positive signals. Both specific and nonspecific amplifications can be distinguished by constructing and analyzing a melting curve (Ririe et al., 1997). The optimization of PCR reaction conditions is required to reduce nonspecific fluorescent signals. Fluorescing *Taqman* probes are more specific than SYBR Green I in real-time PCR (Ward and Roy, 2005). This hydrolysis probe was first developed by Heid et al. (1996). This probe is also designed to hybridize with target sequence, and is labeled with a reporter (fluorophores) at the 5' end and a suppressor (quencher) at the 3' end. The close distance between the fluorophore and the quencher allows the fluorescence resonance energy transfer (FRET) to occur. The production of fluorescent signal from the fluorophore is suppressed by the quencher, in which this signal is only detected at the 3' end. Primers and *Taqman* probes are bound to DNA template during amplification, and the probe specifically hybridizes with the DNA template between the forward and reverse primers. When amplification extends to the binding site of the probe, the 5'–3' exonuclease activity of *Taq* polymerase enables the cleavage of double-strand nucleotides. The

FRET between fluorophore and quencher is broken when the fluorophore labeled at the 5' end of *Taqman* probe is degraded and the fluorescent signal is produced. The obtained fluorescence emission is proportional to the amount of PCR products; the DNA template is determined from the fluorescence intensity (Heid et al., 1996). The use of *Taqman* probe in real-time PCR eliminates the potential false positive results observed using SYBR Green I. The melting curve is not required, thereby reducing the experiment time. However, this method generates nonspecific fluorescent background when the quenching fluorescent is incomplete because the fluorophore and the quencher are far from each other (Bustin, 2005; Mackay, 2004). Modified real-time PCR has been developed to address the nonspecific fluorescent background.

Several studies used minor groove binder (MGB) probe as alternatives to conventional *Taqman* probe in the quantitative analysis of bifidobacteria (Delroisse et al., 2008; Gomez-Donate et al., 2012; Haarman and Knol, 2005; Solano-Aguilar et al., 2008). A non-fluorescent quencher (NFQ) is adopted at the 3' end of the fluorescent probe (Yao et al., 2006). No fluorescence emission is obtained when NFQ absorbs energy from the fluorophores. The background signal is significantly lowered and yields better precision in quantification. Dehydrocyclopyrroindole tripetide (DPI3) is attached to the 3' end of the MGB probe to stabilize the hybridization of the probe and DNA template. DPI3 also increases the melting temperature ($T_m$) of the probe, thereby shortening the probe length (Kutyavin et al., 2000). The closer distance between fluorophore and

quencher in the shortened MGB probe yields better quenching effect, and also lowers the cost of probe synthesis (Yao et al., 2006).

A study conducted molecular beacon (MB) real-time PCR to quantitatively detect viable bifidobacteria in probiotic yogurt (Meng et al., 2010). This beacon is a stem-loop hairpin structure oligoprobe, with designed sequences that are complementary to the target sequence positioned in the loop (Mackay, 2004; Mhlanga and Malmberg, 2001). A fluorophore (FAM) is labeled at one end of the stem, and a non-fluorescent quencher (DABCYL) is labeled at the other end of the stem. The fluorophore and the quencher are held close to each other in the stem by complementary base pairing of nucleotides with short lengths. MB remains a closed hairpin structure with the absence of a target DNA sequence, whereas the fluorophore and the quencher are close enough to allow FRET occurs (Tyagi et al., 1998). The MB oligoprobe specifically binds to the target sequence with its presence, thereby forming a stable double-strand DNA. The fluorophore is spatially apart from the quencher as soon as this MB probe hybridizes with the target sequence; hence, the FRET is broken and fluorescent the signal is produced (Mackay, 2004; Mhlanga and Malmberg, 2001; Tyagi et al., 1998). The stem-loop hairpin structure of the MB probe is more specific than the linear probe because of higher stability (Meng et al., 2010).

Studies adopted the lanthanide probes for real-time PCR quantification of bifidobacteria in human fecal samples (Gueimonde et al., 2007; Gueimonde et al., 2004). Compared with the studies that used only one oligoprobe, a fluorescent probe and a quencher probe were used in this

method (duplex real-time PCR) (Gueimonde et al., 2007; Gueimonde et al., 2004). The fluorescent probe was labeled with isothiocyanate-modified and stable fluorescent europium chelates at the 5' end, and the quencher probe was labeled with the non-fluorescent Dabcyl at the 3' end. The quencher probe was complementary to the fluorescent probe, and absorbed fluorescent signal when hybridized with the fluorescent probe in the absence of specific DNA template (Nurmi et al., 2002). The hybrid of the fluorescent and quencher probes broke when the specific DNA template was present; the fluorescent probe was bound preferentially to the DNA template at a higher temperature to produce a fluorescent signal (Nurmi et al., 2002). This signal is measured in a time-resolved manner and the fluorescent intensity is proportional to the amount of DNA template, allowing the quantification of initial DNA template. No change in fluorescent signal of the quencher probe was observed. The quencher probe is the internal control and enhances further calibration of the fluorescent signal detected from the fluorophore (Nurmi et al., 2002). The unspecific fluorescent background is eliminated at maximum, yielding higher signal-to-noise ratios and lower threshold cycles compared with those obtained using *Taqman* real-time PCR (Gueimonde et al., 2007; Gueimonde et al., 2004; Nurmi et al., 2002).

Real-time reverse transcription PCR (RT-PCR or RT-qPCR) is frequently used in measuring gene expression of microbial communities because it is the most accurate and most specific technique (Postollec et al., 2011). RT-qPCR used in the studies of *Bifidobacterium* is carried out through the reverse transcription of isolated or extracted RNA (mRNA or tRNA) to cDNA,

performed independently of real-time PCR (Fujimoto and Watanabe, 2013; Reimann et al., 2010; Turroni et al., 2011). RT-qPCR was used to quantify viable *B. longum* NCC2705 cells exhibiting different morphologies by measuring the mRNA expression of two housekeeping genes *cysB* and *purB* (Reimann et al., 2010). Luciferase mRNA specific primer and probes are used in RT-qPCR to provide internal reference for monitoring mRNA loss in different experimental conditions, such as heat stress and rifampicin addition. The 400- bp fragment of *purB* has been shown as a suitable biomarker of cell viability by comparing the results obtained from RT-qPCR and plate count (Reimann et al., 2010). Fujimoto and Watanabe (2013) combined PMA treatment of the samples before DNA extraction with RT-qPCR to quantify viable *B. bifidum* BF-1 cells in human feces. Previously developed reverse primer BiBIF-2 was used to produce cDNA at the first step of RT-qPCR (Fujimoto and Watanabe, 2013; Matsuki et al., 1998). A few number of bifidobacterial studies determines that RT-qPCR was developed through the extraction or isolation of intact RNA from complex environmental samples that are more difficult to obtain than DNA (Postollec et al., 2011; Smith and Osborn, 2009). A potentially short half-life determines the labile nature of RNA (Grunberg-Manago, 1999). The amount of isolated or extracted RNA determines the quantity of cDNA in the reverse transcription reaction, in which an insufficient amount of starting RNA lowers the kinetics of this technique (Chandler et al., 1998; Smith and Osborn, 2009). Various RNA extraction methods have been developed and evaluated for RT-qPCR(Tan and Yiap, 2009).

The amount of *Bifidobacterium* in the samples using real-time PCR is mostly determined through the absolute quantification method, in which the amount of nucleic acids in the sample is quantified by interpolating their quantities from a standard curve, rather than an absolute measurement of the nucleic acids present in the sample (Mackay, 2004; Smith and Osborn, 2009). Absolute quantification method reveals the initial amount of nucleic acids in the sample in relation to a specific gene (Freeman et al., 1999). A standard curve is constructed by plotting the threshold cycle ($C_t$) values against the amount of nucleic acids or number of cells on a logarithmic scale (Smith and Osborn, 2009). A linear regression is obtained from the standard curve, and the amplification efficiency is determined by the slope of the linear regression. Similar amplification efficiencies for all test samples and standards should be required (Postollec et al., 2011). The $C_t$ value refers to the cycle number at which the fluorescent signal produced in a real-time PCR reaction crosses the primarily set fluorescent threshold (Jung et al., 2000). The $C_t$ value is proportional to the amount of target sequences in the test samples, and assumptions have been made that each reaction tube has an equal amount of amplicons (Gibson et al., 1996; Mackay, 2004). The logarithmic amount of nucleic acids is determined by predilution of an amplicon before real-time PCR, or repeating real-time PCR using a diluted sample of the selected reference *Bifidobacterium*. Careful choice of the standard curve template is mandatory (Dhanasekaran et al., 2010). The number of bifidobacterial cells in the sample is determined by intersecting the $C_t$ values of the amplified sample to the standard curve. An alternative method to

 

construct a standard curve for absolute quantification is described. Gomez-Donate et al. (2012) generated standards for real-time PCR by constructing a plasmid, which was transformed into a model bacterium. The 16S rRNA gene fragments from the target bifidobacterial strains were cloned with a pGEM-T Easy vector. The standard curve of real-time PCR was constructed by amplifying this vector containing each fragment (Gomez-Donate et al., 2012). The Liu and Saint method is used to determine the relative initial amount of DNA in infants receiving prebiotic infant formula (Haarman and Knol, 2005). This method is a relative quantification method that uses a mathematical model to quantify and standardize gene expression (Liu and Saint, 2002). Relative quantification method is used to estimate changes in gene expression based on its comparison to the expression of an internal reference (IRF) gene (Smith and Osborn, 2009). Housekeeping genes are often chosen for use because IRF genes in relative quantification method as their expressions are constitutive and uniform (Smith and Osborn, 2009; Thellin et al., 2009). Solano-Aguilar et al. (2008) validated the use of RT-qPCR to detect the single-copy *tuf* gene of *B. animalis* subsp. *lactis* as a marker for the differentiation of *B. animalis* subsp. *lactis* strain Bb12 from several *Bifidobacterium* species and *Lactobacillus* species, as well as *E. faecium*. Single-copy Bb12 *tuf* gene is used as an IRF gene in RT-qPCR to study the relationship between the localized accumulation of *B. animalis* subsp. *lactis* strain Bb12 in the GIT of pigs and the modulation of host innate immune response (Solano-Aguilar et al., 2008). Turroni et al. (2011) conducted whole-transcriptome analyses using microarray to identify potential

housekeeping genes suitable for use as IRF genes in *B. bifidum* PRL2010. From the microarray experiments, they identified 22 putative genes, whose expression was not alternated by growth conditions. Among the 22 candidate genes, three housekeeping bifidobacterial genes from the model microorganism have been found suitable as IRF genes by RT-qPCR. *pdxS, uvrD*/Rep, and *gluC* genes displayed the highest level of expression stability and low level of variation associated with gene expression under different growth conditions (Turroni et al., 2011). However, the authors stated that these identified IRF genes are not universal reference genes for *Bifidobacterium* because gene expression varies with different environmental conditions and *Bifidobacterium* species.



### 2.2.4 PCR-ELISA

PCR assay is used together with enzyme-linked immunosorbent assay (ELISA) for the semi-quantitative detection of intestinal bacteria, lactobacilli, and bifidobacteria (Laitinen et al., 2002). PCR-ELISA assay is also used in the semi-quantitative detection of bifidobacterial populations in human fecal samples from a consumption trial (Malinen et al., 2002). This assay involves three main steps: PCR amplification, labeling of PCR products, and hybridization (Laitinen et al., 2002; Malinen et al., 2002). The PCR amplification step was similar to that of the conventional PCR, but the PCR products were labeled with DIG at the 5' end after denaturation. In the hybridization step, oligoprobes are selected either by utilizing published sequence or designed based on 16S rDNA sequence, and all the probes are labeled with biotin at

the 3' end (Laitinen et al., 2002). The biotinylated probes were immobilized on the streptavidin coated wells of the microtiter plate by the biotin-streptavidin cross-links. Denatured DIG-labeled PCR products were added and hybridized with the streptavidin-immobilized oligoprobes on the wells of the microtiter. After several times of washing with buffer and antibody solution, the microtiter plate was incubated at room temperature in the dark. Freshly prepared substrate solution was added to each well to generate chromogenic signals, which were analyzed by measuring the absorbance at 450 nm wavelength (Laitinen et al., 2002). A cut-off value for each oligoprobe is determined separately as twice the mean value of all negative controls. A bifidobacterial species is considered present if the absorbance of the measurements is greater than the cut-off value corresponding to its oligoprobe. *Bifidobacterium*-specific oligoprobes (ado440, b162) have failed to recognize its target sequences due to the preferential amplification of certain specific target that resulted from the PCR amplification step (Laitinen et al., 2002; Markoulatos et al., 2002). The sensitivity of the PCR-ELISA assay is heavily reliant on the specificity level of the PCR primer set used (Laitinen et al., 2002). Bifidobacterium genus-specific primer set and multiple bifidobacterial species-specific probes have been developed to be used in the PCR-ELISA assay (Malinen et al., 2002). All designed oligoprobes are specific to their corresponding bifidobacterial species (Malinen et al., 2002). These species-specific oligoprobes are also used in the comparative study of molecular methods for the identification and genotyping of *Bifidobacterium* (Matto et al., 2004).

### 2.3 Fingerprinting techniques

The techniques described in this section are molecular typing and fingerprinting techniques. Molecular typing or fingerprinting allows identification of different microorganisms at a genomic level. The choice of an proer molecular typing or fingerprinting depends on the meet of demands. PCR has been widely used in the amplification of bifidobacterial nucleic acids since the 1990s. Methods for detection and sizing of PCR products are demanded by researchers. Standard agarose gel electrophoresis is widely used to detect and determine the size of amplification products by separating DNA molecules according to their molecular weight (Voytas, 2001). However, agarose gel electrophoresis has poor resolution of DNA fragments, making it non sensitive and not specific enough to meet the demands of bifidobacterial typing and fingerprinting. Thus several techniques have been developed for typing and fingerprinting of bifidobacteria.



#### 2.3.1 Ribotyping

Ribotyping is based on the differences in location and number of ribosomal RNA present in the bacterial genome (Bouchet et al., 2008). This genotyping method is a form of RFLP typing, consisting of cleavage of bacterial DNA with restriction endonucleases, electrophoretic separation of cleaved restriction DNA fragments, Southern blot transfer, and transferred DNA fragments hybridized with a radiolabeled ribosomal operon probe (Bouchet et al., 2008; Foley et

al., 2009; McCartney, 2002). Southern blot hybridization with rRNA gene regions as probes shows at least three *rrn* loci in *B. breve* (Bourget et al., 1993). The cloned heterologous 23S rDNA probe I41 was developed and labeled with radioactive phosphorus32 in ribotyping of type/collection bifidobacterial strains (Mangin et al., 1994). The screening of the restriction enzymes is essential in RFLP, where the number of restriction fragments can be reduced using rare restriction enzymes (McCartney, 2002). The use of two or more restriction enzymes with different recognition sequences is recommended in characterizing bacterial ribotypes (McCartney and Tannock, 1995). In the Southern hybridization analysis of *rrn* operons of *B. adolescentis* E-981074, four fragments were hybridized with *Eco*RI digests, whereas five fragments were hybridized with *Eco*RV digests, indicating that there are five copies of *rrn* operon in this strain of *B. adolescentis* (Satokari et al., 2001a). Multiple restriction enzymes are used to increase the discriminatory power of ribotyping (Simpson et al., 2002). Three endonucleases (*Bam*HI, *Eco*RV, and *Pvu*II) are used to cleave each of the *Bifidobacterium* strains of DNAs, and the radioactive labeled probe was hybridized with each of the *Bam*HI-, *Eco*RV-, or *Pvu*II- cleaved *Bifidobacterium* strain DNAs in Southern blot hybridization (Mangin et al., 1994). Comparative analysis of the *Bam*HI, *Eco*RV, and *Pvu*II ribosomal patterns for type/collection of *Bifidobacterium* strains show that *Bam*HI revealed most of the common bands within the same species among the three endonucleases (Mangin et al., 1994). The ribosomal patterns allow the differentiation of tested bifidobacterial strains (Mangin et al., 1994). This

ribotyping method has been used in the study of ribosomal DNA polymorphism in *Bifidobacterium* and has been applied in epidemiological study (Mangin et al., 1999; Mangin et al., 1996). In ribotypying, species-specific DNA probes derived from recombinant DNA libraries of *B. longum, B. adolescentis, B. bifidum,* and *B. animalis* were used to study restriction polymorphism in these four *Bifidobacterium* species (Mangin et al., 1995). As an alternative to the probes derived from cloned 23S rRNA gene or recombinate DNA libraries, a 16S rDNA probe was developed using PCR amplification of an approximately 1.5 kb fragment of 16S rDNA from *B. infantis* (McCartney and Tannock, 1995). After the preliminary screening for suitable restriction endonucleases, *Bam*HI, *Nar*I, and *Pvu*II were used to generate ribosomal patterns (McCartney and Tannock, 1995). The importance of using two or more restriction endonucleases was further demonstrated. Identical ribosomal patterns of two bifidobacterial strains were generated by *Bam*HI and *Nar*I, whereas *Pvu*II generated different ribosomal patterns, allowing differentiation between the two bifidobacterial strains(McCartney and Tannock, 1995). Another study showed that *Pvu*II has more discriminating power than *Eco*RI, as *Pvu*II revealed more heterogeneity in *Bifidobacterium* ribotyping (Matto et al., 2004). Procedures including culturing bacterial cells, DNA extraction constructing probes, electrophoretic analysis, and interpretation of generated ribotypes make conventional RFLP or ribotyping time-consuming and labor-intensive, especially when obtaining restriction patterns from different endonucleases by Southern blot hybridization (Scott et al., 2002).



 

### 2.3.2 RFLP

Restriction fragment length polymorphism (RFLP) analysis is based on the heritable differences in the lengths of DNA fragments that occur when bacterial DNA is cleaved with a restriction endonuclease (Tait, 1999). The cleaved DNA fragments, also known as restriction fragments, can be separated by gel electrophoresis, yielding a band pattern. Bacteria can be distinguished by comparing the band patterns after cutting and separating the DNA fragment with restriction endonucleases (Mohania et al., 2008). Conventional RFLP contains cleaved DNA fragments that are electrophoretic, and detected with probes after Southern blot hybridization (Simpson et al., 2002). In the study of bifidobacterial transaldolase gene, a single restriction fragment was hybridized with a 246- bp radiolabeled transaldolase gene probe, indicating that the transaldolase gene is present in bifidobacterial chromosome as a single copy (Requena et al., 2002). Southern blot hybridization, probed with *tuf* gene fragment analysis of *Hind*III-digested genomic DNA of eight bifidobacterial species, show that only one copy of *tuf* gene is present in bifidobacterial chromosome (Ventura et al., 2003a).



### 2.3.3 PCR-RFLP

Cumbersome procedures are minimized in RFLP analysis accompanied with PCR. The principle of PCR-RFLP is the same as that of conventional RFLP or ribotyping, but the procedures involved are modified (Baffoni et al., 2013; Delcenserie et al., 2004; Delcenserie et al., 2013;

Kullen et al., 1997; Ventura and Zink, 2003). PCR-RFLP analysis is more sensitive than conventional PCR because small amounts of DNA are amplified with PCR prior to RFLP analysis (Tait, 1999). The target sequence to be amplified must be determined first for designing primers specific to it (Tait, 1999). The Southern blot hybridization step in conventional RFLP analysis is replaced by the amplification of target sequences with specific primers. The PCR products are cleaved by restriction endonucleases, subsequently restriction patterns are generated by gel electrophoresis. In an early bifidobacterial study, PCR-RFLP analysis of the 16S rRNA gene distinguished four restriction patterns from 46 randomly picked fecal bifidobacterial isolates, and the most dominant restriction patterns were selected for further systematic study using *rec*A gene sequence data (Kullen et al., 1997). PCR-RFLP is used in discriminating *B. lactis* from *B. animalis*. Restriction patterns of amplified 16S-23S rDNA fragments, cleaved with *Sau*3AI for all strains of these two bifidobacterial species, allowed direct identification at species-level (Ventura and Zink, 2003). The use of bifidobacterial species to monitor contamination in food has been proposed because bifidobacterial species are host-specific, and an accurate identification of bifidobacterial species is useful for tracing contamination origin (Delcenserie et al., 2004). Therefore, PCR-RFLP is used in discriminating between human bifidobacterial species and animal origin bifidobacterial species by generating restriction patterns of amplified 16S rDNA fragments cleaved with Alu*I* and Taq*I* (Delcenserie et al., 2004). The PCR-RFLP with 16S rDNA-specific primers and restriction enzyme Alu*I* and Taq*I* is also used

with other phenotypic methods to detect *B. crudilactis* and *B. mongolisense* during the manufacturing process of French raw milk cheeses (Delcenserie et al., 2013). The identification of *Bifidobacterium* spp. using PCR-RFLP was developed and validated (Baffoni et al., 2013). A dichotomous key is constructed by comparing *In silico* digestion and obtained restriction patterns of amplified *hsp*60 gene fragment cleaved with *Hae*III, and the dichotomous key is efficient enough to differentiate 25 *Bifidobacterium* species as well as subspecies belonging to *B. pseudolongum* and *B. animalis* (Baffoni et al., 2013).

### 2.3.4 PCR-T-RFLP

PCR-based terminal restriction fragment length polymorphisms (PCR-T-RFLP) is an adaptable, accessible, and reproducible method for studying complex microbial ecology (Liu et al., 1997). Microbial community structure and dynamic changes in response to different environmental factors can be examined with T-RFLP (Kitts, 2001; Osborn et al., 2000). PCR-T-RFLP based on the variable regions in 16S rRNA gene has been used in distinguishing fecal contamination in coastal water by identifying genetic markers from *Bifidobacterium* and *Bacteroides-Prevotella* group, determining the bacterial composition of commercial probiotic products, and in profiling *Bifidobacterium* composition in the infant gut (Bernhard and Field, 2000; Lewis et al., 2013; Marcobal et al., 2008).

The rapid, sensitive and cost-effective PCR-T-RFLP technique combines DNA sequencing/comparative genomics, PCR, RFLP, and electrophoresis to generate terminal restriction (TRF) pattern (Marsh, 1999). DNA sequencing or comparative genomics provide the information necessary for designing primers homologous to highly conserved region of target sequences. Primer design is a crucial step in PCR-T-RFLP. Designed primers for PCR-T-RFLP are ideally targeting bacterial species present in microbial communities; however, such primers have not been developed yet (Schutte et al., 2008). The database of 16S rRNA sequences are relatively large enough for designing primers in PCR-T-RFLP (Marsh, 1999). Binding sites for forward and reverse primer on the target sequence should have adequate distance, ideally 400 bp to 700bp (Kitts, 2001). If the forward primer is close to the reverse primer, amplicons will be short and no restriction site will be available for restriction endonucleases; thus no terminal restriction fragments (TRF) are formed. Conversely, long amplicons will produce large TRF that may cause inaccurate analysis. Hayashi et al. (2004) evaluated the performance of three different forward primers in conjunction with one reverse primer in PCR-T-RFLP in determining fecal *Bifidobacterium* in healthy individuals. The performance of primer 35F and 529F in PCR-T-RFLP was better compared with 27F; and primer 529F was used to determine fecal *Bifidobacterium* in healthy humans (Hayashi et al., 2004). Forward or reverse primer is labeled with a fluorescent dye at the 5' end, or both forward and reverse primers are labeled, but only fluorescently labeled TRF are visualized in the subsequent analysis by a fluorescence detector



 

attached to a DNA sequencer. In the study of *Bifidobacterium* using PCR-T-RFLP, either forward primer or reserve primer was labeled with fluorescent dye 6-FAM or HEX (Bernhard and Field, 2000; Hayashi et al., 2004; Lewis et al., 2013; Marcobal et al., 2008). The amplified DNA fragment are then cleaved with restriction endonucleases that usually have a tetranucleotide recognition sequence, and the TRF are subjected to electrophoretic analysis either by polyacrylamide gel or capillary gel electrophoresis (Kitts, 2001; Osborn et al., 2000). Two or more restriction endonucleases are used to aid accurate bifidobacterial identification by obtaining better TRF resolution (Bernhard and Field, 2000; Hayashi et al., 2004; Lewis et al., 2013; Marcobal et al., 2008). The obtained TRF size was measured in reference to an internal size standard or internal standard marker that is commercially available. The peak height or peak area represent the abundance of bacteria, and the measurement of TRF size was obtained using an automated fragment analysis program (GeneScan software or Peak Scanner, Applied Biosystems) (Bernhard and Field, 2000; Kitts, 2001; Lewis et al., 2013; Marcobal et al., 2008; Sibley et al., 2012). Several reviews of PCR-T-RFLP have pointed out that the measurement of TRF size is affected by the sequence composition that affects the electrophoresis mobility. In addition, different fluorescent dyes have different electrophoretic properties (Kaplan and Kitts, 2003; Schutte et al., 2008; Sibley et al., 2012; Tu et al., 1998). The completed PCR-T-RFLP analysis resulted in a semi-quantitative profile of the dominant bacteria in a microbial ecology (Sibley et al., 2012).

Specific 16S rDNA TRF peaks were identified in a pattern by directly matching peak patterns constructed from clone libraries parallel to PCR-T-RFLP; however, the cloning and sequencing process increased the expenditure and experiment time (Bernhard and Field, 2000; Hayashi et al., 2004; Kitts, 2001). Web-based *in silico* prediction tool is available online for the identification of *Bifidobacterium* species in PCR-F-RFLP analysis (Kent et al., 2003; Shyu et al., 2007). Marcobal et al. (2008) identified TRF using a previously developed T-RFLP analysis program (TAP) available on the ribosomal database project (RDP) website. TAP enables *in silico* selection of restriction enzymes for T-RFLP to enable the highest discriminating activity, best resolution power, and optimal primer-enzyme combination (Marsh et al., 2000). TAP provides a platform for users to perform *in silico* T-RFLP procedures based on RDP, allowing TRF size to be measured in base pairs (Marsh et al., 2000). The obtained TRF size was compared with the TRF size obtained from TAP to identify bacterial species (Marcobal et al., 2008). Another web-based tool was used in T-RFLP to evaluate *Bifidobacterium* species in the gut of infants (Lewis et al., 2013; Shyu et al., 2007). In this study, an empirical database from pure strains was manually constructed for manual assignment of size standard peaks, and TRF size was predicted using the most common TRF size for a particular *Bifidobacterium* size from the microbial community analysis (MiCA) (Lewis et al., 2013; Shyu et al., 2007). MiCA is a web-based resource that consists of two programs: in silico PCR and Restriction (ISPaR), and a plausible community (APLAUS), utility of former program is to simulate PCR amplification of 16S or

18S rRNA genes using designed primers and restriction digestion of PCR products with different restriction enzymes, whereas the latter program deduces and refines the relative abundances of bacterial populations (Shyu et al., 2007). The algorithmic APLAUS program addresses the issues associated with rounding and size-calling errors caused by multiple factors including double peaks yielded after separation from some double-stranded marker ladders and differences in electrophoresis properties of the different fluorescent dyes (Kitts, 2001; Schutte et al., 2008; Shyu et al., 2007). TRF sizes obtained from the empirical database are compared statistically with those obtained from fecal samples for bifidobacterial identification(Lewis et al., 2013). PCR-T-RFLP studies using a web-based tool for bacterial identification showed discrepancies between the observed and predicted TRF lengths (Lewis et al., 2013). These were mainly due to the inherent issues existing during PCR-T-RFLP experiment and because many sequences in the current databases are unreliable (Sibley et al., 2012). Discrepancies between the observed and predicted fragment length can be overcome by allowing ± one or two base mismatch between the primer and the template sequence, amplification in PCR reaction will tolerate a few base mismatches between primer and template sequence (Nocker et al., 2007; Schutte et al., 2008).

### 2.3.5 PFGE

Pulsed field gel electrophoresis (PFGE) is another form of RFLP typing method in which the separation of very large DNA fragments in a gel matrix is carried out using an alternating electric field (Tannock, 2001). Compared with the ribotyping method, PFGE involves the use of rare

cutting restriction enzymes to generate restriction patterns that represents the whole genome of the bacteria, therefore giving PFGE a greater discriminatory power than that of ribotying (Foley et al., 2009). However, Matto et al. (2004) stated that PFGE has an equally discriminatory power with ribotyping after comparing molecular methods for the identification and genotyping of *Bifidobacterium*. PFGE is an extremely useful tool in epidemiological studies and in the determination of bacterial relatedness because of its good reproducibility and its ability to discriminate between subspecies (Scott et al., 2002; Ward and Roy, 2005). PFGE can be useful in determining genome size in five strains of *B. breve*, obtaining the genomic fingerprint of commercially important *Bifidobacterium* strains, confirming the ribotyping results of *Bifidobacterium* strains, investigating the strain individuality of *B. animalis* and *B. lactis*, characterizing the strains of *B. animalis* subsp. *lactis*, and differentiating the dominant consecutive *Bifidobacterium* isolates after fingerprint comparison an a epidemiological study (Aires et al., 2011; Bourget et al., 1993; Briczinski et al., 2009; McCartney et al., 1996; Roy et al., 1996; Ventura and Zink, 2002). *Bifidobacterium* cells are first embedded and lysed in low-melting-point agarose, allowing the extraction of intact chromosomal DNA. In the agarose plug, the extracted DNA is digested *in situ* by a rare restriction endonuclease that cleaves the DNA. The agarose plug containing digested chromosomal DNA is added to an agarose gel for resolving. Conventional gel electrophoresis cannot resolve DNA molecules larger than 50- kb because long DNA molecules cannot penetrate the pores of agarose (Goering, 2010). Restriction

fragments generated by rare restriction endonucleases are called marcorestriction fragments. These fragments are often too large to be resolved by conventional gel electrophoresis, but can be resolved from one another when the electric field is applied in pulses that are in orthogonal orientation from each other (Goering, 2010). In PFGE, marcorestriction fragments are reoriented to the direction of the new field each time the polarity of the current is switched (Foley et al., 2009; Goering, 2010). With pulsed field, 10- 20 marcorestriction fragments ranging from 20 kb to 800 kb can be separated (Foley et al., 2009; Ward and Roy, 2005). Although PFGE is often considered the gold standard typing methods, it is not suitable for routine laboratory use because of its time consuming process, which required 2-3 days to complete (Donelli et al., 2013; Foley et al., 2009; Ward and Roy, 2005).



### 2.3.6 ARDRA

Amplified ribosomal DNA restriction qnalysis (ARDRA) is a technique developed from RFLD. ARDRA involves entirely or partially amplified 16S rRNA genes (Satokari et al., 2003; Ward and Roy, 2005). In the ARDRA study of *Bifidobacterium,* 16S rRNA genes are amplified using genus- specific or species- specific primers, and amplified products are digested using restriction enzymes. Digested products are then separated by agarose or polyacrylamide gel electrophoresis and visualized with ethidium bromide staining similar to RFLP-PCR. The obtained ARDRA patterns of bifidobacterial species are analyzed and compared with those obtained from the reference strains (Donelli et al., 2013). The discriminatory power of ARDRA is determined by

restriction enzymes used and the amplified 16S rRNA fragments; thus, using multiple of restriction enzymes in ARDRA is crucial because different bacterial species may generate identical ARDRA patterns (Nocker et al., 2007; Satokari et al., 2003; Ward and Roy, 2005). Roy and Sirois (2000) generated ARDRA patterns using *Bifidobacterium* genus-specific primer set Pbi F1/Pbi R2, and a combination of three restriction enzymes (*Bam*HI, *Taq*I, and *Sau*3AI) to characterize *B. infantis*, *B. longum*, and *B. animalis*. Differentiation using ARDRA has good correlation with species-specific primers. Webcutter analysis protocol is used to evaluate ARDRA patterns of *Bifidobacterium* generated by different restriction enzymes, *Sau*3AI generated the clearest and most reliable distinction *in silico* ARDRA patterns with universal 16S rRNA primer set P0/P6 (Ventura et al., 2001a). Sixteen *Bifidobacterium* species were differentiated using experimentally obtained ARDRA patterns by primer set P0/P6 and restriction enzyme Sau3AI, and the results were in agreement with the results obtained by the Webcutter analysis (Ventura et al., 2001a). Venema and Maathuis (2003) generated ARDRA patterns of *Bifidobacterium* species found in the human alimentary tract by primer set Bif164/Bif662, which amplified partial sequence of bifidobacterial 16S rRNA. *In silico* analysis was initially performed to screen for suitable restriction enzymes. Five candidate restriction enzymes (*Sau*3A, *Taq*I, *Rsa*I, *Alu*I and *Sau*96I) were chosen for use in obtaining ARDRA patterns experimentally (Venema and Maathuis, 2003). As previously report, experimentally obtained ARDRA patterns using combination of five restriction enzymes can discriminate 14

*Bifidobacterium* species present in the human alimentary tract (Venema and Maathuis, 2003). Krizova et al. (2006) compared the discriminatory power of ARDRA patterns generated by *Bifidobacterium* genus-specific primer sets Pbi-F1/Pbi-R2 and Bif164/Bif662 with a combination of six restriction enzymes. The discriminatory power of ARDRA patterns generated by primer set Pbi-F1/Pbi-R2 was higher than that of primer set Bif164/Bif662 (Krizova et al., 2006). ARDRA patterns generated by the universal 16S rRNA primer set E8F/E1115R, with the combination of three restriction enzymes (*Alu*I, H*hal* and R*sal*) was able to differentiate three *Bifidobacterium* species (*B. bifidum*, *B. breve* and *B. longum* ssp. *infantis*) and two *Lactobacillus* species (Pal et al., 2012). A comparative study showed that the discriminating power of ARDRA was less than that of RAPD (Carmen Collado and Hernandez, 2007). This study demonstrated that ARDRA can only discriminate probiotic *Lactobacillus*, *Streptococcus* and *Bifidobacterium* at genus-level, whereas, RAPD can discriminate all bacteria present in dairy products at both genus- and strain-levels (Carmen Collado and Hernandez, 2007). In general, PFGE has the most discriminating power and ARDRA has the least discriminating power among the commonly applied molecular typing methods (Satokari et al., 2003). Moreover, ARDRA can be utilized in both confirmation and identification human *Bifidobacterium* species, but it is not recommended to apply ARDRA in large scale studies or in identification of a single *Bifidobacterium* species from an environment that has a mixture of *Bifidobacterium* species (Germond et al., 2002).

### 2.3.7 AFLP

Currently, studies on *Bifidobacterium* using amplified fragment length polymorphism (AFLP) are limited. A previous study used AFLP to exam the transmission of intestinal *B. longum* subsp. *longum* strains from mother to infant (Makino et al., 2011). AFLP is a highly sensitive method based on PCR selective detection of the digests of total genomic DNA, and provides a good image of genetic diversity among bacterial isolates (Mohania et al., 2008; Mueller and Wolfenbarger, 1999; Vos et al., 1995). AFLP allows resolving bacterial DNA fingerprinting in high quality by generating multiple highly replicable markers from bacterial DNA (Janssen et al., 1996; Vos et al., 1995). The usefulness of AFLP has been shown in species resolution and strain differentiation of *B. longum* subsp. *longum* (Makino et al., 2011). The DNA of *B. longum* subsp.*longum* was first digested with restriction enzymes MspI and MesI to generate a large number of restriction fragments. The restriction enzymes used in AFLP were frequent cutters that digest the bacterial genome from multiple restriction sites (Mueller and Wolfenbarger, 1999). The resulting restriction fragments were subjected to a ligation reaction that uses double-stranded adapters, containing sequences complementary to the sticky ends of the restriction enzyme (Makino et al., 2011). PCR amplification of restriction fragments was carried out using primers (preselective primers) targeting the adapter sequences (Makino et al., 2011). A considerably large number of PCR amplified restriction fragments was produced, causing difficulties in subsequent profile analysis (Foley et al., 2009). To reduce the number of amplified restriction fragments, a new set of primer (selective primer) was designed and used in selective

PCR reaction. Both the forward and reverse primers contain an additional nucleotide at the 3' end paired with complementary bases in the target sequence. Under highly stringent PCR conditions, the production of primers bound with mismatched nucleotides was prevented; thus, the total number of PCR products was reduced to a manageable number (Foley et al., 2009). One primer of the selective primer set was labeled with fluorescent dye FAM so that the automated DNA sequencer can separate and detect fluorescent amplified fragments, enabling high output analysis (Foley et al., 2009; Makino et al., 2011). Amplified fragments with an internal size standard were analyzed by automated DNA sequencer, allowing accurate comparison of elution profiles and phylogenies of *B. longum* subsp.*longum* strains (Foley et al., 2009; Makino et al., 2011).



### 2.3.8 PCR-DGGE

PCR-denaturing gradient gel electrophoresis (PCR-DGGE) is the molecular fingerprinting method for studying microbial diversity and monitoring microbial population dynamics in complex microbial ecology (Muyzer, 1999). PCR-DGGE was established by Fischer and Lerman (1983) to screen for mutation. Since its introduction, PCR-DGGE have been modified and applied in microbial community studies. Table 5 lists the PCR primers used in PCR-DGGE. Muyzer et al. (1993) was the first study that used PCR-DGGE in the analysis of genetic diversity of complex microbial populations. PCR-DGGE is a gel electrophoresis technique based on melting properties of the amplified DNA fragments, those that have same size but differ in base

composition are separated (Muyzer, 1999). In PCR-DGGE, a fragment of 30-50 GC base is attached to the 5' end of either forward primer or reverse primer so that one end of the PCR products contains the GC-clamp (Ercolini, 2004). The GC-clamp developed by Walter et al. (2000) was used in the PCR-DGGE analysis of *Bifidobacterium*. The amplified double-stranded DNA is loaded to the polyacrylamide gel containing a linear denaturing gradient formed by mixture of urea and formamide (Muyzer, 1999; Muyzer et al., 1993). Direction of the DNA fragment migration in the gel is perpendicular to the denaturing gradient, and the initial separation of DNA fragments is according to molecular weight. As the DNA fragments migrate towards to an increasingly strong denaturing condition, the point where the denaturant is strong enough to melt the double-stranded DNA is ultimately reached (Muyzer, 1999; Muyzer et al., 1993). At this point, migration stops and the double-stranded DNA is partially separated. Given that the GC-clamp has a higher melting temperature ($T_m$) than the discrete regions of the DNA fragment, the GC-clamp remains double-stranded, whereas, the discrete region is melted, forming a butterfly-shaped molecule (Muyzer, 1999; Muyzer et al., 1993). The butterfly-shaped DNA molecule migrates very slowly in the gel. The partially melted discrete region of the DNA fragment is called "melting domain" (Ercolini, 2004). The melting property of the melting domain of each DNA fragment varies as their $T_m$ differ. The difference in melting properties of the DNA fragments under DGGE mainly depends on the differences in base sequences, i.e. the melting property of a DNA fragment is sequence-specific (Ercolini, 2004). DNA fragments with

the same molecular weight but different base composition can be separated in DGGE gel. Theoretically, PCR-DGGE can detect the differences in base composition by as little as one nucleotide (Nocker et al., 2007). The PCR-DGGE separated DNA fragments can then be cloned and sequenced for further phylogenetic analysis. Cloning and sequencing of PCR-DGGE separated DNA fragments ensure better phylogenetic analysis compared to direct sequencing (Nocker et al., 2007).

Satokari et al. (2001a) utilized PCR-DGGE in the analysis of *Bifidobacterium* diversity in human feces, wherein primer set Bif164/Bif662 was modified to amplify an 520-bp bifidobacterial 16S rDNA. Better separation of different Bifidobacteria species was obtained when the GC-clamp was attached to the reverse primer. In addition, PCR-DGGE using primer set Bif164/Bif662 failed to separate the closely related bifidobacterial species and the different strains of the same *Bifidobacterium* species (Satokari et al., 2001a). PCR-DGGE analysis using bifidobacterial transaldolase gene-specific primers cannot separate bacterial species or strains with highly similar transaldolase gene (Requena et al., 2002). Several studies conducted nested PCR-DGGE in the analysis of community bifidobacterial diversity (Hoyles et al., 2013; Martin et al., 2009; Temmerman et al., 2003a). The length of amplicons produced from *Bifidobacterium* genus-specific primer set lm26/lm3 (1,417bp) were not analyzed because they exceeded the 500-bp limit for PCR-DGGE analysis; thus, the amplicons were used as DNA template for the second PCR to generate amplicons less than 500-bp (Temmerman et al., 2003a). Nested PCR-DGGE

was conducted to prevent low amplicon yield (Martin et al., 2009). Significant differences were observed between PCR-DGGE profiles obtained with universal primers and those obtained by the *Actinobacteria* nested PCR (Hoyles et al., 2013). Nested PCR-DGGE was adopted in the study of the diversity of bifidobacterial diversity in wastewaters (Balleste and Blanch, 2011). *Bifidobacterium* species from different sources (human, poultry, pig and bovine) were analyzed in this study. The result showed that the DGGE profiles of human *Bifidobacterium* and poultry *Bifidobacterium* were similar; whereas, the DGGE profiles of pig *Bifidobacterium* and bovine *Bifidobacterium* were more heterogeneous (Balleste and Blanch, 2011). PCR-DGGE allows culture-independent and direct differentiation of probiotic bacteria isolated from commercial probiotic products. The tested products have been found to have incorrect labels of probiotic bacteria (Temmerman et al., 2003b; Theunissen et al., 2005). PCR-DGGE has been widely used in monitoring bifidobacterial dynamics because of its ability of simultaneous analyze multiple samples (Favier et al., 2002; Joossens et al., 2011; Piacentini et al., 2010; Satokari et al., 2001b; Stsepetova et al., 2007). Recently, PCR-DGGE analysis of bifidobacterial diversity and dynamics has been assisted with computer band matching software (Gomez-Donate et al., 2012; Joossens et al., 2011). Band patterns generated by PCR-DGGE are visualized by staining; however, background staining may be encountered (Nocker et al., 2007). Distinguishing weak bands from poor PCR procedure or less abundant species in the samples is difficult. A threshold is set by the computer software to reduce background staining, making the interpretation and

positioning of band patterns more accurate (Nocker et al., 2007). Despite the usefulness of computer software in the analysis of DGGE profiles, the final image may no longer reflect the abundance of bacterial species in the sample when a smeary image is digitally converted to pattern with sharp bands (Nocker et al., 2007).

### 2.3.9 RAPD-PCR



Random amplified polymorphism DNA (RAPD) technique is a modification of PCR that generates RAPD profiles through random amplification of genomic DNA with short arbitrary primers that are usually 9- 10 nucleotides long (Williams et al., 1990). The short arbitrary primers are designed and synthesized to amplify the unknown target sequences under low-stringency annealing conditions. Amplified DNA fragments are random in size and separated by agarose gel electrophoresis and visualized by ethidium bromide staining (Donelli et al., 2013; Mohania et al., 2008). RAPD-PCR has been used in many aspects, including detection of polymorphism in genetic mapping, taxonomy and phylogenetic analysis, and in studying genotoxicity and carcinogenesis (Atienzar and Jha, 2006). In bifidobacterial studies using RAPD-PCR, genomic variation between *Bifidobacterium* species was revealed by specific band patterns generated from RAPD-PCR, and the identification of *Bifidobacterium* species was based on the differences in size and numbers of DNA fragments in the generated DNA profiles (Vincent et al., 1998). As an extension of PCR technology, the RAPD-PCR technique has its own characteristics, different from conventional PCR assay. First, designing primers specifically

for RAPD amplification is not required, and any randomly designed oligonucleotide primers with a length of 9- 10 bases can be adopted in RAPD-PCR. Therefore, knowledge on the targeted genome sequences is not required in RAPD-PCR (Vincent et al., 1998). Although the conventional PCR requires knowledge on the targeted genome sequences prior to the specific primer design, unlike conventional PCR using forward primers and reverse primers, only one primer is added to each of RAPD-PCR reaction and the added primer has many binding sites on the target genome. The primers randomly bind on the complementary sequence on the double-stranded genome in a particular orientation so that two primers are pointing towards each other within a distance of 0.3–4 kilobases (Atienzar and Jha, 2006; Satokari et al., 2003). The DNA fragment between two primers (one on the opposite strand) is amplified, and the length of amplified fragment is equal to the length between two primers (Donelli et al., 2013; Satokari et al., 2003). The differences in the number and location of the primer binding sites determine the genomic variation among bacterial species or strains belonging to the same species (Donelli et al., 2013; Satokari et al., 2003). A low annealing temperature ($30^{o}C–36^{o}C$) is used for the initial primer annealing process (Atienzar and Jha, 2006). The low stringent condition ensures stable base pairing between the short arbitrary primers and template DNA, allowing appropriate mismatches that increase the primer pairing randomness and improve the discriminatory power (Foley et al., 2009).



 

Although the RAPD-PCR technique is a simple and rapid bacterial typing method, criticisms have been garnered because of for its poor reproducibility (Foley et al., 2009; McCartney, 2002; Mohania et al., 2008; Satokari et al., 2003; Ward and Roy, 2005). The reproducibility of RAPD-PCR generated DNA profiles are sensitive to reaction conditions, and also requires optimization and standardization of the reaction conditions including thermal cyclers, primers, DNA extraction methods, thermal cycling conditions, and concentration of PCR chemicals (Atienzar and Jha, 2006; Satokari et al., 2003). In the RAPD-PCR analysis of *Bifidobacterium*, multiple primers are designed and subjected to rigorous testing for suitability in characterizing *Bifidobacterium* species and strains (Krizova et al., 2008; Srutkova et al., 2011; Toshimitsu et al., 2013; Vincent et al., 1998). Vincent et al. (1998) assessed 100 primers in the characterization of *Bifidobacterium* by RAPD-PCR; six primers (OPA-02, OPA-18, OPL-07, OPA-13, OPL-16 and OPM-05) were found suitable for subsequent optimization of RAPD-PCR assay. Dairy-related *Bifidobacterium* species (*B. bifidum*, *B. breve* and *B. adolescentis*) were distinguished under optimized five single-primer reactions (Vincent et al., 1998). Primer OPA-02 was found be discriminatory enough to identify *B. lactis* Bb-12 from other *Bifidobacterium* strains in RAPD-PCR genotyping of isolates from a feeding trial (Alander et al., 2001). RAPD-PCR with primer OPA-02 had the least discriminatory power among the three adopted genotyping methods (ribotyping, RAPD and PFGE), and RAPD-PCR with primer OPA-02 was considered as unreliable genotyping method for *Bifidobacterium* identification at the species level (Matto et al.,

2004). The RAPD-PCR technique is only suitable for initial screening of representative isolates (Matto et al., 2004). RAPD-PCR with primer OPA-18 was used to generate a general picture of the *Bifidobacterium* species diversity among the intestinal populations (Delgado et al., 2006). Krizova et al. (2008) characterized *Bifidobacterium* species using newly designed and retrieved RAPD-PCR primers from previous studies. Good discriminatory power of RAPD-PCR is achieved with a combination of several primers in the single reaction (Krizova et al., 2008). DNA profiles obtained with a combination of seven 10-mer primers (P2, P15, P16, P17, PER1, CC1, and CORR1) were able to discriminate between close related *Bifidobacterium* species of *B. longum*/*B. infantis*, *B. animalis* ssp. *animalis*/*B. animalis* ssp. *lactis*, and *B. catenulatum*/*B. pseudocatenulatum* (Krizova et al., 2008). However, strains of *B. longum* ssp. *longum* and *B. longum* ssp. *infantis* cannot be discriminated against one another using RAPD-PCR with five primers (P15, P16, P17, PER1, and CC1) (Srutkova et al., 2011). ARDRA using *Sau*3AI restriction enzyme is the first choice for discriminating *B. longum* ssp. *longum* and *B. longum* ssp. *infantis* (Srutkova et al., 2011). RAPD-PCR analysis was used to provide information needed in designing *Bifidobacterium* strain-specific real-time PCR primers (Fujimoto et al., 2011; Fujimoto and Watanabe, 2013; Toshimitsu et al., 2013). Specific RAPD-derived primer pairs p1285-1F/p1285–1151R, pBbrY-F/pBrY-R, pBF-1f/pBF-1r, and 1B-F1/1B-R3 were developed and used in the quantitative detection of strains, namely, *B. breve*, *B. breve* Yakult, *B.*

*bifidum* BF-1, and *B. bifidum* OLB6378, respectively (Fujimoto et al., 2011; Fujimoto and Watanabe, 2013; Toshimitsu et al., 2013).

Arbitrarily primed- polymerase chain reaction (AP-PCR) was developed by Welsh and McClelland (1990), and this method is technically similar to RAPD-PCR which involves initial random amplification with arbitrary primers at low stringent conditions (Cusick and O'Sullivan, 2000; McCartney, 2002; Pereira et al., 2008; Welsh and McClelland, 1990). AP-PCR has longer primer length, higher primer concentration, and higher annealing temperature in AP-PCR than those of RAPD-PCR (Atienzar and Jha, 2006; Welsh and McClelland, 1990). Cusick and O'Sullivan (2000) developed a single triplicate AP-PCR (TAP-PCR) to generate information-rich fingerprint as representative isolates from genera of lactic acid bacteria. Multiple factors affect the reproducibility of RAPD-PCR analysis, and some of these factors can be controlled by optimization and standardization; however, it is impossible to control minute changes in the reaction mixture components and slight variations in thermocycler temperature profiles (Cusick and O'Sullivan, 2000). The production of band patterns are greatly influenced by minor changes in the annealing temperature during PCR process (Ellsworth et al., 1993). Hypotheses were made on which bands are more reproducible and remain constant at three slightly different annealing temperatures (Cusick and O'Sullivan, 2000). Three different annealing temperatures (38, 40, and 42$^{o}$C) were simultaneously used in a triplicate reaction to test these hypotheses. No unstable bands were observed; thus, bands that are temperature sensitive were eliminated. In the study

conducted by Cusick and O'Sullivan (2000), the concentration ratio of two primers (P32-A and P32-T) and the $MgCl_2$ concentration were optimized. Clear fingerprints of various *B. breve* and *B. infantis* strains were generated using a 1:1 ratio of two primers and 5 mM $MgCl_2$ concentration.

### 2.3.10 Rep-PCR

Dispersed repetitive sequence elements are found in both prokaryotic and eukaryotic genomes that separate longer single-copy DNA sequences (Lupski and Weinstock, 1992). Repetitive element sequence-based PCR (rep-PCR) is a DNA fingerprinting technique that involves the amplification of repetitive elements present in bacterial genomic DNA using outwardly facing oligonucleotide primers (Versalovic et al., 1994). These primers are complementary to interspersed repeated sequences that enable the amplification of DNA fragments in different sizes. The amplified DNA fragments contain sequences lying between repetitive sequence elements, and different sized DNA fragments are separated in gel electrophoresis to generate DNA fingerprint patterns (Versalovic et al., 1994). Each bacterial strain has a specific DNA fingerprint pattern generated by rep-PCR, which is compared to those obtained from reference species patterns, allowing classification and typing. However, rep-PCR requires establishing a large database of reference species pattern for the identification of unknown species (Simpson et al., 2002). Four major bacterial repetitive elements are utilized in bifidobacterial rep-PCR fingerprinting, namely, repetitive extragenic palindromic (REP), enterobacterial repetitive

intergenic consensus (ERIC), BOX components, and polytrinucleotides $(GTG)_5$ (Gómez Zavaglia et al., 2000; Krizova et al., 2008; Kulagina et al., 2010; Masco et al., 2003; Shkoporov et al., 2008; Shuhaimi et al., 2001; Srutkova et al., 2011; Ventura et al., 2003b; Ventura and Zink, 2002). These interspersed repetitive elements are conserved in the bacterial genome; thus, the knowledge of bacterial genomic sequence is not required and the designed primer sets targeted by these repetitive elements can be used for DNA fingerprinting of different bacteria and fungi (Balajee et al., 2007; Versalovic et al., 1994).

The rep-PCR fingerprinting technique has been successfully applied in several bifidobacterial studies. Gómez Zavaglia et al. (2000) utilized BOX-PCR (rep-PCR with single nucleotide BOX primer BOXA1R) to generate fingerprint patterns for characterizing bifidobacterial strains. Cluster analysis of rep-PCR generated fingerprint patterns and separated Bifidobacteria strains into two major clusters and seven subclusters (Gómez Zavaglia et al., 2000). *B. bifidum* strains were grouped in a well-differentiated cluster, this was in agreement with studies conducted by Kullen et al. (1997) and Leblond-Bourget et al. (1996), whose results were based on RFLP of *rec*A gene and sequence analysis of ribosomal RNA gene, respectively. ERIC-PCR (rep-PCR with two nucleotide ERIC primers ERIC-1R and ERIC-2) was used to fingerprint bifidobacterial genome as well as other probiotic bacteria (Shuhaimi et al., 2001). It has been firstly reported that ERIC elements are present in *Bifidobacterium* species and other Gram positive probiotic bacteria. ERIC-PCR was able to generate highly reproducible bifidobacterial fingerprint patterns,

allowing discrimination of five *Bifidobacterium* species (*B. pseudocatenulatum*, *B. infantis*, *B. longum*, *B. animalis* and *B. indicum*). Moreover, ERIC-PCR is capable of distinguishing wild type strains of *B. pseudocatenulatum* (Shuhaimi et al., 2001). In the study conducted by Ventura and Zink (2002), the fingerprint patterns generated by ERIC-PCR show that several *B. lactis* strains (NCC363, NCC383, NCC402, and NCC311) and *B. animalis* strains (ATCC27536, ATCC27673, and ATCC27674) were incomparable to other *B. animalis* strains. Findings from both ERIC-PCR result and 16S-23S ITS sequence data obtained in this study were consistent with those from other DNA fingerprinting methods (PFGE and ARDRA) and PCR-based methods (analysis of amplified *ldh* gene and *hsp*60 gene) (Jian et al., 2001; Roy and Sirois, 2000; Roy et al., 1996). Ventura and Zink (2002) proposed new taxonomic classification of *B. lactis*, namely, *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis*. ERIC-PCR has been shown useful in bifidobacterial phylogenetic and taxonomical analysis, as well as *Bifidobacterium* species composition analysis in various environments (Ventura et al., 2003b). ERIC-PCR allows the simultaneous handling and comparison of many isolates without repeated PCR using *Bifidobacterium* species-specific primers (Ventura et al., 2003b). Masco et al. (2003) evaluated the applicability of rep-PCR fingerprinting with each of the BOXA1R, ERIC, $(GTG)_5$, and REP primers for the discrimination of *Bifidobacterium* species. BOX-PCR is most suitable for bifidobacterial identification at species- and subspecies-level in terms of discriminating power, complexity of patterns, and the taxonomic correlation (Masco et al., 2003). Questions have been

raised on the reproducibility of BOX-PCR. In another study, BOX-PCR and $(GTG)_5$ were tested for subspecies discrimination of the *B. longum* strains, it was demonstrated that BOX-PCR generated fingerprint patterns were not clear enough to resolve analyzed strains at subspecies-level (Srutkova et al., 2011). Krizova et al. (2008) compared the performance of RAPD and BOX-PCR for discriminating bifidobacterial species, and found that both methods produced similar result, but RAPD resulted higher inter-strain heterogeneity. However, from the practical point of view, BOX-PCR is preferred in bifidobacterial fingerprinting because RAPD-PCR generates poor reproducible results and require the optimization of reaction conditions (Krizova et al., 2008). However, the fact that rep-PCR produces more reproducible results compared with RAPD-PCR remains controversial. In the study comparing four PCR-based methods (species- and subspecies-specific PCR, RAPD, rep-PCR with BOXA1R/$(GTG)_5$ primers, and ARDRA) for discriminating *B. longum* ssp. *longum* and *B. longum* ssp. *infantis* strains, RAPD showed no discriminating power for resolving two closely related strains (Srutkova et al., 2011). Nevertheless, rep-PCR was able to differentiate type/collection strains into *B. longum* and *B. infantis* at subspecies level, but it was unable to discriminate newly isolated strains (Srutkova et al., 2011).



### 2.3.11 MLVA

Multiple-locus variable-number of tandem repeat analysis (MLVA) has been used as a typing method for *B. longum* subsp. *longum* (Matamoros et al., 2011). MLVA is a fast and relatively

cheap bacterial typing method that combines the polymorphous nature of the variable-number tandem repeat loci (VNTR) and the PCR (Lindstedt, 2005). MLVA bacterial typing method is based on the variation in the number of tandem repeated elements present in many different loci in the genome of bacteria (Matamoros et al., 2011). Strains of the same bacterial species have different number of tandem repeats at a particular locus, and the number of tandem repeats at multiple VNTR loci in the bacterial genome are determined by MLVA (van Belkum, 2007). In the MLVA conducted by (Matamoros et al., 2011), tandem repeats were identified in the genome of two type/collection strains of *B. longum* subsp. *longum* (NCC2705 and DJ010A) from the microorganism tandem repeat database which is available online. It was imperative that selection of VNTR loci for ensuring MLVA typing was under optimal operating parameters (Lindstedt, 2005). If unstable loci was included, the actual distribution of bacterial genotype would not be properly revealed (Lindstedt, 2005). There were six VNTR loci (VNTR12, 15,21,23,25 and 26) that were initially selected for MLVA typing, five of them were discriminatory enough for typing except VNTR 15, which showed no discriminatory power (Matamoros et al., 2011). After identifying regions that are more likely to be repeated, multiple PCR primer sets were designed on the basis of satellite flanking regions. A total of five fluorescently primer sets were used in a single multiplex PCR to amplify five identified VNTR loci. The PCR products were then separated using capillary electrophoresis, which revealed the size variation of the PCR products. The product sizes were determined with the aid of a software used to detect the number of

tandem repeats. Different strains were distinguished according to the differences in the number of tandem repeats present (Matamoros et al., 2011). Sequencing of each different allele (VNTR loci) was performed with MLVA non-labeled primers to verify the number of tandem repeats detected. A MLVA profile was formed by the string of alleles from multiple loci which is the combination of calculated numbers of tandem repeats for multiple loci (Lindstedt, 2005; Matamoros et al., 2011). Ultimately, a total of 19 MLVA profiles were identified from 44 isolates of *B. longum* subsp. *Longum* (Matamoros et al., 2011). The MLVA with single multiplex PCR is capable of differentiating isolates and identifying new strains of *B. longum* subsp. *longum* (Matamoros et al., 2011). MLVA is faster and less labor intensive than PFGE, and it has the same discriminating power as that of PFGE (Matamoros et al., 2011).



## 3. Conclusion

The nucleic acid-based molecular techniques described in the present review all have different strengths and weaknesses that affect their applicability as a nucleic acid-based tool for the detection, identification, and characterization of *Bifidobacterium*. There is no perfect techniques, and a choice of a particular technique depends on multiple factors including availablity of facilities, budget, time, and, most importantly, the research purpose. There are three different applications for nucleic acid-based molecular techniques in *Bifidobacterium* research. The first application is aimed at phylogenetic analysis of the members of Genus *Bifidobacterium*, which reveals that *Bifidobacterium* is a relatively coherent genus. The second application is aimed at

bifidobacterial dynamics and diversity among complex microbial ecology, focusing on the human gastrointestinal tract. The third application is aimed at either qualitative or quantitative evaluation of bifidobacterial composition in probiotic products. In many cases, compromises have been made between operation time and reproducibility, complexity and output, experimental cost and discriminating power, or quality and quantity. In summary, the benefits of nucleic acid-based molecular techniques are obvious, used either directly or in conjunction with phenotypic approaches, in acquisition of new or more detailed information about Genus *Bifidobacterium*. There is no single technique that is considered "universal" for research related to *Bifidobacterium*. The choice of technique used depends on research purposes, personal familiarity with the techniques, instrumentation, accessibility, cost, and time constraints. In addition, use of nucleic-based techniques has facilitated the improvement of detection and characterization of *Bifidobacterium* beyond classical plating and phenotypic methods. The usefulness and necessity of plating and phenotypic methods cannot be negated, there are hardly any studies described in this review that are based solely on nucleic-based molecular techniques alone. Most of the studies adopted a polyphasic strategy, wherein, both phenotypic and genotypic approaches are performed in order to obtain a reliable finding. For example, most of the fingerprinting techniques require prior cultivation of bacterial cells in order to obtain pure culture for generating reference fingerprints. Moreover, when isolating bifidobacteria from complex microbial community such as fecal sample, phenotypic tests are often performed for preliminary

screening. Therefore, apart from genetic profile, a valid bifidobacterial identification, detection and characterization should be performed initially, based on their morphological, physiological, and biochemical characteristics.

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Table 1. Hybridization-based techniques for use in identification and detection of Bifidobacteria

| **Techniques** | **Bifidobacteria strains** | **Probe codes** | **Sequences (5'→3')** | **Reference** |
|---|---|---|---|---|
| Dot-blot hybridization | *B. adolescentis* | PAD | $^{32}$P-GCTCCCAGTCAAAAGGG | (Yamamoto et al., 1992) |
| | *B. bifidum* | PBI | GCAGGCTCCGATCCGA | |
| | *B. breve* | PBR | AAGGTACACTCAACACA | |
| | *B. infantis* | PIN | TCACGCTTGCTCCCCGATA | |
| | *B. longum* | PLO | TCTCGCTTGCTCCCCGATA | |
| | Genus *Bifidobacterium* | Bif228 | GATAGGACGCGACCCCAT | (Marteau et al., 2001) |
| | *B. lactis* | N/A | GTGGAGACACGGTTTCCCTT | (Malinen et al., 2003) |
| | *B. longum* | N/A | GTTCCAGTTGATCGCATGGTCTT | |
| Colony hybridization | Genus *Bifidobacterium* | lm3 | CGGGTGCTICCCACTTTCATG | (Kaufmann et al., 1997) |
| | *B. adolescentis* | N/A | CTCCAGTTGGATGCATGTCCTTCTGGG | (Lynch et al., 2002) |
| | *B. lactis* LAFTI | Laf94p | GCGTGGAAGAGTCGTATTGGATTGTG | (Su et al., |

| | | | | |
|---|---|---|---|---|
| | B94 | | | 2005) |
| | Human Bifidobacteria | HM-B | GACTGGGAGCAAGCCCTTCGGGGTGAGTGTACCTT | (Ballestee and Blanch, 2011) |
| | Poultry Bifidobacteria | PL | CTTTTATCGGGGAGCAAGCGAGAGTGAGTGTACC | |
| | Pig Bifidobacteria | PG-B | GGGTTGTAAACCGCTTTTGATTGGGAGCAAGCG | |
| | Bovine Bifidobacteria | CW-E | CGCTTTTGTTCAAGGGCAAGGCACGGTTTCGGCCGT | |
| Microarray-based techniques | *B. longum* | BIL-1 | CTCCATCACACCGCATGGTGTGTTGGGAAAGCCTTTGCGGC | (Wang et al., 2002a) |
| | | BIL-2 | GGCTTGACATGTTCCCGACGATCCCAGAGATGGGGTTTCC | |
| | | BIL-3 | AGCCGGTGGCCTAACCCCTTGCGGGAGGGAGCCGTCTAATG | |
| | *B. adolecentis* | BIA-1 | GGATCGGCTGGAGCTTGCTCCGGCCGTGAGAGTGGCGAA | |
| | | BIA-2 | CTCCAGTTGGATGCATGTCCTTCTGGGAAAGATTCTATCGGT | |
| | | BIA-3 | CAACGGGATGCGACCTCGTGAGGGGGAGCGGATCCCTT | |


ACCEPTED MANUSCRIPT

| | | | | |
|---|---|---|---|---|
| | *B. infantis* | BIN-1 | ACCGGATGCTCCGCTCCATCGCATGGTGGGGTGGGAAAT | |
| | | BIN-2 | GGCTTGACATGTGCCGGATCGCCGTGGAGACACGGTTTCC | |
| | | BIN-3 | GGTAGACACCCGAAGCCGGTTGGCCCGACCCTTGTTGGG | |
| Fluorescence in situ hybridization | *Bifidobacterium* spp. | Bif164 | CATCCGGCATTACCACCC | (Langendijk et al., 1995) |
| | | Bif662 | CCACCGTTACACCGGGAA | |
| | | Bif1278 | CCGGTTTTCAGGGATCC | |
| | Genus *Bifidobacterium* | Bp153 | GAGGACCTTTGCCCACCA | (Matsuki et al., 2004) |
| | *B. adolescentis* | PAD | GCGAAAACTGACCCTCG | |
| | *B. catenulatum* group | pBiCATg | ACACCCCATGCGAGGAGT | |
| | *B. longum* | pBiLON | AGCCGTATCTCTACGACCGT | |
| | *B. bifidum* | pBiBIF | CCA CAATCACATGCGATCATG | |
| | Genus *Bifidobact* | Bif153 | ACCACCCGTTTCCAGGAG | (Takada et al., |

| | | | | |
|---|---|---|---|---|
| | *erium* | | | 2004) |
| | *B. adolescentis* | Bado434 | GCTCCCAGTCAAAAGCG | |
| | *B. angulatum* | Bang198 | AATCTTTCCCAGACCACC | |
| | *B. bifidum* | Bbif186 | CCACAATCACATGCGATCATG | |
| | *B. breve* | Bbre198 | AAAGGCTTTCCCAACACACC | |
| | *B. catenulatum group* | Bcat187 | ACACCCCATGCGAGGAGT | |
| | *B. dentium* | Bden82 | ACTCTCACCCGGAGGCGAA | |
| | *B. longum* | Blon1004 | AGCCGTATCTCTACGACCGT | |

Table 2. PCR primers in published papers (in chronological order)

| Bifidobacteria strains | Primer codes | Primer sequences (5'→3') | Target locations | Applications | References |
|---|---|---|---|---|---|
| Universal primer | P0[a] | GAGAGTTTGATCCTGGCTCAG | 16S rDNA | PCR/Identification/ ARDRA | (Di Cello, 1996; Ventura et al., 2001a) |
| | P6 | GTACGGCTACCTTGTTACGA | 16S rDNA | | |
| *B. breve* | BreU3 | CTTCCAGCTCGACTGTCGC | 16S rDNA | PCR/Identification | (Roy et al., 1996) |
| | BeL4 | GCACTTTGTGTTGAGTGTACCTTTCG | | | |
| Universal primer | L | GCTGGATCACCTCCTTTCT | 16S rDNA | PCR/Sequencing | (Leblond-Bourget et al., 1996) |
| | R | CTGGTGCCAAGGCATCCA | 23S rDNA | | |
| *B. infantis* | InfU5 | CCATCTCTGGGATCGTCGG | 16S rDNA | PCR/Identification | (Roy et al., 1996) |

| | | | | | |
|---|---|---|---|---|---|
| *B. indicum* | InfL6 | TATCGGGGAGCAAGCGTGA | | | |
| *B. longum* | LonU7 | GCCGTATCTCTACGACCGTCG | 16S rDNA | PCR/Identification | (Roy et al., 1996) |
| *B. pseudolongum* | LonL8 | TATCGGGGAGCAAGCGAGAG | | | |
| *B. adolescentis* | BIA-1 | GGAAAGATTCTATCGGTATGG | 16S rDNA | PCR/Identification | (Wang et al., 1996) |
| | BIA-2 | CTCCCAGTCAAAAGCGGTT | | | |
| *B. longum* | BIL-1 | GTTCCCGACGGTCGTAGAG | 16S rDNA | PCR/Identification | (Wang et al., 1996) |
| | BIL-2 | GTGAGTTCCCGGCATAATCC | | | |
| Genus *Bifidobacterium* | Bif164 | GGGTGGTAATGCCGGATG | 16S rDNA | PCR/Identification/ PCR-ARDRA/Sequencing | (Kok et al., 1996; Venema and Maathuis, 2003) |
| | Bif662 | CCACCGTTACACCGGGAA | | | |
| Genus *Bifidobacterium* | Bif16S3[b] | AGGGTTCGATTCTGGCTCAG | 16S rDNA | PCR/Identification/ Sequencing | (Kok et al., 1996) |



 

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|---|---|---|---|---|---|
| | Bif16S4 | ACGGTTACCTTGTTACGACTT | 16S rDNA | | |
| | Bif23S1 | GCCAAGGCATCCACCGT | 23S rDNA | | |
| *Bifidobacterium* sp. strain LW420 | LW420A | GCACGGTTTCGGCCGTG | 16S rDNA | PCR/Sequencing | (Kok et al., 1996) |
| | LW420C | GGATGCTCCGCTCCATCG | 16S rDNA | | |
| | LW420D[c] | GGGAAACCGTGTCTCCAC | 16S rDNA | | |
| Genus *Bifidobacterium* | lm26 | GATTCTGGCTCAGGATGAACG | 16S rDNA | PCR/Nested-PCR colony hybridization | (Kaufmann et al., 1997; Temmerman et al., 2003a) |
| | lm3 | CGGGTGCTI[d]CCCACTTTCATG | | | |
| Genus *Bifidobacterium* | RCL | TTY[e]ATH[f]GAY[e]GCN[g]GAR[h]CAY[e]GC | *rec*A gene | PCR-RFLP / Sequencing | (Kullen et al., 1997) |
| | RCR | TGR[h]TTD[i]ATR[h]AAD[i]ATN[g]GC | | | |
| *B. adolescе* | BiADO-1 | CTCCAGTTGGATGCATGTC | 16S rDNA | PCR/Identification/ real-time | (Matsuki et al., |



 

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|---|---|---|---|---|---|
| *ntis* | | | | PCR | 1998) |
| | BiADO-2 | CGAAGGCTTGCTCCCAGT | | | |
| *B. angulatum* | BiANG-1 | CAGTCCATCGCATGGTGGT | 16S rDNA | PCR/Identification/ real-time PCR | (Matsuki et al., 1998) |
| | BiANG-2 | GAAGGCTTGCTCCCCAAC | | | |
| *B. bifidum* | BiBIF-1 | CCACATGATCGCATGTGATTG | 16S rDNA | PCR/Identification/ real-time PCR | (Matsuki et al., 1998) |
| | BiBIF-2 | CCGAAGGCTTGCTCCCAAA | | | |
| *B. breve* | BiBRE-1 | CCGGATGCTCCATCACAC | 16S rDNA | PCR/Identification/ real-time PCR | (Matsuki et al., 1998) |
| | BiBRE-2 | ACAAAGTGCCTTGCTCCCT | | | |
| *B. catenulatum* and | BiCATg-1 | CGGATGCTCCGACTCCT | 16S rDNA | PCR/Identification/ real-time PCR | (Matsuki et al., 1998) |
| *B. pseudocatenulat* | BiCATg-2 | CGAAGGCTTGCTCCCGAT | | | |

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|---|---|---|---|---|---|
| *um* | | | | | |
| *B. longum* and *B. infantis* | BiLONg-1 | TTCCAGTTGATCGCATGGTC | 16S rDNA | PCR/Identification | (Matsuki et al., 1998) |
| | BiLONg-2 | TCS[i]CGCTTGCTCCCCGAT | | | |
| *B. longum* | BiLON-1 | TTCCAGTTGATCGCATGGTC | 16S rDNA | PCR/Identification/ real-time PCR | (Matsuki et al., 1999) |
| | BiLON-2 | GGGAAGCCGTATCTCTACGA | | | |
| *B. infantis* | BiINF-1 | TTCCAGTTGATCGCATGGTC | 16S rDNA | PCR/Identification/ real-time PCR | (Matsuki et al., 1999) |
| | BiINF-2 | GGAAACCCCATCTCTGGGAT | | | |
| *B. dentium* | BiDEN-1 | ATCCCGGGGGTTCGCCT | 16S rDNA | PCR/Identification/ real-time PCR | (Matsuki et al., 1999) |
| | BiDEN-2 | GAAGGGCTTGCTCCCGA | | | |
| *B. gallicum* | BiGAL-1 | TAATACCGGATGTTCCGCTC | 16S rDNA | PCR/Identification | (Matsuki et al., 1999) |
| | BiGA | ACATCCCCGAAAGGACGC | | | |

| | | | | | |
|---|---|---|---|---|---|
| | L-2 | | | | |
| *B. bifidum* | PBI245f[d] | GCTTGTTGGTGAGGTAACGGCT | 16S rDNA | mPCR/Identification | (Dong et al., 2000) |
| *B. breve* | PBR442f[d] | AGGGAGCAAGGCACTTTGTGT | 16S rDNA | mPCR/Identification | (Dong et al., 2000) |
| *B. infantis* | PIN710f[d] | CTGTTACTGACGCTGAGGAGCT | 16S rDNA | mPCR/Identification | (Dong et al., 2000) |
| *B. adolescentis* | PAD805f[d] | GTGGGGACCATTCCACGGTC | 16S rDNA | mPCR/Identification | (Dong et al., 2000) |
| *B. longum* | PLO965f[d] | TCCCGACGGTCGTAGAGATAC | 16S rDNA | mPCR/Identification | (Dong et al., 2000) |
| Genus *Bifidobacterium* | LDH F1 | TACATGCTCATCACCAACCCGGTCGAC | *ldh* gene | PCR-ARDRA/ Sequencing | (Roy and Sirois, 2000) |
| | LDH R1 | CATGCCGATGGCGTAGTTGGTGGCACCCTT | *ldh* gene | | |
| *Bifidobacterium* spp. | Pbi F1 | CCGGAATAGCTCC | 16S rDNA | PCR-ARDRA | (Roy and Sirois, 2000) |
| | Pbi R2 | GACCATGCACCACCTGTGAA | | | |

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|---|---|---|---|---|---|
| *B. animalis* | Ban F2 | AACCTGCCCTGTG | 16S rDNA | PCR/Identification | (Roy and Sirois, 2000) |
| | Pbi R1 | GCACCACCTGTGAACCG | | | |
| *B. infantis* | Bil F3 | AGTTGATCGCATGGTCTTCT | 16S rDNA | PCR/Identification | (Roy and Sirois, 2000) |
| | Inf R5 | CCATCTCTGGGATC | | | |
| *B. longum* | Pbi F1 | CCGGAATAGCTCC | 16S rDNA | PCR/Identification | (Roy and Sirois, 2000) |
| | Lon R4 | CGTATCTCTACGACC | | | |
| Genus *Bifidobacterium* | Bif164 F | GGGTGGTAATGCCGGATG | 16S rDNA | PCR-T-RFLP | (Bernhard and Field, 2000) |
| Genus *Bifidobacterium* | Bif601 R | TAAGCGATGGACTTTCACACC | | | |
| *B. breve* | Bre-BV.R | GCAAGAACGAGGAATCAAAGG | 16S-23S ITS region | PCR/Identification | (Brigidi et al., 2000) |
| | L | GCTGGATCACCTCCTTTCT | 16S | | |

| | | | | | |
|---|---|---|---|---|---|
| | | | rDNA | | |
| *B. infantis* Y1 | InfY-BV.L | TGTGGTTGCCAGTGTGTCC | 16S-23S ITS region | PCR/Identification | (Brigidi et al., 2000) |
| | R | CTGGTGCCAAGGCATCCA | 23S rDNA | | |
| *B. breve* Y8 | BreY-BV.R | GCAAGAACGAGGAATCAAACC | 16S-23S ITS region | PCR/Identification | (Brigidi et al., 2000) |
| | L | GCTGGATCACCTCCTTTCT | 16S rDNA | | |
| Bacteria | BOXA1R | TACGGCAAGGCGACGCTGACG | repetitive element | rep-PCR fingerprinting | (Gómez Zavaglia et al., 2000; Krizova et al., 2008; Masco et al., 2003; Shkoporov et al., 2008; Srutkova et al., 2011) |

| | | | | | |
|---|---|---|---|---|---|
| *B. lactis* DSM10140 | Bflact2 | GTGGAGACACGGTTTCCC | 16S rDNA | mPCR/Identification | (Ventura et al., 2001b) |
| *B. lactis* NCC311,NCC363 | | | | | (Ventura et al., 2001b) |
| | Bflact5 | CACACCACACAATCCAATAC | 16S-23S ISR | | |
| Genus *Bifidobacterium* | H60F | GG(ATGC)GA(CT)GG(ATGC)AC(ATGC)AC(ATGC)AC(ATGC)GC(ATGC)AC(ATGC)GT | *hsp*60 gene | PCR/PCR-RFLP/ sequencing | (Baffoni et al., 2013; Delcenserie et al., 2005; Jian et al., 2001) |
| | H60R | TC(ATGC)CC(AG)AA(ATGC)CC(ATGC)GG(ATGC)GC(CT)TT(ATGC)AC(ATGC)GC | | | |
| Bacteria | ERIC-1 | ATGTAAGCTCCTGGGGATTCAC | repetitive element | rep-PCR fingerprinting | (Masco et al., 2003; Shuhaimi et al., 2001; Ventura |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | et al., 2003b; Ventura and Zink, 2002) |
| | ERIC-2 | AAGTAAGTGACTGGGGTGAGCG | | | |
| Genus *Bifidobacterium* | g-Bifid-F | CTCCTGGAAACGGGTGG | 16S rDNA | PCR/Identification/ real-time PCR | (Matsuki et al., 2002) |
| | g-Bifid-R | GGTGTTCTTCCCGATATCTACA | | | |
| Genus *Bifidobacterium* | ForTal | CGTCGCCTTCTTCTTCGTCTC | Transaldolase gene | PCR-DGGE/ Identification | (Requena et al., 2002) |
| | RevTal | CTTCTCCGGCATGGTGTTGAC | | | |
| *B. animalis* ATCC 25527 | Ban2 | CATATTGGATCACGGTCG | 16S-23S ITS region | mPCR-PFGE | (Ventura and Zink, 2002) |
| *B. animalis* ATCC 27672 | 23Si | CATTCGGACACCCTGGGATC | | | |
| *B. lactis* | 16S-fo | GCTAGTAATCGCGGATCA | 16S-2 | PCR-ARD | (Ventur |

| strains[k] | r | | 3S ITS region | RA | a and Zink, 2002) |
|---|---|---|---|---|---|
| *B. breve* | Bbr[l] | GATGCGACAGTGCGAGC | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| *B. angulatum* | Bag[l] | CGTGTTGCCAGCACATG | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| *B. longum* | Blo[l] | GACATGTTCCCGACGGT | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| *B. adolescentis* | Bad[l] | GGGACCATTCCACGGTC | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| *B. bifidum* | Bbi[l] | AAGGGCTCGTAGGCGGC | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| *B. infantis* | Bin[l] | TATCGGGGAGCAAGCGT | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| *B. catenulatum* | Bc/p[l] | CGGATGCTCCGACTCCT | 16S rDNA | mPCR/Identification | (Germond et al., |

 

| | | | | | |
|---|---|---|---|---|---|
| group | | | | | 2002) |
| *B. catenulatum* | Bca[l] | AAGTCGAACGGGATCAG | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| *B. dentium* | Bde[l] | CATCGCTTAACGGTGGG | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| *B. pseudocatenulatum* | Bps[l] | GACAGCCGTAGAGATAT | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| *B. lactis* | Bla[l] | TGGCCGGTACAACGCGG | 16S rDNA | mPCR/Identification | (Germond et al., 2002) |
| Bacteria | $(GTG)_5$ | GTGGTGGTGGTGGTG | repetitive element | rep-PCR fingerprinting | (Krizova et al., 2008; Masco et al., 2003) |
| Bacteria | REP1R-I | IIII[b]CGICGICATCIGGC | repetitive element | rep-PCR fingerprinting | (Kulagina et al., 2010; Masco et al., 2003; |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | Srutkova et al., 2011) |
| | REP2-I | $I^{b}CGI^{b}CTTATCIGGCCTAC$ | | | |
| *B. infantis* Y1 | Y116Sl | GTCAAGTCATGAAAGTGGGTA | 16s rDNA | PCR/Identification/ real time PCR | (Vitali et al., 2003) |
| | Y1ITSr | GGACACACTGGCAACCACA | 16s-23S rDNA | | |
| *B. breve* Y8 | Y816Sl | TACAACGGGATGCGACAGC | 16s rDNA | PCR/Identification/ real time PCR | (Vitali et al., 2003) |
| | Y8ITSr | GAACGAGGAATCAAACCCCGTCT | 16s-23S rDNA | | |
| *B. longum* Y10 | Y1016Sl | GCAAGGCACTTTGTGTTGAG | 16s rDNA | PCR/Identification/ real time PCR | (Vitali et al., 2003) |
| | Y10ITSr | AAGAACGAGGAATCAAAGGAAACC | 16s-23S rDNA | | |
| *B. breve* | AB006658[m] | GGGAGCAAGGCACTTTGTGT | 16S rDNA | mPCR/Identification | (Mullie et al., 2003) |

| | | | | | |
|---|---|---|---|---|---|
| | | GAAACCCCATCTCTGGGATC | | | |
| *B. infantis* | D86184[m,n] | GAAACCCCATCTCTGGGATC | 16S rDNA | mPCR/Identification | (Mullie et al., 2003) |
| *B. angulatum* | D86182[m] | ATCGGCTGGAGCTTGCTCC | 16S rDNA | mPCR/Identification | (Mullie et al., 2003) |
| | | CATGCGATGGACTGGAGCAT | | | |
| *B. denticolens* | D89331[m] | GGAAAGCTTTTGCGCCATGG | 16S rDNA | mPCR/Identification | (Mullie et al., 2003) |
| | | GGCGATCGTCAACATGTCAA | | | |
| *B. scardovii* | AJ307005[m] | GTGATGGCCTACCGTGGCTT | 16S rDNA | mPCR/Identification | (Mullie et al., 2003) |
| | | CGTGAGTTCCCATCCAAAATG | | | |
| *B. gallicum* | D86189[m,o] | ACACAGGACCCGGAAAAAGG | 16S rDNA | mPCR/Identification | (Mullie et al., 2003) |
| nine *Bifidobacterium* strains[p] | Bif-1 | GAGTACGACTTCAACCAG | *tuf* gene | PCR/Sequencing | (Ventura et al., 2003a) |
| | Bif-2 | CAGGCGAGGATCTTGGT | | | |
| *B. animalis* and | 16S-for | GCTAGTAATCGCGGATCAG | 16S-23S ITS | PCR-RFLP | (Ventura and Zink, |

| | | | | | |
|---|---|---|---|---|---|
| *B. lactis* | | | region | | 2003) |
| | 23Si | CATTCGGACACCCTGGGATC | | | |
| Genus *Bifidobacterium* | 16S direct | AATAGCTCCTGGAAACGGGT | 16S rDNA | PCR-RFLP | (Delcenserie et al., 2004; Delcenserie et al., 2013) |
| | 16S reverse | CGTAAGGGGCATGATGATCT | | | |
| *Bifidobacterium* spp. | 27F[q] | AGAGTTTGATCCTGGCTCAG | 16S rDNA | PCR-T-RFLP<br>PCR-T-RFLP<br>PCR-T-RFLP | (Hayashi et al., 2004; Marcobal et al., 2008) |
| *Bifidobacterium* spp. | 35F[q] | CCTGGCTCAGGATGAACG | 16S rDNA | | (Hayashi et al., 2004) |
| *Bifidobacterium* spp. | 529F[q] | ACGTGCCAGCAGCCGCGG | 16S rDNA | | (Hayashi et al., 2004) |
| *Bifidobacterium* spp. | 1492R | GGTTACCTTGTTACGACTT | 16S rDNA | PCR-T-RFLP | (Hayashi et al., 2004; Marcobal et al., 2008) |



 

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|---|---|---|---|---|---|
| *B. infantis* | Bifinfant-F | TGTCTCCATCGAAGTTGATC | Not stated | PCR/Identification | (Drisko et al., 2005) |
| | Bifinfant-R | AACTTCTTCTCGAAGAGCTC | | | |
| *B. lactis* | Bifi.Lac-F | ATGCACAAACGACTGAGTCATA | Not stated | PCR/Identification | (Drisko et al., 2005) |
| | Bifi.Lac-R | ACGCATGTAGTCATCCGTAATA | | | |
| *B. bifidum* | Bifbif-F | TACGAGATTTGAGCCACTGT | Not stated | PCR/Identification | (Drisko et al., 2005) |
| | Bifbif-R | CGCTGGCAACACAAATCATC | | | |
| *B. bifidum* | BIFI-GAL-F | TGTCATGTACTTCTTCCGCGAC | β-galactosidase gene | PCR/Identification | (Drisko et al., 2005) |
| | BIFI-GAL-R | AGGTTGATGGTGAAGGTCTTGC | | | |
| Genus *Bifidobacterium* | IDBC 1R | ATCCGAACTGAGACCGGTT | 16S rDNA | mPCR/Identification | (Kwon et al., 2005) |
| | IDBC 2F | ATCGCAGTCTGCAACTCGA | | | |
| *B. adolescе* | IDB11 | ATCGGCTGGAGCTTGCT | 16S | mPCR/Iden | (Kwon et al., |

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|---|---|---|---|---|---|
| *ntis* | F[r] | | rDNA | tification | 2005) |
| *B. bifidum* | IDB21 F[r] | TGAGGTAACGGCTCACCAAGGCT | 16S rDNA | mPCR/Identification | (Kwon et al., 2005) |
| *B. breve* | IDB31 F[r] | TAGGGAGCAAGGCACTTTGTGT | 16S rDNA | mPCR/Identification | (Kwon et al., 2005) |
| *B. pseudolongum* | IDB41 F[r] | CCCTTTTTCCGGGTCCTGT | 16S rDNA | mPCR/Identification | (Kwon et al., 2005) |
| *B. longum* | IDB51 F[r] | CGGTCGTAGAGATACGGCTT | 16S rDNA | mPCR/Identification | (Kwon et al., 2005) |
| *B. animalis* and *B. lactis* | IDB61 F[r] | GCATGTTGCCAGCGGGTGA | 16S rDNA | mPCR/Identification | (Kwon et al., 2005) |
| *B. lactis* | IDB71 R | CCACACCACACAATCCAATACG | 16S rDNA | mPCR/Identification | (Kwon et al., 2005) |
| *B. infantis* and *B. longum* | IDB81 R | AGCAACACACACCATGAAGGTG | 16S rDNA | mPCR/Identification | (Kwon et al., 2005) |
| *B. longum* group | Not given(F) | TTCCAGTTGATCGCATGGTCTTCT | 16S rDNA | PCR/Identification/ real-time PCR | (Rinne et al., 2005) |

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|---|---|---|---|---|---|
| | Not given(R) | GGCTACCCGTCGAAGCCACG | | | |
| *B. adolescentis* | Not given(F) | GGATCGGCTGGAGCTTGCTCCG | 16S rDNA | PCR/Identification/ real-time PCR | (Rinne et al., 2005) |
| | Not given(R) | CCCCGAAGGCTTGCTCCCAGT | | | |
| *B. breve* | Not given(F) | AATGCCGGATGCTCCATCACAC | 16S rDNA | PCR/Identification/ real-time PCR | (Rinne et al., 2005) |
| | Not given(R) | GCCTTGCTCCCTAACAAAAGAGG | | | |
| *B. bifidum* | Not given(F) | TGACCGACCTGCCCCATGCT | 16S rDNA | PCR/Identification/ real-time PCR | (Rinne et al., 2005) |
| | Not given(R) | CCCATCCCACGCCGATAGAAT | | | |
| *B. catenulatum* group | Not given(F) | GCCGGATGCTCCGACTCCT | 16S rDNA | PCR/Identification/ real-time PCR | (Rinne et al., 2005) |
| | Not | ACCCGAAGGCTTGCTCCCGAT | | | |

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|---|---|---|---|---|---|
| | given(R) | | | | |
| *B. angulatum* | Not given(F) | GGATCGGCTGGAGCTTGCTCCG | 16S rDNA | PCR/Identification/ real-time PCR | (Rinne et al., 2005) |
| | Not given(R) | TCACCCGAAGGCTTGCTCCCCAA | | | |
| *B. dentium* | Not given(F) | ATCCCGGGGGTTCGCCTCC | 16S rDNA | PCR/Identification/ real-time PCR | (Rinne et al., 2005) |
| | Not given(R) | ATACCGATGGAACCTTTCCCGG | | | |
| *B. animalis* group | Not given(F) | ACCAACCTGCCCTGTGCACCG | 16S rDNA | PCR/Identification | (Rinne et al., 2005) |
| | Not given(R) | CCATCACCCCGCCAACAAGCT | | | |
| Genus *Bifidobacterium* | B11 up | GTS[h]CAY[c]GAR[f]GGY[c]CTS[h]AAGAA | *hsp* 60 | PCR/Identification | (Delcenserie et al., 2005) |
| Genus *Bifidobacterium* | B12 down | CCR[f]TCCTGGCCR[f]ACCTTGT | *hsp* 60 | | |

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|---|---|---|---|---|---|
| *B. breve* UCC 2003 | DnaJ1-uni | GAGAAGTTCAAGGACATCTC | *dnaJ1* gene | Sequencing | (Ventura et al., 2006) |
| | DnaJ1-rev | GCTTGCCCTTGCCGG | | | |
| *B. longum* DJ010A | PurF-uni | CATTCGAACTCCGACACCGA | *purF* gene | Sequencing | (Ventura et al., 2006) |
| | Puf-rev | GTGGGGTAGTCGCCGTTG | | | |
| *B. longum* DJ010A | RpoC-uni | GTGCACTCGGTCCACAG | *rpoC* gene | Sequencing | (Ventura et al., 2006) |
| | RpoC-rev | CATGCTCAACAACGAGAAG | | | |
| *B. longum* DJ010A | DnaB-uni | GTGCTTGGCCATGATGAT | *dnaB* gene | Sequencing | (Ventura et al., 2006) |
| | DnaB-rev | ACCTCGACAAGGTCGG | | | |
| *B. longum* DJ010A | DnaG-uni | CTGTGCCCGTTCCACGAC | *dnaG* gene | Sequencing | (Ventura et al., 2006) |
| | DnaG-rev | CTCGATGCGCAGGTCGCA | | | |
| *B. animalis* spp, | Xfp-uni | CTTCGG(AGCT)CC(AGCT)GA(AGCT)GAGAC | *xfp* gene | Sequencing | (Ventura et al., |

| | | | | | |
|---|---|---|---|---|---|
| *lactis* DSM 10140 | | | | | 2006) |
| | Xfp-rev | AGCCACTT(AGCT)GC(AGCT)TGCTG | | | |
| *B. breve* UCC 2003 | ClpC-uni | GAGTACCGCAAGTACATCGAG | *clpC* gene | Sequencing | (Ventura et al., 2006) |
| | ClpC-rev | CATCCTCATCGTCGAACAGGAAC | | | |
| Genus *Bifidobacterium* | BifF | ATTTGAGCCACTGTCTGGTG | 16S-23S ISR | mPCR/Identification | (Sul et al., 2007) |
| | BifR | CATCCGGGAACGTCGGGAAA | | | |
| *B. thermophilum* | Bthermᵃ | GATGTGCCGGGCTCCTGCATG | 16S rDNA | PCR/Identification | (Mathys et al., 2008) |
| *B. adolescentis* strains[s] | Bits-1 | GGTTTTCTGTGGCGCGGTTC | 16S-23S ITS region | PCR/Identification | (Tsai et al., 2008) |
| | Bits-2 | CTGGTGCCAAGGCATCCA | | | |
| *B. longum* | BLoF | GTATCCGTCCGACCCAGCAG | *tuf* gene | mPCR/Identification | (Sheu et al., 2009) |
| | BloR | GGTGACGGAGCCCGGCTTG | | | |

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|---|---|---|---|---|---|
| *B. longum* | F-long-IS | TGGAAGACGTCGTTGGTTT | 16S-23S ITS region | mPCR/Real-time PCR | (Sheu et al., 2009) |
| | R-long-IS | ATCGCGCCAGGCAAA | | | |
| *Bifidobacterium* spp. | Bifseq-tF | GCCCACATCGAGTACCAG | *tuf* gene | PCR/Identification | (Sheu et al., 2010) |
| | Bifseq-tR | CCACCGACGTCACCGGCG | | | |
| *B. animalis subsp. animalis / B. animalis* subsp.*lactis* | Bani-tF | TCACGACAAGTGGGTTGCCA | *tuf* gene | PCR/Identification/ real-time PCR | (Sheu et al., 2010) |
| | Bani-tR | GTTGATCGGCAGCTTGCCG | | | |
| *B. bifidum* | Bbif-tF | GTCAGGTGGGTGTCCCGCGT | *tuf* gene | PCR/Identification | (Sheu et al., 2010) |
| | Bbif-tR | ATGCCGACGATCTCGACCGG | | | |
| *B. breve* | Bbre-tF | CTGGCCGTCAACACTCCG | *tuf* gene | PCR/Identification | (Sheu et al., 2010) |
| | Bbre-tR | TGGCCACGCTCGACAGCT | | | |

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|---|---|---|---|---|---|
| *B. longum* subsp.*infantis* | Binf-tF | ATCCGTCCGACCCAGACC | *tuf* gene | PCR/Identification/ real-time PCR | (Sheu et al., 2010) |
| | Binf-tR | CTCGACATCCTCACGGCC | | | |
| *Bifidobacterium spp.* | Bif-tF | GTCCGTGACCTCCTCGAC | *tuf* gene | PCR/Identification | (Sheu et al., 2010) |
| | Bif-tR | GTGGAAGGTCTCGATGGAG | | | |
| *B. breve* strain Yakult | p1285-1F | AGCCAGTTTCGAGGTATGGC | Not stated | PCR/Identification | (Fujimoto et al., 2011) |
| | p1285-1151R | AGCCAGTTTCCGAAGTTACC | | | |
| *B. breve* strain Yakult | pBbrY-F | ATGGCAAAACCGGGCTGAA | Not stated | PCR/Identification/ real-time PCR | (Fujimoto et al., 2011) |
| | pBbrY-R | GCGGATGAGAGGTGGG | | | |
| VNTR 12 | Forward | AGGTATTCGGGGATGTTCGC | tandem repeat | MLVA | (Matamoros et al., 2011) |
| | Reverse | GTATGGCGACGGCACATTCC | | | |



 

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|---|---|---|---|---|---|
| VNTR 21 | Forward | GCCTGTCATTCGCCGTTCTG | tandem repeat | MLVA | (Matamoros et al., 2011) |
| | Reverse | ATCACCCAGCGTGTTTCGG | | | |
| VNTR 23 | Forward | TCGATCTTGAACTCGCACACC | tandem repeat | MLVA | (Matamoros et al., 2011) |
| | Reverse | TGCTTGACCTGCCATTCACC | | | |
| VNTR 25 | Forward | AATCGTGATTCCAGGTGCGG | tandem repeat | MLVA | (Matamoros et al., 2011) |
| | Reverse | CGTCGCATTCATACGGTCGG | | | |
| VNTR 26 | Forward | ACTGCATGTTCCGCAATACCC | tandem repeat | MLVA | (Matamoros et al., 2011) |
| | Reverse | ATGTCGGCGGCTTTGTAGTG | | | |
| Universal primer | E8F | AGAGTTTGATCCTGGCTCAG | 16S rDNA | PCR-ARDRA | (Pal et al., 2012) |
| | E1115 | AGGGTTGCGCTCGTTG | | | |

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|---|---|---|---|---|---|
| | R | | | | |
| Genus *Bifidobacterium* | Not given | GGCTN[e]GAGCTTGCTCCGGCT | 16S rDNA | cPCR/quantification | (Tang et al., 2012) |
| | | GN[e]CTCACCTTAGACGGCTCC | | | |
| *B. bifidum* BF-1 | pBF-1f | ATGGCAAAACCGGGCTGAA | Not stated | PCR/Identification/ real-time PCR | (Fujimoto and Watanabe, 2013) |
| | pBF-1r | GCG GAT GAG AGG TGG G | | | |
| *B. bifidum* OLB6378 | 1B-F1 | TCCCACGTTTGGGTAAAGGAT | Not stated | PCR/Identification/ real-time PCR | (Toshimitsu et al., 2013) |
| | 1B-R3 | TCAATTCTGGAGGTTCCTTGTTAT | | | |
| *B. bifidum* OLB6378 | C89 | TCCCACGTTTGG | Not stated | PCR-RADP | (Toshimitsu et al., 2013) |
| Genus *Bifidobacterium* | NBIF389 | GCCTTCGGGTTGTAAAC | 16S rDNA | PCR-T-RFLP | (Lewis et al., 2013) |
| | NBIF1018REV | GACCATGCACCACCTGTG | 16S rDNA | | |

[a] used in conjunction with reverse primer lm3 for Bifidobacteria genus-specific; [b] Bif16S3/Bif16S4 target to 16S rDNA, Bif16S3/Bif23S1 target to 16S-23S ISR; [c] used in conjunction with either forward primer LW420A or LW420C; [d] Inosine, matched nucleotide A, C, T and G; [e] C/T; [f] A/C/T; [g] A/G/C/T; [h] A/G; [i] A/G/T; [j] G/C; [k] *B.lactis* NCC363, *B.lactis* NCC 311, *B.lactis* NCC 239, *B.lactis* NCC 387, *B.lactis* NCC 383, and *B.lactis* NCC 402; [l] each of these forward primers are used in conjunction with reverse primer lm3; [m] primer names are not given, only accession number is given; [n] used in conjunction with forward primer BiINF-1; [o] used in conjunction with forward primer BiGAL-1; [p] *B.longum* ATCC 15707, *B. longum* NCC 2705, *B. infantis* ATCC 15697, *B. bifidum* ATCC 29521, *B. lactis* DSM 10140, *B. catenulatum* DSM 20103, *B. adolescentis* ATCC 15703, *B. breve* ATCC 15700, and *B. animalis* ATCC 25527; [q] used in conjunction with reverse primer 1492R; [r] used in conjunction with either reverse primer IDB71R or IDB81R; [s] *B. adolescentis* BCRC 14606, 14607, 14658, and 14608.



 

Table 3. Real-time PCR primers and probes in published papers (in chronological order)

| **Bifidobacteria strains** | **Primer/ probe codes** | **Primer/probe sequence (5'→3')** | **Target locations** | **References** |
|---|---|---|---|---|
| Genus *Bifidobacterium* | g-Bifid-F | CTCCTGGAAACGGGTGG | 16S rDNA | (Requena et al., 2002) |
| | g-Bifid-R | GGTGTTCTTCCCGATATCTACA | | |
| Genus *Bifidobacterium* | TAQfor<br>TAQrev<br>Not given (P) | GCGTCCGCTGTGGGC<br>CTTCTCCGGCATGGTGTT<br>FAM[a]-TCCACCGGCACCAAGAACGC-TAMRA[b] | Transaldolase gene | (Requena et al., 2002) |
| *B. infantis* Y1 | Y116Sl<br>Y1ITSr | GTCAAGTCATGAAAGTGGGTA | 16S rDNA | (Vitali et al., 2003) |
| | | GGACACACTGGCAACCACA | 16S-23S rDNA | |
| *B. breve* Y8 | Y816Sl<br>Y8ITSr | TACAACGGGATGCGACAGC | 16S rDNA | (Vitali et al., 2003) |
| | | GAACGAGGAATCAAACCCCGTCT | 16S-23S rDNA | |
| *B. longum* Y10 | Y1016Sl<br>Y10ITSr | GCAAGGCACTTTGTGTTGAG | 16S rDNA | (Vitali et al., 2003) |
| | | AAGAACGAGGAATCAAAGGAAACC | 16S-23S rDNA | |
| *B. lactis* | Not | CCCTTTCCACGGGTCCC | 16S | (Malinen |

| | | | | |
|---|---|---|---|---|
| | given (F) | | rDNA | et al., 2003) |
| | Not given (R) | AAGGGAAACCGTGTCTCCAC | | |
| | Not given (P) | HEX-AAATTGACGGGGGCCCGCACAAG C-DABCYL | | |
| *B. longum* | Not given (F) | CAGTTGATCGCATGGTCTT | 16S rDNA | (Malinen et al., 2003) |
| | Not given (R) | TACCCGTCGAAGCCAC | | |
| | Not given (P) | FAM-TGGGATGGGGTCGCGTCCTATCAG -TAMRA | | |
| *Bifidobacte rium* spp. | Not given(F) | TCGCGTC(C/T)GGTGTGAAAG | 16S rDNA | (Rinttila et al., 2004) |
| | Not given(R) | CCACATCCAGC(A/G)TCCAC | | |
| Genus *Bifidobacte rium* | Bifido5' | GATTCTGGCTCAGGATGAACGC | 16S rDNA | (Gueimon de et al., 2004) |
| | Bifido3' | CTGATAGGACGCGACCCCAT | | |
| | Bifidopr obe | CATCCGGCATTACCACCCGTTTCC**TC**[c] | | |
| *B.* | BiADOg | CTCCAGTTGGATGCATGTC | 16S | (Matsuki |

| | | | | |
|---|---|---|---|---|
| *adolescentis* group (genotype A and B) | -1a[d] | | rDNA | et al., 2004) |
| | BiADOg -1b[d] | TCCAGTTGACCGCATGGT | | |
| | BiADO-2 | CGAAGGCTTGCTCCCAGT | | |
| *B. angulatum* | BiANG-1 | CAGTCCATCGCATGGTGGT | 16S rDNA | (Matsuki et al., 2004) |
| | BiANG-2 | GAAGGCTTGCTCCCCAAC | | |
| *B. bifidum* | BiBIF-1 | CCACATGATCGCATGTGATTG | 16S rDNA | (Matsuki et al., 2004) |
| | BiBIF-2 | CCGAAGGCTTGCTCCCAAA | | |
| *B. breve* | BiBRE-1 | CCGGATGCTCCATCACAC | 16S rDNA | (Matsuki et al., 2004) |
| | BiBRE-2 | ACAAAGTGCCTTGCTCCCT | | |
| *B. catenulatum* group | BiCATg -1 | CGGATGCTCCGACTCCT | 16S rDNA | (Matsuki et al., 2004) |
| | BiCATg -2 | CGAAGGCTTGCTCCCGAT | | |
| *B. longum* | BiLON- | TTCCAGTTGATCGCATGGTC | 16S | (Matsuki |



 

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|---|---|---|---|---|
| biotype longum | 1 | | rDNA | et al., 2004) |
| | BiLON-2 | GGGAAGCCGTATCTCTACGA | | |
| *B. longum* biotype infantis | BiINF-1 | TTCCAGTTGATCGCATGGTC | 16S rDNA | (Matsuki et al., 2004) |
| | BiINF-2 | GGAAACCCCATCTCTGGGAT | | |
| *B. dentium* | BiDEN-1 | ATCCCGGGGGTTCGCCT | 16S rDNA | (Matsuki et al., 2004) |
| | BiDEN-2 | GAAGGGCTTGCTCCCGA | | |
| Universal | F_eub | TCCTACGGGAGGCAGCAGT | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_eub | GGACTACCAGGGTATCTAATCCTGTT | | |
| | P_eub | FAM[a]-CGTATTACCGCGGCTGCTGGCAC-NFQ[e] | | |
| Genus *Bifidobacterium* | F_allbif_IS | GGGATGCTGGTGTGGAAGAGA | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_allbif_IS | TGCTCGCGTCCACTATCCAGT | | |
| | P_allbif_IS | FAM[a]-TCAAACCACCACGCGCCA-NFQ[e] | | |



 

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|---|---|---|---|---|
| *B. adolescentis* | F_adol_IS | ATAGTGGACGCGAGCAAGAGA | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_adol_IS | TTGAAGAGTTTGGCGAAATCG | | |
| | P_adol_IS | FAM[a]-CTGAAAGAACGTTTCTTTTT-NFQ[e] | | |
| *B. angulatum* | F_angul_IS | TGGTGGTTTGAGAACTGGATAGTG | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_angul_IS | TCGACGAACAACAATAAACAAAACA | | |
| | P_angul_IS | FAM[a]-AAGGCCAAAGCCTC-NFQ[e] | | |
| *B. bifidum* | F_bif_IS | GTTGATTTCGCCGGACTCTTC | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_bif_IS | GCAAGCCTATCGCGCAAA | | |
| | P_bif_IS | FAM[a]-AACTCCGCTGGCAACA-NFQ[e] | | |
| *B. breve* | F_breve_IS | GTGGTGGCTTGAGAACTGGATAG | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_breve_IS | CAAAACGATCGAAACAAACACTAAA | | |
| | P_breve_IS | FAM[a]-TGATTCCTCGTTCTTGCTGT-NFQ[e] | | |

| | | | | |
|---|---|---|---|---|
| *B. catenulatum* | F_cate_IS | GTGGACGCGAGCAATGC | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_cate_IS | AATAGAGCCTGGCGAAATCG | | |
| | P_cate_IS | FAM[a]-AAGCAAACGATGACATCA-NFQ[e] | | |
| *B. dentium* | F_dent_IS | CCGCCACCCACAGTCT | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_dent_IS | AGCAAAGGGAAACACCATGTTT | | |
| | P_dent_IS | FAM[a]-ACGCGTCCAACGGA-NFQ[e] | | |
| *B. infantis* | F_inf_IS | CGCGAGCAAAACAATGGTT[f] | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_inf_IS | AACGATCGAAACGAACAATAGAGTT | | |
| | P_inf_IS | FAM[a]-TTCGAAATCAACAGCAAAA-NFQ[e] | | |
| *B. longum* | F_long_IS | TGGAAGACGTCGTTGGCTTT | 16S-23S ISR | (Haarman and Knol, 2005) |
| | R_long_IS | ATCGCGCCAGGCAAAA[f] | | |
| | P_long_IS | FAM[a]-CGCACCCACCGCA-NFQ[e] | | |

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|---|---|---|---|---|
| *Bifidobacterium* spp. | Not given(F) | GCGTGCTTAACACATGCAAGTC | 16S rDNA | (Penders et al., 2005) |
| | Not given(R) | CACCCGTTTCCAGGAGCTATT | | |
| | Not given(P) | TCACGCATTACTCACCCGTTCGCC | | |
| *B. longum* group | Not given(F) | TTCCAGTTGATCGCATGGTCTTCT | 16S rDNA | (Gueimonde et al., 2007) |
| | Not given(R) | GGCTACCCGTCGAAGCCACG | | |
| | Not given(P) | GCGACCCCATCCCATACCGCGA**TT**[c] | | |
| *B. adolescentis* | Not given(F) | GGATCGGCTGGAGCTTGCTCCG | 16S rDNA | (Gueimonde et al., 2007) |
| | Not given(R) | CCCCGAAGGCTTGCTCCCAGT | | |
| | Not given(P) | CTCCAGTTGGATGCATGTCCTTCTGG**CT**[c] | | |
| *B. breve* | Not given(F) | AATGCCGGATGCTCCATCACAC | 16S rDNA | (Gueimonde et al., 2007) |
| | Not given(R) | GCCTTGCTCCCTAACAAAAGAGG | | |
| | Not | CATGCCGCAAAGGCTTTCCCAACAC**TG**[c] | | |


ACCEPTED MANUSCRIPT

| | | | | |
|---|---|---|---|---|
| | given(P) | | | |
| *B. bifidum* | Not given(F) | TGACCGACCTGCCCCATGCT | 16S rDNA | (Gueimonde et al., 2007) |
| | Not given(R) | CCCATCCCACGCCGATAGAAT | | |
| | Not given(P) | TGTTCCACATGATCGCATGTGATTGTG**C C**[c] | | |
| *B. catenulatum* group | Not given(F) | GCCGGATGCTCCGACTCCT | 16S rDNA | (Gueimonde et al., 2007) |
| | Not given(R) | ACCCGAAGGCTTGCTCCCGAT | | |
| | Not given(P) | TACCGATGAAATCTTTCCCGACACCC**G T**[c] | | |
| *B. angulatum* | Not given(F) | GGATCGGCTGGAGCTTGCTCCG | 16S rDNA | (Gueimonde et al., 2007) |
| | Not given(R) | TCACCCGAAGGCTTGCTCCCCAA | | |
| | Not given(P) | ATCTTTCCCAGACCACCATGCGATGGA C**AC**[c] | | |
| *B. dentium* | Not given(F) | ATCCCGGGGGTTCGCCTCC | 16S rDNA | (Gueimonde et al., 2007) |
| | Not given(R) | ATACCGATGGAACCTTTCCCGG | | |

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|---|---|---|---|---|
| | Not given(P) | TGCTCCGGTTGGATGCATGTCCTTCC**CC**[c] | | |
| *B.* spp. | rec-A-F | CGTY[g]TCB[h]CAGCCGGAY[g]AAC | *recA* gene | (Masco et al., 2007) |
| | recA-R | CCAR[i]V[j]GCR[i]CCGGTCATC | | |
| Genus *Bifidobacterium* | F-bifido | CGCGTCY[g]GGTGTGAAAG | 16S rDNA | (Delroisse et al., 2008) |
| | R-bifido | CCCCACATCCAGCATCCA | | |
| | MGB-bifido[k] | FAM[a]-AACAGGATTAGATACCC-NFQ[e] | | |
| *B. thermophilum* | bthermRTF | TTGCTTGCGGGTGAGAGT | 16S rDNA | (Mathys et al., 2008) |
| | bthermRTR | CGCCAACAAGCTGATAGGAC | | |
| | bthermTqM | FAM[a]-ATGTGCCGGGCTCCTGCAT-TAMRA[b] | | |
| *B. animalis* subsp. *lactis* strain Bb12 | Not given(F) | GTGTCGAGCGCGGCAA | *tuf* gene | (Solano-Aguilar et al., 2008) |
| | Not given(R) | CTCGCACTCATCCATCTGCTT | | |
| | BGB probe | 6-TET-phosphoramidite[l]-ATCAACACGAACGTCGAGA-BHQ1[m] | | |
| *B. animalis* | Not | CGACAAGAAGCTCGAGGAGAT | transald | (Solano- |

| | | | | |
|---|---|---|---|---|
| | given(F) | | olase gene | Aguilar et al., 2008) |
| | Not given(R) | CGGATCCTCGGCGAACT | | |
| | Not given(P) | 6-TET-phosphoramidite[l]-CCTTGCCTTCGAG ACCCTTGGCCT-BHQ1[m] | | |
| *B. suis* *B. breve* *B. longum* | Not given(F) | GCGTCCGCTGTGGGC | transald olase group gene | (Solano-Aguilar et al., 2008) |
| | Not given(R) | CTTCTCCGGCATGGTGTT | | |
| | Not given(P) | 6-TET-phosphoramidite[l]-TCCACCGGCACC AAGAACGC-BHQ1[m] | | |
| *B. animalis* *B. animalis* subsp. *lactis* | Not given(F) | TTGGCCCAGGTCGTCGT | *groES* gene | (Solano-Aguilar et al., 2008) |
| | Not given(R) | AGGTATTCCTCGCCCTTGAAGT | | |
| | Not given(P) | 6-TET-phosphoramidite[l]-AGGGCGAGCGTG TTCCCATGGA-BHQ1[m] | | |
| | BGB probe | 6-TET-phosphoramidite[l]-CGTGTTCCCATGG AC-BHQ1[m] | | |
| *B. animalis* *B. animalis* subsp. *lactis* | Not given(F) | TTTGCCGAGTGCGATGGT | 16S-23S ISR | (Solano-Aguilar et al., 2008) |

| | | | | |
|---|---|---|---|---|
| | Not given(R) | GTGGCGGCCAGGGAAC | | |
| | Not given(P) | 6-TET-phosphoramidite[l]-CCTGGCTTGCTGG CGTGGAAGAG-BHQ1[m] | | |
| *B. animalis* *B.* subsp. *lactis* | Not given(F) | GAAGGCGATATGGGTGACAG | *recA* gene | (Solano-Aguilar et al., 2008) |
| | Not given(R) | GCCGATCTTCTCTCGCAACT | | |
| | BGB probe | 6-TET-phosphoramidite[l]-CACAGGCGAACA CGA-BHQ1[m] | | |
| Genus *Bifidobacterium* | Bif164F | GGGTGGTAATGCCGGATG | 16S rDNA | (Martin et al., 2009) |
| | Bif662R | CCACCGTTACACCGGGAA | | |
| *B. animalis* subsp. *animalis*/*lactis* | Bani-tF | TCACGACAAGTGGGTTGCCA | *tuf* gene | (Sheu et al., 2010) |
| | Bani-tR | GTTGATCGGCAGCTTGCCG | | |
| *B. longum* subsp. *longum* | BloF | GTATCCGTCCGACCCAGCAG | *tuf* gene | (Sheu et al., 2010) |
| | BloR | GGTGACGGAGCCCGGCTTG | | |
| *Bifidobacterium* spp. | xfp-fw | ATCTTCGGACCBGAY[g]GAGAC | *xfp* gene | (Centanni et al., |

| | | | | |
|---|---|---|---|---|
| | xfp-rv | CGATV[j]ACGTGV[j]ACGAAGGAC | | 2013; Cleusix et al., 2010) |
| | Xfp-T | FAM[a]-ACGGCATCTGGAGCTCCTA-TAMRA[b] | | |
| *B. breve* strain Yakult | pBbrY-F | ATGGCAAAACCGGGCTGAA | Not stated | (Fujimoto et al., 2011) |
| | pBbrY-R | GCGGATGAGAGGTGGG | | |
| *B. longum* NCC2705 | Not given (F) | CAACCGCCGCGATCTTC | *cysS* gene 57bp | (Reimann et al., 2010) |
| | Not given (R) | CCAGCTGTGAAAGCAACGTATT | | |
| *B. longum* NCC2705 | Not given (F) | CATGGGCGGCCTTGAGT | *purB* gene 57bp | (Reimann et al., 2010) |
| | Not given (R) | TCAAGCTCACGCTCGATGAC | | |
| *B. longum* NCC2705 | Not given (F) | AGCAGCGGCATATCCTTGAA | *purB* gene 400bp | (Reimann et al., 2010) |
| | Not given (R) | TTCTGGCCAACGGCTTTG | | |
| Genus *Bifidobacte* | Bif-F | TCTGGCTCMGGATGAACGC | 16S rDNA | (Meng et al., 2010) |



 

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| *rium* | | | | |
| | Bif-R | CACCGTTACACCGGGAATTC | | |
| | Not given (P) | FAM[a]-CCAGGCATCCGGCATTACCACCC GTCCTGG-DABCYL[n] | | |
| *B. bifidum* PRL2010 | 375_fw | ATGATCAAGGGCATTCAGGA | *pdxS* gene | (Turroni et al., 2011) |
| | 375_rev | CGTCGAACTGTGTCTTGTCG | | |
| *B. bifidum* PRL2010 | 429_fw | ATACTCCGAGAATGCGGATG | *uvrD*/Rep gene | (Turroni et al., 2011) |
| | 429_rev | ACGACATCCCGCTCATATTC | | |
| *B. bifidum* PRL2010 | 670_fw | CGCATCTCTCCGATATCCTC | *gluC* gene | (Turroni et al., 2011) |
| | 670_rev | ACAGACTCAGTCCCGTCACC | | |
| *B. adolescentis* | B_ado-f | CTCCGCCGCTGATCCGGAAGTCG | *groEL* gene | (Junick and Blaut, 2012) |
| | B_ado-r | AACCAACTCGGCGATGTGGACGACA | | |
| *B. angulatum* | B_ang-f | CTGTCCTCCCAGCAGGACGTGGTC | *groEL* gene | (Junick and Blaut, 2012) |
| | B_ang-r | GCGCTTCGCCGTCAACGTCTTCGG | | |
| *B. animalis* | B_ani-f | CACCAATGCGGAAGACCAG | *groEL* | (Junick and Blaut, |

| | | | | |
|---|---|---|---|---|
| | | | gene | 2012) |
| | B_ani-r | GTTGTTGAGAATCAGCGTGG | | |
| *B. bifidum* | B_bif-f | CTCCGCAGCCGACCCCGAGGTT | *groEL* gene | (Junick and Blaut, 2012) |
| | B_bif-r | TGGAAACCTTGCCGGAGGTCAGG | | |
| *B. breve* | B_bre-f | GCTCGTCGTTGCCGCCAAGGACGTT | *groEL* gene | (Junick and Blaut, 2012) |
| | B_bre-r | ACAGAATGTACGGATCCTCGAGCACG | | |
| *B. catenulatum* | B_cat-f | GGCTATCGTCAAGGAGCTCA | *groEL* gene | (Junick and Blaut, 2012) |
| | B_cat-r | AGTCCAGATCCAAACCGAAAC | | |
| *B. dentium* | B_den-f | GGCCCAGTCTTTGGTGCATGAAGGCC | *groEL* gene | (Junick and Blaut, 2012) |
| | B_den-r | GTCTTCGAGCACCGCGGTCTGGTCC | | |
| *B. gallicum* | B_gal-f | AGCTCGTCAAGTCCGCCAAGC | *groEL* gene | (Junick and Blaut, 2012) |
| | B_gal-r | CATACCTTCGGTGAACTCGAGG | | |
| *B. longum* | B_lon-f | CGGCGTY$^{g}$GTGACCGTTGAAGAC | *groEL* gene | (Junick and Blaut, 2012) |



 

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|---|---|---|---|---|
| | B_lon-r | TGY[g]TTCGCCR[i]TCGACGTCCTCA | | |
| *B. pseudocatenulatum* | B_pcat-f | AGCCATCGTCAAGGAGCTTATCGCAG | *groEL* gene | (Junick and Blaut, 2012) |
| | B_pcat-r | CACGACGTCCTGCTGAGAGCTCAC | | |
| *B. pseudolongum* | B_plon-f | CRATY[g]GTCAAGGAACTY[g]GTGGCCT | *groEL* gene | (Junick and Blaut, 2012) |
| | B_plon-r | GCTGCGAM[o]GAK[p]ACCTTGCCGCT | | |
| *B. thermophilum* | B_the-f | ACTGGTCGCTTCCGCCAAGGATG | *groEL* gene | (Junick and Blaut, 2012) |
| | B_the-r | CCARGTCAGCM[o]AGGTGRACGATG | | |
| Genus *Bifidobacterium* | Bif-F | TTCGGGTTGTAAACCGCTTTT | 16S rDNA | (Gomez-Donate et al., 2012) |
| | Bif-R | TACGTATTACCGCGGCTGCT | | |
| Human *Bifidobacterium* | HMprobe | VIC[q]-TCGGGGTGAGTGTACCT-NFQ[e] | 16S rDNA | (Gomez-Donate et al., 2012) |
| Poultry *Bifidobacterium* | PLprobe | FAM[a]-GAGAGTGAGTGTACCCGTT-NFQ[e] | 16S rDNA | (Gomez-Donate et al., 2012) |
| Pig *Bifidobacterium* | PGprobe | FAM[a]-CGCAAGTGAGTGTACCTT-NFQ[e] | 16S rDNA | (Gomez-Donate et al., 2012) |

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|---|---|---|---|---|
| Cattle *Bifidobacterium* | CWprobe | FAM[a]-TTCGGCCGTGTTGAGT-NFQ[e] | 16S rDNA | (Gomez-Donate et al., 2012) |
| *B. bifidum* OLB6378 | 1B-F1 | TCCCACGTTTGGGTAAAGGAT | Not stated | (Toshimitsu et al., 2013) |
| | 1B-R3 | TCAATTCTGGAGGTTCCTTGTTAT | | |
| *B. bifidum* BF-1 | BiBIF-1 | CCACATGATCGCATGTGATTG | 16S rDNA | (Fujimoto and Watanabe, 2013) |
| | BiBIF-2 | CCGAAGGCTTGCTCCCAAA | | |
| *B. animalis* subsp. *lactis* | Not given (F) | GTGGAGACACGGTTTCCC | 16S rDNA | (Tobin et al., 2013) |
| | Not given (R) | CACACCACACAATCCAATAC | 16S-23S ISR | |
| | Not given (P) | FAM[a]-TTCACAGGTGGTGCATGGTCGT-BHQ1[m] | | |
| *B. longum* subsp. *infantis* | Not given (F) | TTCCAGTTGATCGCATGGTC | 16S rDNA | (Tobin et al., 2013) |
| | Not given (R) | GGAAACCCCATCTCTGGGAT | | |
| | Not given (P) | CY5[r]-TCAAg[s]CCCAg[s]g[s]TAAg[s]g[s]TTCTTCg[s]C-BHQ3[t] | | |

[a] FAM: 6-carboxyfluoroscein; [b] TAMRA: 6-carboxytetramethylrhodamine; [c] Bases that are not complementary to the target are shown in bold letters; [d] PCR mixtures contains both BiADOg-1a and BiADOg-1b, BiADOg-1a is used for *B. adolescentis* genotype A detection, and BiADOg-1b is used for *B. adolescentis* genotype B detection; [e] NFQ: non-fluorescent quencher; [f] Concessions to these probes and primers design were made because of more than three consecutive nucleotides are the same of amplicon length is greater than 150 bp; [g] Y: C/T; [h] B: G/C/T; [i] R: A/G; [j] V: A/G/C; [k] MGB: minor groove binding probe; [l] 5'-tetrachloro-fluoresceinphosphoramidite; [m] BHQ1: Black hole quencher 1; [n] DABCYL: DABCYL quencher; [o] M: A/C; [p] K: G/T; [q] VIC: VIC fluorophore; [r] CY5: CY5 fluorophore; [t] BHQ3: Black hole quencher 3.



 

Table 4. Primers and probes used in PCR-ELISA

| Bifidobacteria strains | Primer/probe codes | Primer/probe sequence (5'→3') | Target locations | References |
|---|---|---|---|---|
| Genus *Bifidobacterium* | lm26-forward | GATTCTGGCTCAGGATGAACG | 16S rDNA | (Laitinen et al., 2002; Malinen et al., 2002) |
| | lm3-reverse | CGGGTGCTICCCACTTTCATG | 16S rDNA | (Laitinen et al., 2002; Malinen et al., 2002) |
| *B. adolescentis* | ado440[a] | CGCTTTTGACTGGGAGC | 16S rDNA | (Laitinen et al., 2002) |
| *Bifidobacterium spp.* | b162[a] | CATGAAAGTGGGI[b]AGCACCCG | 16S rDNA | (Laitinen et al., 2002) |
| *B. bifidum* | bcun180[a] | CCACATGATCGCATGTGATTG | 16S rDNA | (Malinen et al., 2002) |
| *B. breve* | bre456[a] | GCACTTTGTGTTGAGTGTACCT | 16S rDNA | (Malinen et al., 2002) |
| *B. catenulatum* and | caps165[a] | CGGATGCTCCGACTCCTC | 16S rDNA | (Malinen et al., |

| | | | | |
|---|---|---|---|---|
| *B. pseudocatenulatum* | | | | 2002) |
| *B. denticolens* | denc60[a] | GGATCCTCTCCAGCTTGCTGG | 16S rDNA | (Malinen et al., 2002) |
| *B. dentium* | dent16S[a] | ATCCCGGGGGTTCGCCT | 16S rDNA | (Malinen et al., 2002) |
| *B. infantis* and *B. longum* | ilp440[a] | AAGCGTGAGTGAGTTTACC | 16S rDNA | (Malinen et al., 2002) |
| *B. lactis* | lac980[a] | CCGGATCGCCGTGGAGACAC | 16S rDNA | (Malinen et al., 2002) |
| *B. longum* and *B. suis* | lsp960[a] | GACGGTCGTAGAGATACGGCTT | 16S rDNA | (Malinen et al., 2002) |

[a]biotinylated oligoprobes; [b] I: A/C/T/G.



 

Table 5. PCR primers used in PCR-DGGE

| Target | Primer codes | Primer sequences (5'→3') | Target locations | References |
|---|---|---|---|---|
| Genus *Bifidobacterium* | Bif164-GC-f | GGGTGGTAATGCCGGATG | 16S rDNA | (Martin et al., 2009; Satokari et al., 2001a) |
| | Bif662-GC-r | CCACCGTTACACCGGGAA | | |
| Genus *Bifidobacterium* | Bif164-mod-f | GGGTGGTAATACCGGATG | 16S rDNA | (Gomez-Donate et al., 2012; Satokari et al., 2001b) |
| | Bif662-GC-r | CCACCGTTACACCGGGAA | | |
| Genus *Bifidobacterium* | ForTal | CGTCGCCTTCTTCTTCGTCTTC | transaldolase gene | (Requena et al., 2002) |
| | RevTal-GC | CTTCTCCGGCATGGTGTTGAC | | |
| Bacteria | U968-GC-f | AACGCGAAGAACCTTAC | 16S rDNA | (Favier et al., 2002; Piacentini et al., 2010; Stsepetova et al., 2007) |
| | L1404-r | CGGTGTGTACAAGACCC | | |
| Bacteria | F357-GC | TACGGGAGGCAGCAG | 16S rDNA | (Joossens et al., 2011; Temmerman |

| | | | | |
|---|---|---|---|---|
| | | | | et al., 2003a; Temmerman et al., 2003b) |
| | 518R | ATTACCGCGGCTGCTGG | | |
| Bacteria | U968F-GC | AACGCGAAGAACCTTAC | 16S rDNA | (Temmerman et al., 2003a) |
| | L1401R | GCGTGTGTACAAGACCC | | |
| Bacteria | HDA1 | ACTCCTACGGGAGGCAGCAGT | 16S rDNA | (Theunissen et al., 2005) |
| | HDA2 | GTATTACCGCGGCTGCTGGCA | | |
| Actinobacteria | Eub338F | ACTCCTACGGGAGGCAGC | N/A | (Hoyles et al., 2013) |
| | Act1159R | TCCGAGTTRACCCCGGC | | |
| Universal | P2 | ATTACCGCGGCTGCTGG | N/A | (Hoyles et al., 2013) |
| | P3 | CCTACGGGAGGCAGCAG | | |