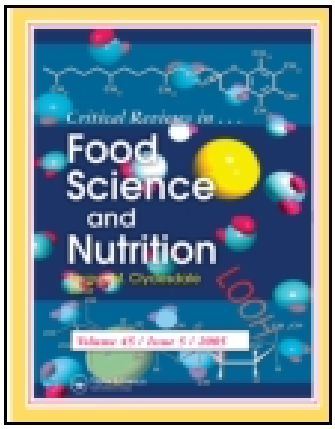

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Comparison of Molecular Techniques with other Methods for Identification and Enumeration of Probiotics in Fermented Milk Products

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Comparison of Molecular Techniques with other Methods for Identification and Enumeration of Probiotics in Fermented Milk Products

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Nowadays, an increasing attention is being given to fermented milk products including yogurt, kefir, buttermilk, and acidophilus milk. Fermented milks, especially the ones containing probiotics, are claimed to be useful for health of host (such as intestinal- and immune-associated diseases). Their healthful effects could be significantly enhanced by incorporating probiotic microorganisms; those have healthful advantages for host when consumed in an appropriate viable number in food products. Probiotic dairy products have stepped to the market and are being commercially produced under various brand names. In addition, these products are legislatively obliged to be labeled for the microorganisms contained. Therefore, identification and enumeration of their microorganisms are a cause of concern. Several culture-dependent methods have been introduced and used to identify the microorganisms, in which the researchers have experienced multiple difficulties. Thereby, molecular approaches were present as an alternative, offering advantages such as accuracy, sensitivity, specificity, and speed. This article reviews the molecular approaches employed for identification and enumeration of probiotics in fermented milk products.

Keywords Fermented milk products, yogurt, molecular techniques, probiotics

INTRODUCTION

Fermented milk as defined by Codex Alimentarius (Anonymous, 2003) is a milk product obtained by fermentation of milk by the action of suitable microorganisms and resulting in reduction of pH with or without coagulation. These starter microorganisms shall be viable, active, and abundant in the product to the date of minimum durability, except for those exposed to postfermentation heat treatment. Fermented milks are very popular due to their specific organoleptic properties, health benefits, and extended shelf life, especially the ones containing probiotics.

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Probiotics are defined as "live microorganisms able to confer a health benefit on the host when administered in adequate amount" (FAO/WHO, 2002). Several health benefits have been attributed to probiotics such as antimutagenic and anticarcinogenic properties, immune system stimulation or immunomodulation, anti-infection properties, reduction of serum cholesterol, alleviation of lactose intolerance/lactose maldigestion, and nutritional enhancement (Mortazavian et al., 2005; Mortazavian and Sohrabvandi, 2006; Mortazavian et al., 2008). The quantity of probiotics in probiotic products when consumed, in order to present pharmaceutical effectiveness, is matter of importance. Despite the fact that there is a gap on universal agreement regarding the minimum account of viable probiotic cells per milliliter or gram of final product at the moment of consumption, the values of 10^6 cfu mL^{-1} or cfu g^{-1} as minimal and $>10^7$ (generally 10^7–10^8 cfu mL^{-1} or cfu g^{-1}) as satisfactory levels have been regarded, respectfully. Moreover, it is worth noting that

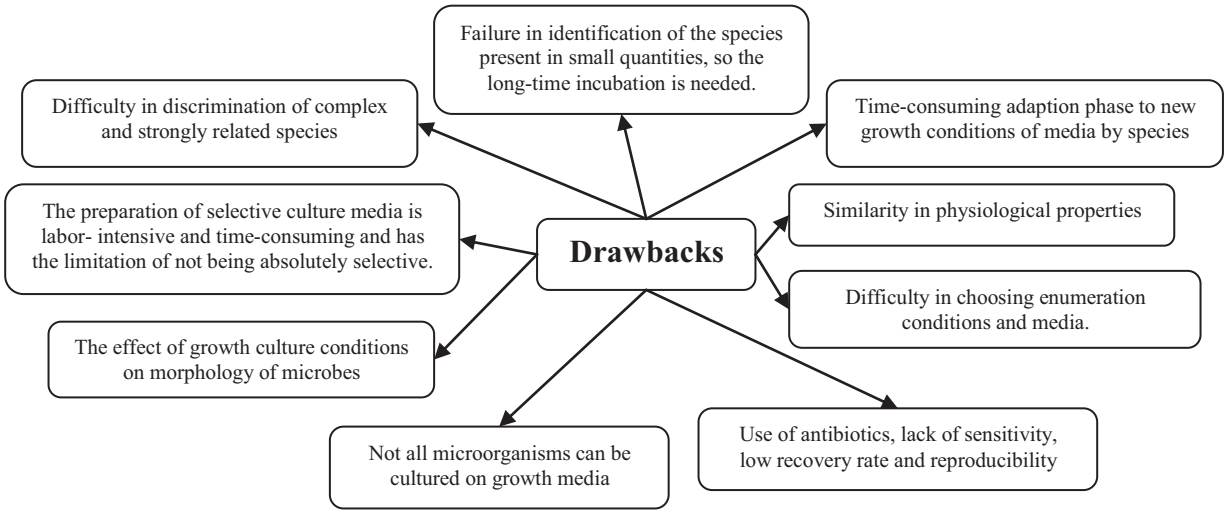

Figure 1 Drawbacks of culture-based techniques for microbial identification and/or enumeration.

the regular consumption of probiotic products has been recommended, by meeting a daily intake of approximately 10^9 viable cells (Sohrabvandi et al., 2010). However, the minimum counts of probiotics based on revealing more details on them would be likely to be altered in functionality of strain and the health effect preferred (Roy, 2005).

Therefore, identification and enumeration of probiotic strains present in fermented milk products is matter of importance (Hamilton-Miller et al., 1999; Ouwehand et al., 2002). Also, identification of probiotic strains is an important issue due to discover the useful species for industrial, technological, and probiotic applications (Klein et al., 1998; Wouters et al., 2002; Collado et al., 2006).

In order to perform the identification and enumeration precisely the researchers have developed and improved various culture-dependent and culture-independent methods. As given in Fig. 1, the classical methods faced investigators with drawbacks which led them to culture-independent methods based on genetic analysis as a valuable tool, since these techniques have offered unique advantages such as analysis the entire product without need to previously isolation (Tabasco et al., 2007); accuracy, sensitivity, simplicity, and the chance of robotic automation (Powell et al., 2006). However, these methods can be expensive, sophisticated, and demand the personnel who are well trained (Lim et al., 2009). Based on mentioned advantages related to molecular approaches, their application has conferred revolution in food microbiology.

Application of molecular methods for identification and enumeration of microorganisms has been reviewed by others from different aspects (Charteris et al., 1997; Vaughan et al., 1999; Giraffa and Neviani, 2000; Dubernet et al., 2002; Reutera et al., 2002; Coeuret et al., 2003; Lick, 2003; Randazzo et al., 2009). In this article, the authors have focused on molecular methods used in identification and enumeration of probiotics (and adjunct/support) cultures present in fermented milk products.

MOLECULAR APPROACHES MOST USED TO IDENTIFY AND/OR ENUMERATE THE PROBIOTICS IN FERMENTED MILKS

Several and different phenotypic techniques have been applied for microbial identification of fermented milk products which are mainly grouped in three categories: PCR-based, non-PCR-based techniques, and combination of two or more methods. The most used approaches have been illustrated in Fig. 2 that will be discussed in more details later. In addition, the dominant probiotics identified in yogurt and other fermented milk products along with the identification method employed and ideas of the authors on applied molecular techniques have been summarized in Tables 1 and 2, respectively; wherein most authors have proposed the employed approaches as rapid, simple, reliable, and effective tools in characterization of species compared to classical methods.

PCR-Based Molecular Techniques

Specific Polymerase Chain Reaction

The rapid and reliable detection of species by polymerase chain reaction (PCR)-based techniques, equipped with species-specific primers targeting 16S rRNA gene sequence diversity (Coeuret et al., 2003). 16S rRNA sequencing was not the best to classify the analogous strains and intraspecies; for instance, the high similarity present between *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. *lactis* led to failure in discrimination of these two subspecies (Drake et al., 1996b; Tilsala-Timisjärvi and Alatossava, 1997). In order to overcome this problem, Drake et al. (1996b) designed species-specific primers for PCR to amplify DNA from *Lactobacillus* species and Torriani et al. (1999) introduced specific primers designed based on *pep IP* and *pep I* genes encoding

Table 1 Selected publications on molecular methods to identify and/or enumerate the probiotics and/or adjunct cultures in yogurt

Product type	Molecular method*	Primer/probe	Species	Notes	Reference
Yogurt	Species-specific DNA probes in dot-blot hybridization	IS 1201, pY85, and LL	*Lactobacillus delbrueckii* subsp. *bulgaricus*	Enhancement of differentiation between strongly related strains and detection of atypical isolates by combining various molecular methods.	Andrighetto et al. (1998)
	PCR using species-specific oligonucleotide primers	DB1, SS1, HE1, and SS2			
	ARDRA analysis of the 16S rRNA gene	The same PCR protocol		ARDRA was not able to identify all strains present.	
	PCR fingerprinting	G1 and M13		PCR fingerprinting enabled reliable enumeration at species or subspecies level.	
Mild and probiotic yogurt products, and yogurt starter culture	DNA–DNA hybridization followed by spectrophotometry	–	*Lactobacillus acidophilus, Lactobacillus johnsonii, Lactobacillus crispatus, Lactobacillus casei, Lactobacillus paracasei,* and *Lactobacillus rhamnosus*		Schillinger (1999)
Bulgarian yogurt and fermented milks	Specific PCR	LB1 and LLB1	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. *lactis*	Suitable characterization at subspecies level by using 1,065- and 1,600-bp fragments specific for *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. *lactis*, respectively.	Torriani et al. (1999)
	RAPD-PCR	M13 and 1254		Possibility of specific amplification with primers LB1 and LLB1. Potential use of RAPD-PCR assay with primer M13 to apply either separately or in combination for greater reliability. RAPD-PCR with primer 1254 was superior for discrimination individual strains, but not applicable at subspecies.	
Yakult, yogurt, and fermented milk	RFLP using the 23S–5S rRNA intergenic spacer regions (ISRs) (ribotyping)	23-Fl and 5-Ru	*Lactobacillus paracasei, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus fermentum,* and *Lactobacillus plantarum,*	Polymorphism within the spacer sequences between 23S and 5S rRNAs among bacteria in the *Lactobacillus casei*-related group found to be useful for detection.	Chen et al. (2000)

Yogurt	Species-specific PCR amplifying a 1,340-bp fragment within the variable V1 region of the 16S rRNA gene	Forward primer corresponded to positions 25–42 of the *L. brevis* 16S rRNA gene and reversed primer corresponded to positions 1,348 ± 1,365 of the *L. brevis* 16S rRNA gene	*Lactobacillus brevis*		Guarneri et al. (2001)
Bulgarian yogurt and homemade yogurt	tDNA-PCR in combination with capillary electrophoresis	T5Aand T3 Roy, B	*Lactobacillus delbrueckii* subsp. *bulgaricus*	The tDNA fingerprints from lactobacilli were specific to distinguish lactobacilli from over 180-Gram positive and 170 Gram-negative species.	Baele et al. (2002)
Bulgarian yogurt	PCR-based method analyzing similarities between the nucleotide sequence of the spacer between the 16S and 23S rRNA genes	LbLMA1-rev and R16-1	*Lactobacillus delbrueckii* subsp. *bulgaricus*	LbLMA1-rev/R16-1-based PCR could be a trustful means to characterize the members belonging to *Lactobacillus* genus.	Dubernet et al. (2002)
Yogurt, Bulgarian yogurt, and fermented milks	PCR-DGGE technique	HDA1-GC, HDA2, Lc1, and Lc2	*Bifidobacterium lactis, Lactobacillus acidophilus* and *Lactobacillus casei*	Rapid and reliable screening of the microbial population in probiotic products.	Fasoli et al. (2003)
Yogurt and Bulgarian yogurt	PCR-RFLP of protein-coding genes	β-gal 2596, β-gal 3599, Lp 222, Lp 1778, PepQ 2475, and PepQ 3523	*Lactobacillus delbrueckii* subsp. *bulgaricus*	PCR-RFLP of protein-coding genes applied to *Lactobacillus delbrueckii* seemed to be a useful tool to evaluate microbial variation occurring in the bacterial subspecies.	Giraffa et al. (2003)
	Species-specific PCR. The amplification of the V2–V3 region of the 16S rDNA gene.				
Yogurt	Multiplex PCR, combining the sequences corresponding to the 16S and 23S rRNA genes and their ISR	R16-1 and LbLMA1-rev	*Lactobacillus delbrueckii*	Multiplex primer set was an appropriate means to run rapid, simple, and reliable characterization approach for seven *Lactobacillus* species.	Kwon et al. (2004)
Yogurt	Repetitive DNA element rep-PCR fingerprinting using the BOX primer (BOX-PCR)	BOX primer (BOX-PCR)	*Bifidobacterium acidophilus Lactis, Bifidobacterium longum biotype longum NB, Bifidobacterium bifidum,* and *Bifidobacterium breve*		Masco et al. (2005)
	DGGE analysis of 16S rDNA nested-PCR products	Bifidobacterium-specific 16S rDNA primers		Fast and reproducible for taxonomical analysis of probiotic products.	

(Continued on next page)

Table 1 Selected publications on molecular methods to identify and/or enumerate the probiotics and/or adjunct cultures in yogurt *(Continued)*

Product type	Molecular method*	Primer/probe	Species	Notes	Reference
	PFGE			PFGE of a number of bifidobacterials isolates at strain level clarified a relatively high degree of genomic homogeneity among the *Bifidobacterium* strains.	
South African yogurts	PCR-based DGGE analysis combined with species-specific PCR detection using approximately 200 base pairs (bp) of the V2–V3 variable region of the 16S rRNA gene	HDA1-GC and HDA2	*Lactobacillus acidophilus, Bifidobacterium bifidum, Streptococcus thermophilus, Lactobacillus bulgaricus,* and *Bifidobacterium longum*	Accurate, effective, and rapid method for detection of microbial composition contained in probiotic products.	Theunissen et al. (2005)
Yogurt	Species-specific PCR sequencing of the 16S *rRNA*	Acid, Delbr, Casei, Para, Rhamn, and Lac	*Lactobacillus delbrueckii*	Rapid and accurate for identification of probiotic lactobacilli.	Kao et al. (2007)
	Real-time PCR/melting curve analysis	RhamF/RhamR and RhamA/RhamS		Fast, simple, and precise technique in screening the strongly related strains of lactobacilli, within less than 40 min.	
Commercial yogurt	CE	–	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*	Reliable, simple, cost and labor efficient technique for quantitive analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in yogurt (3 samples/h compared to 2 d/sample by plate count method).	Lim et al. (2009)

Table 2 Selected publications on molecular methods to identify and/or enumerate the probiotics and adjunct cultures in other fermented milks than yogurt

Product type	Molecular method*	Primer/probe	Species	Notes	Reference
Fermented milk products	PCR followed by amplified RAPD	OPA-02, OPA-13, OPA-18, OPL-07, OPL-16, and OPM-05	*Bifidobacterium animalis*	Trustworthy and swift technique for the characterization of bifidobacteria.	Vincent et al. (1998)
Fermented milk products	ARDRA-PCR	GWM, LBL R1, LBL R2, Lho, Ldl, Lgj, LCS, LRH, LPA, and LCZ	*Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus,* and *Lactobacillus casei*		Roy (2001)
Sour milk products	PFGE profiling	–	*Bifidobacterium animalis and Bifidobacterium lactis*		Grand et al. (2003)
Commercial fermented milk products	PCR amplification of the V3 region of the 16S ribosomal DNA, and separation of the amplicons on DGGE	Forward primer F357-GC contained a GC clamp and reverse primer 518R	*Lactobacillus casei, Bifidobacterium lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus, Lactococcus lactis, Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum, Enterococcus faecium,* and *Lactobacillus helveticus*	The reliable and quick molecular approach to analyze the probiotic products.	Temmerman et al. (2003)
	SDS-PAGE protein profiling				
Fermented milk products	Real-time quantitative PCR using an alignment of LAB 1080-bp 16S rRNA gene	La1, La2, Lj1, Ld1, Ld2, Lc3, Lc4, Lc1, Lc2, Lp,1 Lr1, P1, and P2	*Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus acidophilus,* and *Lactobacillus johnsonii*	Possible enumeration of lactic acid bacteria species present in fermented milk products at a minimum threshold of 10^3 cells per mL.	Furet et al. (2004)
Commercial fermented milks	PFGE, partial sequence analysis of the 16S rRNA V1–V2 gene region	Y1 and Y2	*Lactobacillus paracasei, Lactobacillus johnsonii, Lactobacillus zeae/casei, Lactobacillus acidophilus,* and *Bifidobacterium animalis/lactis*	Good differentiation between stains by similar macro-restriction status and different carbohydrate fermentation profiles by a combining the macro-restriction analysis and carbohydrate fermentation patterns.	Gueimonde et al. (2004)
Commercial fermented milks	Multiplex PCR	Bflact2 and Bflact5	*Bifidobacterium acidophilus* subsp. *lactis*	Good, rapid, and precise detection of bifidobacteria present in dairy products.	Collado et al. (2006)
	ARDRA-PCR amplifying specific 1,350 bp 16S rRNA gene fragment	Lm26 and Lm3		ARDRA-PCR technique was a promising tool for characterization of bifidobacteria in dairy products.	

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Table 2 Selected publications on molecular methods to identify and/or enumerate the probiotics and adjunct cultures in other fermented milks than yogurt *(Continued)*

Product type	Molecular method*	Primer/probe	Species	Notes	Reference
	RAPD-PCR	Bif			
	AFLPs	ADH1 and ADH2			
Kefir	RFLP analyses	P3, P4, P3Lb, P4i, PLc1, and PLc2	*Lactobacillus kefir, Lactobacillus kefiranofaciens, Lactococcus lactis* subsp. *Lactis,* and *cremoris, Leuconostoc mesenteroides* subsp. *cremoris*	Inability to demonstrate strain diversity within the *Lactobacillus kefir* species.	Mainville et al. (2006)
Fermented milk products	Rep-PCR fingerprinting technique	$(GTG)_5$ primer	*Lactococcus lactis*, *Leuconostoc* subsp., *Enterococcus* subsp., and *Enterococcus saccharominimus*		Zamfir et al. (2006)
	SDS–PAGE of whole-cell proteins				
	Fragment of the 16S rDNA gene (corresponding with the positions 8-1541 in the *Escherichia coli* numbering system) was amplified by PCR	pA and pH			
	PCR–RFLP				
Commercial fermented milk products	ARDRA	16S1a and 16S1b	*Lactobacillus delbrueckii, Streptococcus thermophilus,* and *Bifidobacterium animalis*	Precious approaches for a fast and precise discrimination of strains usually added as probiotics.	Collado and Hernández (2007)
Gioddu, a traditional Sardinian fermented milk	PCR based on 16S rRNA gene amplification and sequencing	Universal primers	*Lactobacillus* subsp., *Lactobacillus paracasei, Lactobacillus plantarum,* and *Lactobacillus reuteri*		Ortu et al. (2007)
	RAPD-PCR	M13 and D8635			
	RAPD analysis	Primer 5′AGTCAGCCAC3′ and primer 5′CCGCAGCCAA3′			
Fermented milk products	PCR	Thermfor, Thermrev, Bulgfor, Bulgrev, Acidfor, Acidrev, Casfor, Casrev, Forlac, Revlac, SacI-POmod, P3rev, 16Smidfor, and SalI-T7-PC5	*Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus acidophilus, Lactobacillus paracasei* subsp. *Paracasei,* and *Bifidobacterium lactis*	Quick and efficient detection of the species by using species-specific primer pairs designed and the PCR conditions developed in their study.	Tabasco et al. (2007)
	PCR and DGGE analysis	Thermfor-GC and Thermrev, Bulgfor and Bulgrev-GC, Acidfor-GC and Acidrev, Casfor-GC and Casrev, and Forlac and Revlac-GC		A good identification and detection capability for confirmation of accurate labeling in fermented milk products without isolation of bacteria in advance.	

Kajmak (an artisanal Serbian dairy product made by fermentation of milk fat)	$(GTG)_5$-PCR identification	$(GTG)_5$-primer	*Leuconostoc mesenteroides, Enterococcus faecium, Lactococcus lactis, Lactococcus raffinolactis, Lactococcus garvieae, Lactobacillus plantarum, Lactobacillus paracasei,* and *Lactobacillus kefiri*		Jokovic et al. (2008)
	Sequencing of 16S rDNA amplicons	U968 and L1401			
Fermented milk products	Combined use of PMA with species-specific quantitative real-time PCR (PMA–RTi-PCR) based on the 16S rRNA gene for lactic acid bacteria species and the transaldolase gene for *Bifidobacterium lactis.*	Thermfor, Thermrev, Bulgfor, Bulgrev, Acidfor, Acidrev, Casfor, Casrev, Forlac, Revlac, SacI-POmod, P3rev, 16Smidfor, and SalI-T7-PC5	*Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus casei* subsp. *casei, Lactobacillus acidophilus,* and *Bifidobacterium lactis*	Time effective, since it could be performed in 3 h, whereas enumeration by selective plate counts required 3 days.	García-Cayuela et al. (2009)

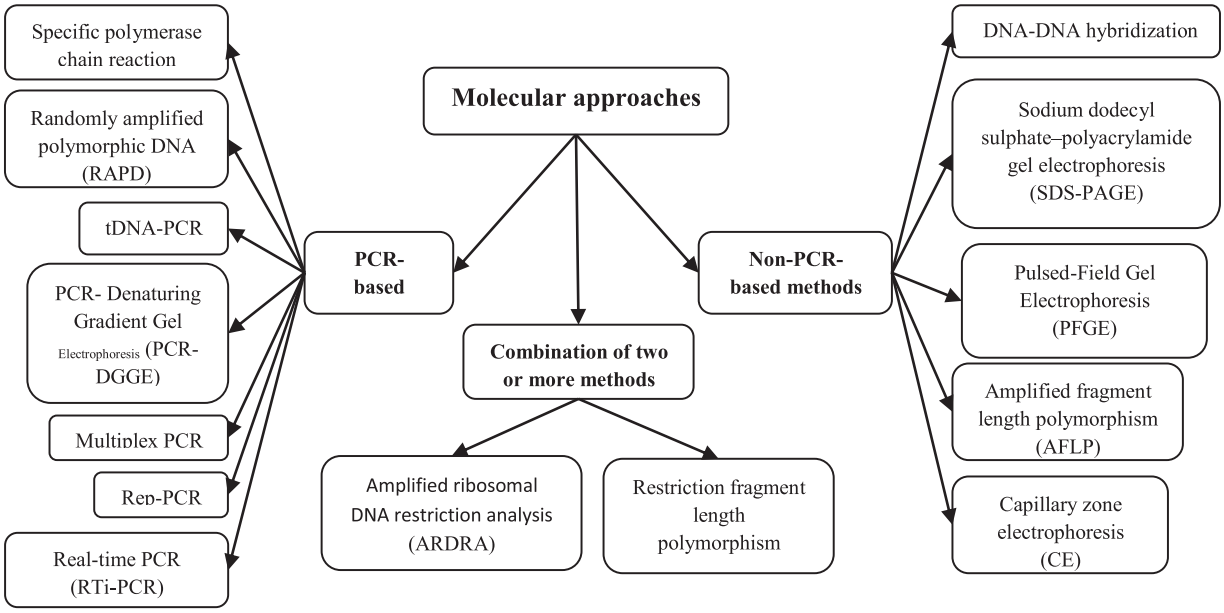

Figure 2 Illustration of the most used molecular techniques for identification and/or enumeration of probiotics in fermented milk products.

same proline iminopeptidases from *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. *lactis*, respectively. They also reported the detection limit of their technique to be 1–10 cell/reaction tube and demonstrated that the method was rapid and sensitive to differentiate these two subspecies which were completed within 4 h. In addition, two other suggested ways were to employ another more variable sequence like 16S–23S intertranscribed space sequence or other housekeeping gene as *rec A* and *atp D* protein-coding sequence and to utilize different strategies, such as DGGE (Ventura et al., 2004).

Randomly Amplified Polymorphic DNA

Randomly amplified polymorphic DNA (RAPD)-PCR is hybridization of short random sequence primers, 9 or 10 bases in length, with adequate affinity to chromosomal DNA sequences at low annealing temperatures. This approach was developed by Williams et al. (1990) which found to be successful in genomic fingerprinting of some lactobacilli. Several advantages have been counted for this methodology including rapid method in comparison to the other genetic techniques; good differentiation without requirement for having the knowledge over the genome beforehand; and high sensitivity and specificity due to applying the complete genome to generate DNA profile (Vincent et al., 1998).

The RAPD technique extensively has been used to identify LAB to species or strain level. Cancilla et al. (1992) employed the RAPD method to achieve genomic fingerprinting of *Lactobacillus lactis* wherein it was found to be efficient and convenient method which could be used for other LAB, as well. In addition, there are literatures employing RAPD to evaluate genomic diversity within other lactobacilli (Du Plessis and Dicks, 1995; Drake et al., 1996a; Tailliez et al., 1996; Torriani et al., 1996; Daud Khaled et al., 1997). In the field of bifidobacteria, Roy et al. (1996) found RAPD to be applicable for classification of industrial strains into specific clusters (either *Bifidobacterium longum/infantis* or *Bifidobacterium animalis/lactis*). However, Vincent et al. (1998) reported the failure of RAPD in presenting the obvious discrimination between *Bifidobacterium longum* and *Bifidobacterium infantis*, and considered this lack of distinction to be associated with variation in RAPD profiles and the wide subclustering present within these strains.

tDNA-PCR

tDNA-PCR firstly was presented by Welsh and McClelland (1991) which consists of amplifying the spacer regions within tRNA genes to produce significant species-specific DNA probs. This method has been applied for variety of bacteria involving *Acinetobacter* (Wiedmann-al-Ahmad et al., 1994; Ehrenstein et al., 1996), staphylococci (Maes et al., 1997), streptococci (De Gheldre et al., 1999), and legionella (De Gheldre et al., 2001). Also, tDNA-PCR in combination with capillary electrophoresis has been employed for listeria species (Vaneechoutte et al., 1998), enterococci (Baele et al., 2000), and streptococci (Baele et al., 2001). In addition, automated capillary electrophoresis has been found rapid detection tool for large number of strains by enjoying exchangeable tDNA-PCR fingerprint databanks and easy-to use data-analysis software (Baele et al., 2000, 2001). Moreover, the user is not required to have prior knowledge on genus identity of the bacteria, because the technique is effective for any type of bacterium.

The efficiency of tDNA-PCR technique for discrimination between bacterial species coming from wide range of genera which has been proved by preliminary studies (Welsh and McClelland, 1991; McClelland et al., 1992; Maes et al., 1997;

Vaneechoutte et al., 1998). The yield tDNA fingerprint revealed to be species specific for most *Lactobacillus* strains. However, tDNA-PCR showed to be week in distinguishing some *Lactobacillus* strains; 16S ribosomal DNA sequencing placed the species with similar tDNA-PCR profiles into the same phylogenetic group (Schleifer and Ludwig, 1996). Other approaches were offered as alternative to discriminate between species with identical tDNA patterns were DNA–DNA hybridization, 16S rDNA sequencing and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) whole cell electrophoresis (Kandler and Weiss, 1986; Fujisawa et al., 1992; Pot et al., 1994).

PCR-Denaturing Gradient Gel Electrophoresis

PCR-denaturing gradient gel electrophoresis (PCR-DGGE) is a two-step technique: (1) amplification of the genes encoding the 16S rRNA and (2) separation of DNA fragments based on the decreased electrophoretic mobility of PCR amplified, partially melted, double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants. PCR-DGGE has been proved to be a priceless approach for analysis of complex microbial composition by which the prior separation of the individual microorganisms is not prerequisite (Muyzer and Smalla, 1998). Separation of genus or species-specific PCR products by DGGE to detect and identify the lactobacilli and bifidobacteria available in fermented products has turned out to be common molecular technique (Temmerman et al., 2003; Ercolini, 2004). In addition, DGGE technique has been reported successful in discrimination of *Lactobacillus* species occurring in the gastrointestinal tract (Walter et al., 2000), monitoring microbial changes in natural habitats (Zoetendal et al., 1998; Omar and Ampe, 2000), and differentiation between pathogenic listeria species (Manzano et al., 2000).

Walter et al. (2000) have showed the possible differentiation at species level by PCR-DGGE method which was applied to the V2–V3 region of 16S rDNA. Fasoli et al. (2003), by using such target region, observed typical position of the band in the DGGE for bacterial species and demonstrated that it was reproducible for different strains of the same species. In the study conducted for identification of probiotic microorganisms in South African products, it was showed that much better and brighter DGGE fingerprint could be achieved by adding a pretreatment step before a repeated DNA isolation. This accomplishment was related to DNA extraction and elimination of enhanced components as possible inhibitors of PCR reaction (Theunissen et al., 2005). However, the authors showed inefficiency of PCR-based DGGE and specific PCR to detect *Bifidobacterium* species, despite the expectations based on labeling. The presence of potassium sorbate as preservative in some of their samples and the resistance of *Bifidobacterium* to it, which is not certain, were possible explanations for reported absence or low content. The presence of *Lactobacillus delbrueckii* subsp. *bulgaricus*, as well, may have been the reason for this observation (Samona and Robinson, 1994; Kailasapathy and Chin, 2000).

Moreover, DGGE approach suffer from weakness in differentiation between species with high phylogenetic relationships, so that sequencing the bands in DGGE profile found to be necessary (Ercolini et al., 2001). Therefore, two proposed suggestions to fill this gap were: (1) applying narrower denaturing gradient, based on an increase in the band position and (2) employment of other primers that might lead to products with easy separation on DGGE gels, would enable identification the species with overlapping bands with V3 primers. However, every change in the gradient, primer set, or electrophoresis conditions will cause new parameters to demand the new database to be constructed. This indicates that DGGE is just applicable for microbial ecosystems which are simple in nature, such as probiotic products; and by increase in complexity of a microbial population more parameters should be modified which would make this technique time-consuming (Temmerman et al., 2003). In addition, DGGE is not powerful enough in providing the data on the metabolic status or strain variation (Masco et al., 2005).

Muyzer and Smalla (1998) and Murray et al. (1996) showed that detection limit of PCR-DGGE would be resulted from DNA templates competition during the amplification reactions. Felske et al. (1998) and Zoetendal et al. (1998) found that PCR-DGGE, in complex microbial mixture, failed to detect organisms less than 1% of total population. Fasoli et al. (2003) confirmed this finding for simple microbial communities such those in probiotic yogurts, containing few bacterial components and established the detection limit of 10^7 cfu mL^{-1}. Although, Temmerman et al. (2003) selected the quantity of 10^4 cfu mL^{-1} as detection limit and presumed the level of 10^7 cfu mL^{-1} may not be able to detect species that are occurring at lower levels. Theunissen et al. (2005) used *Bifidobacterium longum* as reference strain to indicate the lowest bacterial concentration and chose 10^5 cfu mL^{-1} as minimum concentration.

Multiplex PCR

Multiplex PCR is a technique which is able to detect and distinguish several microorganisms simultaneously (Song et al., 2000; Dubernet et al., 2002). Its principle is using primers based on the species-specific sequences of 16S rRNA or 16S–23S rRNA intergenic spacer region (ISR) containing its flanking 16S rRNA or 23S rRNA, respectively (Schleifer and Ludwig, 1996). 16S rDNA- or 23S rDNA-targeted primers have been found being successful for detection and identification of the related *Lactobacillus* species (Nour, 1998; Ward and Timmins, 1999). However, there are limitations in the employment of extra priming sites from each rRNA gene (Gurtler and Stanisich, 1996; Berthier and Ehrlich, 1998) which restrict the differentiation of many species at the same time.

PCR systems mainly are able to apply five species instantaneously, resulted from the use of primer sets based on every specific sequence of 16S, 23S rRNA, or ISR (Tilsala-Timisjärvi and Alatossava, 1997; Song et al., 2000; Heilig et al., 2002). While, Kwon et al. (2004) developed a multiplex primer set to identify various *Lactobacillus* species with a single PCR assay.

They enabled differentiation of all type strains belonging to the seven target species by the multiplex PCR primer set containing nine primers in one-step assay. In addition, they claimed that their innovated approach could improve following issues: (i) insufficiency of priming targets, (ii) difficulty in optimization of PCR conditions, and (iii) generation of unanticipated PCR product.

Repetitive Element Sequence-Based-PCR

In repetitive element sequence-based-PCR (Rep-PCR), primers anneal to repetitive parts of the chromosome and amplification takes place provided that the distance between primers binding sites to be short enough to enable this event. In Rep-PCR, DNA fragments with variety of sizes are obtained by amplification which is separated by agarose gel electrophoresis (Versalovic et al., 1991). Rep-PCR has been applied to characterize LAB isolated from fresh Sausages (Cocolin et al., 2004). In another study performed by Sohier et al. (1999), RAPD and Rep-PCR were shown to be more efficient in comparison with phenotypic or biochemical trends for the discrimination of *Lactobacillus hilgardii* and *Lactobacillus brevis* at the species level. Švec et al. (2005) evaluated $(GTG)_5$-PCR for identification of *Enterococcus* species. The study, according to vast and diverse selection of entrococcal reference strains and field isolates covering all well-described entrococcal species, demonstrated that PCR fingerprinting equipped with $(GTG)_5$ primer was suitable, reliable, and fast tool to identify entrococci. Ouadghiri et al. (2005) studied the bacterial diversity present in traditional Moroccan soft white cheese. The authors were enabled to isolate, purify, and identify a total of 164 lactic acid bacteria by whole-cell protein fingerprinting and rep-PCR using the $(GTG)_5$ primer. Terzic-Vidojevic et al. (2007) applied rep-PCR and API 50 CH system to characterize the microflora occurring in homemade semi-hard white Zlatar cheese. The authors showed that the main groups present during the ripening of cheese were *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus brevis*, *Lactococcus lactis* subsp. *lactis*, *Enterococcuus faecium*, and *Enterococcus faecalis*. Nikolic et al. (2008) analyzed the presence of lactic acid bacteria in Bukuljac cheese, a homemade goat's milk cheese, by using rep-PCR with $(GTG)_5$ primer wherein the *Lactobacillus paracasei* subsp. *paracasei* was found to be dominant strain in the microflora of cheese.

Real-Time PCR

Real-time PCR (RTi-PCR) follows the general principle of PCR in which amplified DNA during reaction is qualified in real time and in order to determine the original amount of DNA/RNA of unknown samples is compared with a series of standard samples. This technique has been employed extensively for direct enumeration of bacteria in complex habitats (Justé et al., 2008). RTi-PCR is being increasingly used for quantification of microorganisms like bacteria, yeasts, and molds. Furet et al. (2004) utilized real-time quantitive PCR (qPCR) to quantify the lactic acid bacteria in commercial fermented milk products. This technique enabled the authors to identify the species of lactic acid bacteria strains and to accurate quantify them in these products with detection limit of 10^3 cells per mL of product. Neeley et al. (2005) employed the real-time PCR technique to enumerate the lactic acid bacterial populations in wine and reported that this approach could be exploited to access the spoilage risk of juice or wine caused by lactic acid bacteria. Finally, Friedrich and Lenke (2006) were the first to study utilization of a multiplex qPCR strategy to enumerate bacteria. They designed the multiplex qPCR approach to quantify the target DNA simultaneously proportional to total bacterial DNA. The developed strategy was assigned to enumerate members of *Lactobacillus lactis* subsp. *cremoris*, *Lactobacillus lactis* subsp. *Lactis*, and *Leuconostoc* species, in mesophilic starter cultures, and was proved to be an efficient and accurate tool for bacterial community analysis of complex starter cultures.

The amount of viable bacteria is considerable mainly because of meeting the minimum viable counts of probiotics in products and molecular approaches such as DGGE and RTi-PCR have not been able to quantify the viable bacteria in probiotic products efficiently. Therefore, several attempts have been made to improve these methods with potential to distinguish dead and alive microorganisms. There are parameters influencing molecular quantification by PCR such as efficiency of bacterial lysis, DNA extraction, and whether the PCR inhibitors in the DNA solutions are present or not (Ludwig and Schleifer, 2000; Hein et al., 2001). The interaction agents that prevent DNA from amplification have made a great advancement in RTi-PCR to enable quantitive and differential identification of viable and dead bacteria by penetrating into cells with damaged membrane (García-Cayuela et al., 2009). Among the investigated agents, ethidium monoazide (EMA) and propidium monoazide (PMA) have been proved to be efficient in distinguish viable/nonviable gram-positive and gram-negative bacteria (Nogva et al., 2003; Rudi et al., 2005a, b; Nocker and Camper, 2006a; Nocker et al., 2006b; Nocker et al., 2007a, b). However, Flekna et al. (2007) and Nocker et al. (2006b) have found EMA as weak indicator of cell viability, and Pan and Breidt (2007) reported EMA to be toxic for living cells, but not PMA. García-Cayuela et al. (2009) expanded a Real-Time PCR with PMA (PMA–RTi-PCR) which permitted an easy, simple, and precise enumeration of living bacteria within three hours with a quantification range of 5 log units in complex bacterial mixtures occurring in fermented milk products.

Several detection limits for variety of microorganisms have been recommended such as 10^3 cfu mL^{-1} for *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis*, and 10^4 cfu mL^{-1} for *Streptococcus thermophilus* and *Lactobacillus casei* (García-Cayuela et al., 2009). These values are similar to the results of the Real-Time PCR with EMA (EMA–RTi-PCR) analysis of *Campylobacter jejuni* reported by Rudi et al. (2005b) and the PMA–RTi-PCR assays of *Listeria monocytogenes* described by Pan and Breidt (2007). Baele et al. (2002), in order to detect and quantify actin mRNA from yeast

and moulds, used Reverse transcription PCR (RTi-PCR) and Real-time RT-PCR assay. The authors found actin mRNA being suitable indicator of cell viability and indicated that the procedure, having the specificity, sensitivity, speed, reliability as well as its potential automatically present advantages to routine analyze the presence and viability of fungi in food commodities.

Non-PCR-Based Molecular Methods

DNA-DNA Hybridization

For the dot-blot assay, the procedure is composed of extraction of target nucleic acid from the cell followed by the immobilization on a membrane and use of probes marked either radioactively or nonradioactively to hybridize with immobilized nucleic acid. For DNA-DNA hybridization, M13 was introduced as successful primer for typing of lacto-strains (Miteva et al., 1992). The authors conducted a study on use of M13 DNA fingerprinting to classify of *Lactobacillus* species and reported that it might be fruitful tool in the taxonomy of *Lactobacillus* species. Meile et al. (1997), applying DNA fingerprinting, described a new species of *Bifidobacterium* named *Bifidobacterium lactis*. They also noted weak similarly between *Bifidobacterium infantis* and *Bifidobacterium animalis* by DNA-DNA hybridization. However, this technique still have the disadvantages of being time-consuming and labor-intensive (Song et al., 1999). DNA-DNA hybridization was also reported unable to discriminate the strains of *Bifidobacterium infantis* and *Bifidobacterium longum* (Bahaka et al., 1993).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SDS-PAGE of whole cell proteins (WCPs) has been applied successfully for the detection of lactobacilli (Pot et al., 1994) and isolation of *Bifidobacterium animalis* strains found in commercial fermented milks was possible with this technique (Roy et al., 1996); however, SDS-PAGE was not efficient enough for routine identification. SDS-PAGE of WCPs and ribotyping were shown to be effective methods for discrimination of *Lactobacillus delbrueckii* subsp. *bulgaricus* from *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *delbrueckii* (Hertel et al., 1993; Moschetti et al., 1997). Villani et al. (1997) analyzed variations present among *Leuconostoc mesenteroides* strains which were obtained from dairy and nondairy environments. Rossi et al. (1998), employing SDS-PAGE of WCPs, identified propionibacteria present in different dairy sources. Silva et al. (2003) isolated the enterococci from an artisanal Portuguese cheese with this technique. Delgado and Mayo (2004) used SDS-PAGE of WCPs to characterize the lactococci and enterococci contained in Spanish starter-free cheeses. Piraino et al. (2005) applied SDS-PAGE of WCPs to detect LAB available in Caciocavallo cheeses and Romanian dairy products. Piraino et al. (2006) employed unsupervised and supervised artificial neural networks to identify the LAB (*Lactobacillus, Leuconostoc, Enterococcus, Lactococcus,* and *Streptococcus*) on the basis of their SDS-PAGE of the WCP pattern and Gatti et al. (2004) used SDS-PAGE of surface proteins to differentiate *Lactobacillus helveticus* strains isolates occurring in natural whey starter cultures. Tsakalidou et al. (1994) used the SDS-PAGE technique of whole-cell proteins and phenotypic identification to examine a total of 148 strains of LAB isolated from traditional Greek dairy products such as yogurt and cheese in which SDS-PAGE approach, as an advanced tool to identify LAB, confirmed the phenotypic identification results.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) has been verified to be an efficient means for differentiating strains. Roy et al. (1996) used PFGE after DNA digestion with *Xba I* and *Spe I*, and PCR amplification of 16S rDNA to identify several important commercial species of *Bifidobacterium*. In this study, the PFGE approach showed that only 5 distinct strains of *Bifidobacterium longum* and 1 strain of *Bifidobacterium animalis* were present in commercial preparations. O'Riordan and Fitzgerald (1997) reported that PFGE was an efficient tool to differentiate 25 strains belonging to *Bifidobacterium* obtained from culture collections. Bouton et al. (2002) exhibited good discrimination between homo-fermentative lactobacilli, playing role over Comté cheese ripening, by PFGE. However, some species were distinguishable by RAPD or REP-PCR as well as PFGE, there were reported some discrepancies. Various exploration of DNA polymorphism, the whole DNA chromosome for PFGE, region amplified by primers for RAPD, and the REP-PCR could explain the observed variation and, also, using the second restriction enzymes would certainly be helpful in this case.

Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP) involves restriction of total bacterial DNA with two endonucleases of different cutting frequencies, followed by ligation of the fragments to oligonucleotide adapters complementary to the sequences of the restriction site. The AFLP approach enables amplification of just two genomic regions by hiring selective primers and results in findings with more reproducibility (Vos et al., 1995; Janssen et al., 1996). This method has been introduced recently; thereby, there is not much information on application of it to type microbes occurring in foods. AFLP has been successfully applied to fingerprint microbial population such as *Clostridium perfringens, Listeria monocytogenes*, and vancomycin-resistant *Enterococcus faecium* (Aarts et al., 1999; Antonishyn et al., 2000; McLauchlin et al., 2000). RAPD and AFLP profiles allowed distinguish between species high related phenotypically of *Lactobacillus plantarum, Lactobacillus pseudoplantarum*, and *Lactobacillus pentosus* along with infra specific discrimination of 30 silage and cheese isolates (Torriani et al., 2001). Gancheva et al. (1999) developed automation in AFLP; this innovation was composed of fluorescently dye-labeled fragments by

capillary electrophoresis under denaturing circumstances along with laser detection of AFLP fragments by an automated analyzer. A new development within AFLP is simplification of this technique under the name SAU-PCR. Similarly, this method employs primers on basis of the restriction enzyme recognition sequence without need to add linkers, and then the products are resolved on agarose gels. The suggested trend was described as digestion of genomic DNA with the restriction endonuclease *Sau3AI* followed by amplification with primers whose core sequence is based on the *Sau3AI* recognition site (Corich et al., 2005).

Capillary Zone Electrophoresis

More recent introduced technique in the field of microorganisms' identification is capillary zone electrophoresis (CE). CE preliminary was applied to detect chemical components (Vallejo-Cordoba et al., 2004), in fermented milk products (Ligor et al., 2008), furosine in dairy products (Vallejo-Cordoba et al., 2004), and citrate and inorganic phosphate in milk (Izco et al., 2003). First attempt on applying the CE for microorganisms' identification was made by Hjertén et al. (1987) who came up with ability of CE for virus and bacteria analysis, which was followed by other studies such as detection and enumeration of bacteria responsible for urinary tract infection (Armstrong and Schneiderheinze, 2000), cell viability (Armstrong and He, 2001a), active bacteria in dietary supplements (Armstrong et al., 2001b), food samples bacterially contaminated (Palenzuela et al., 2004), and sterility test (Rodriguez et al., 2006).

The mechanism of CE to identify and separate the bacteria is considered to be based on this fact that these microorganisms vary in electrophoretic mobilities, which itself is linked to their electrically charged membrane (Palenzuela et al., 2004). In order to reach desired result in microbial identification by CE, there are some underlying parameters. For instance, the separation of different *Pseudomonas* species requires the minimum concentration of tris-borate buffer to an ionic strength of 0.2 mM, achieving peaks with improved shapes and high efficiency in separation procedure using the BGE containing polyethylene oxide (PEO) is recommended (Pfetsch and Welsch, 1997) and having appropriate procedures for preparations of bacterial samples (i.e., vortex or sonication) before injection which significantly would decline bacterial aggregation and prevent the reduction in number of peaks (Armstrong et al., 1999; Armstrong and Schneiderheinze, 2000; Schneiderheinze et al., 2000; Armstrong et al., 2001b; Yu and Li, 2005).

Recently, a rapid CZE technique has been introduced by Kłodzińiska et al. (2006) to identify *Escherichia coli* in human urine and the examination of pure culture of *Helicobacter pylor*i. This method utilizes PEO (as focusing agent), calcium, and myoinositol hexakisphosphate (as specific ions). The later were for interaction with the bacterial surface and to alter their electrical properties and electrophoretic mobilities. As well as all methods, regarding CZE, there are variety of factors considered essential for optimization of CZE conditions including BGE compositions and several instrumental parameters (e.g., injection technique, capillary length, separating voltage, and temperature) which were investigated by Lim et al. (2009) and they presented some suggestions.

Approaches Based on Combination of Molecular Techniques

Amplified Ribosomal DNA Restriction Analysis

Amplified ribosomal DNA restriction analysis (ARDRA) has been useful means to identify various microorganisms. Salzano et al. (1994) classified twenty strains of *Streptococcus thermophilus* into five different types using ribotyping after DNA digestion with *Hind III* and hybridization with *cDNA* from 16S-23srDNA of *Escherichia coli*. In addition, ribotyping after digestion with *Hae III* and *Xhol* placed the same strains into three groups as a result of declined impact of the flanking sequence within the analysis of rRNA operons. Vaneechoutte et al. (1992) compared PCR-based DNA fingerprinting techniques for identification of 93 strains belonging to 13 well-known taxa from eubacterial family comamonadaceae (β subclass of the proteobacteria or rRNA superfamilly III) and indicated that ARDRA was a reliable and simple means to identify the bacterial species. The potential of this method to distinguish at species or subspecies level also has been noted. Moschetti et al. (1997) used three different techniques whole-cell protein profiles, PCR analysis of rDNA genes, and ribotyping to characterize thirty one strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* present in deferent dairy products and three type strains belonging to *Lactobacillus delbrueckii* subsp. *delbrueckii, Lactobacillus delbrueckii* subsp. *Lactis,* and *Lactobacillus delbrueckii* subsp. *bulgaricus* wherein ribotyping was reported as most reliable approach to characterized the subspecies of *Lactobacillus delbrueckii* in comparison with two other methods. Grifoni et al. (1995) obtained DNA fragments of several *Azospirillum* strains (from five well-described species of *Azospirillum amazonense, Azospirillum brasilense, Azospirillum halopraeferens, Azospirillum irakense,* and *Azospirillum lipoferum*) by restriction analysis of the amplified 16S rDNA and by restriction fragment length polymorphisem of the histidine biosynthetic genes. Their results showed both ARDRA and RFLP being easy, fast, reproducible, and reliable tool to identify the *Azospirillum* strains especially at the species level. Collado and Hernández (2007) reported the efficiency of ARDRA, with only one PCR reaction and one restriction enzyme to discriminate between bacteria present in fermented milk products. Aquilanti et al. (2006) identified enterococci by analyzing *Mbo II, Msp I* and *Rsa I* PCR-ARDRA patterns. However, Villani et al. (1997) analyzed *EcoR I* and *Hind III* ARDRA-PCR patterns from *Leuconostoc* species and found it not to be reliable method for species discrimination.

Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) includes whole-genome DNA extraction, its digestion with restriction endonucleases and separation of the resulting array of DNA fragments by gel electrophoresis. Giraffa et al. (2003) reported that PCR-RFLP compared to by 16S rDNA sequencing was more reliable to cluster *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus delbrueckii* subsp *bulgaricus.* It is worth noting that using 16S rDNA sequence analysis to identify *Lactobacillus delbrueckii* at subspecies level had been found to be difficult way (Vandamme et al., 1996), which was corresponded to this fact that the progressive rate of gene coding for proteins contained in the bacterial metabolism was higher than ribosomal genes (Palys et al., 1997). More recently, the ability of protein-coding housekeeping genes has been discovered for expressing the phylogenetic relationships between bacteria (Ludwig and Schleifer, 1999). Although, Mainville et al. (2006) showed good differentiation between different lactobacilli species present in kefir grains achieved by RFLP, but identification of *Lactobacillus Kefir* species at strain level encountered with failure. This phenomenon was attributed to high genomic homology which RFLP was unable to detect. Alternatively, using genomic plasmid profiles, due to owning many different patterns was proposed. De las Rivas et al. (2006) also reported an inconsistency in differences within ISR-RFLP-PCR patterns of *Lactobacillus plantarum* strains.

CONCLUSION

Fermented milk products have stepped to the market and are being commercially produced under variety of brand names. The ones containing probiotics are claimed to be able to offer health benefits to the host (such as intestinal- and immune-associated diseases). The importance of meeting the recommended minimum amounts of probiotics and accurate labeling in these products has urged the need for methods being able to identify and enumerate precisely the microorganisms they contain. In this regards, several culture-dependent techniques have been proposed and utilized, but the lack of sensitivity and specificity of classic methods has resulted in increased demand for alternative approaches. Therefore, the researchers have moved toward culture-independent techniques which were found to be rapid, accurate and reliable methods, and were proved to be efficient tools for identification and enumeration of microorganisms (used either alone or combined with each other). Ultimately, the use of molecular techniques in dairy microbiology has enhanced the knowledge on the sequence of microorganisms at strain and species level. The culture-independent techniques can provide a rapid assessment of the microbial diversity while culture-dependent methods are encountering the researchers with several drawbacks. In the nearest future, the major outlook is the possibility to identify not only the microorganisms, but also their activities during fermentation.

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