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Identification of Lactic Acid Bactria in Dairy Products using Culture-independent Methods: A Review

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ABSTRACT

The microbiota of dairy products is composed of starter and non-starter lactic acid bacteria, as well as other bacteria, yeasts and filamentous fungi that form secondary microbiota and play an important role in cheese ripening. The use of combined LAB strains based on compatibility and/or coexistence tests plays an important role in food production and preservation, while combinations of strains that exhibit mutually inhibitory properties must be avoided. For centuries, researchers have focused on the exploration of autochthonous LAB biodiversity from various ecological dairy niches in order to address its role in texture and flavour formation and to select strains for use as starter cultures. Recent advanced technological tools like next-generation sequencing machines have played a significant role in the fast and accurate characterization and isolation of microbial communities from dairy products. However, the characterization and isolation of LAB using culture-independent methods is also a reliable and cost-effective method for studying a bacterial community without prior cultivation. These methods include single-strand conformation polymorphism (SSCP-PCR), denaturing gradient gel electrophoresis (DGGE), thermal gradient gel electrophoresis (TGGE), real-time PCR (qPCR), terminal restriction fragment length polymorphism (T-RFLP), length heterogeneity PCR (LH-PCR), automated ribosomal intergenic spacer analysis (ARISA) and fluorescence in situ hybridization (FISH). Therefore the goal of this review is to compare and describe the most recent culture-independent molecular-based methods for the characterization and isolation of LAB in dairy products.

Key words: Culture-independent, Isolation, Lactic acid bacteria, Milk, PCR.

The dairy microbial ecosystem is very complex and responsible for the broad diversity of tastes, aromas and textures of dairy products (Jany and Barbier, 2008). Many bacteria make a positive contribution to the organoleptic quality of cheeses or fermented milk, while others may have adverse effects, or may even present a health risk. Starter, probiotic and adjunct microorganisms are widely selected and used in the production of dairy foods to improve quality and safety; they may be formulated as monostrain or multistrain cultures. Lactic acid bacteria are the most important groups of starter and probiotic microorganisms; they play a vital role in the improvement of required characteristics. LABs can convert lactose to lactic acid and are also responsible for the formation of small peptides and amino acids combined with the activity of their proteolytic enzymes during cheese production. Lactic acid bacteria are naturally found in milk as contaminants from various sources such as the udder surface, milking equipment, dairy factory environment, transport and filling operations and storage surfaces (Jany and Barbier, 2008). Thus, exploring the microbial composition of milk and milk products has a paramount role in answering the questions “which microorganisms are present ?” and “what can the community do?” and different methods of characterization and identification were used by researchers (Palmiro Poltronieri, 2018).

Molecular methods provide an outstanding tool for the detection, identification and characterization of microorganisms found in environmental samples, foods and other complex ecosystems. The composition of dairy microflora is examined using both culture-dependent and culture-independent methods. The molecular methods applied exclusively depend on whether bacterial DNA was extracted directly from a sample, or from bacterial colonies grown on culture media. The choice of the method depends on several factors, such as 1) the purpose or context of the research: building a taxonomic inventory of microbial species present in a dairy matrix or monitoring dynamics during production and/or storage, searching for microorganisms with interesting functional and/or biotechnological properties, source tracking and microbial forensics, quality control; 2) the required taxonomical information level, which is strongly associated with the purpose and context: genus, species or strain level; 3) available time: long-term vs. real-time analysis 4) financial resources: small-scale or large-scale studies, low-cost or high-cost applications; 5) What

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methods are currently state-of-the-art? However, these methods have their own limits, which can be overcome by combining them so that they give more information on microbial complexity (Palmiro Poltronieri, 2018).

Literature review

Culture- In dependent approaches

Culture-independent methods enable the identification of microbes that are difficult or impossible to grow on media, revolutionizing our understanding of the microbial ecology of dairy foods. These methods are faster, more sensitive and less prone to bias than culture-dependent ones. As a result, studies on the diversity and dynamics of dairy ecosystem microbiota are increasingly relying on culture-independent methods based on direct analysis of DNA (or RNA) without prior cultivation and polymerase chain reaction (PCR) amplification of the 16S rRNA gene. Their application to the dairy matrix provides valuable insight into specific isolates and microbial populations, as well as the evolution and nature of the microbial groups in these ecosystems (Jany and Barbier, 2008).

Moreover, to study microbial diversity and dynamics, qualitative analyses of dairy microbiota can be conducted using PCR-based tools such as denaturing gradient gel electrophoresis (PCRDGGE), temperature gradient gel electrophoresis (PCRTTGE), single-strand conformation polymorphism PCR (SSCPPCR), terminal restriction fragment length polymorphism (TRFLP), length heterogeneity PCR (LHPCR) and automated ribosomal intergenic spacer analysis (ARISA) techniques (Ndoye *et al*., 2011). Other community fingerprinting techniques used in dairy food microbiology include denaturing high-performance liquid chromatography (Delavenne *et al*., 2011) and amplified ribosomal DNA restriction analysis (ARDRA). Furthermore, reverse-transcribed (RT) RNA analyses, such as RT-PCR-DGGE, RT-PCR-SSCP and LH-RT-PCR, can be performed to profile metabolically active community members. DGGE and TGGE are the commonly used community fingerprinting approaches for studying a microbial population in dairy products.

Culture-independent polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a vital feature of molecular biology and many PCR-based molecular methods for studying microbial communities have been developed. To amplify target sequences of a given population, DNA is used as a template for PCR amplification of genetic targets using universal, genus, or species-specific primers. Basically, PCR consists of three major steps: the first step is denaturing the DNA strand at 94-95°C; annealing of the nucleotide primers at 37-70°C and polymerization (elongation) of the second DNA strand from nucleotides at 60-72°C (Kuchta *et al*., 2004). It should also be noted that in every experiment, optimization of PCR conditions for each step is unavoidable; experimental trials must be carried out to establish optimal PCR conditions (Justé *et al*., 2008). A successful culture-independent molecular approach based on PCR requires the efficient direct extraction of total bacterial DNA from a sample.

Several authors revealed different total bacterial DNA extraction and purification protocols were described, however, whichever extraction protocol is used, the DNA from all bacteria present in the complex cheese matrix might not be recovered or the extraction of DNA from all bacteria present in the sample might not be equally efficient. Therefore, PCR amplification might not result in the amplicons of all LAB present in the sample (Bonaïti *et al*., 2006).

Some genotypes may go undetected due to low species abundance in the sample. This could be due to insufficient homogenization of the cheese matrix, or incomplete cell lysis which inhibits PCR amplification (Bonati *et al*., 2006). Besides, choosing a gene or a genetic marker is an important step in the identification of microbial communities. Currently, the bacterial 16S ribosomal RNA operon (16S rRNA and 23S rRNA genes) is the most commonly used molecular marker in microbial ecology studies. The 16S rRNA gene exhibits discrimination flaws in the identification of closely related species. Therefore, the other target genes, such as the elongation factor Tu gene, rpoB gene, rpoA gene, the DNA recombinase gene (recA) and pheS gene, have also been exploited for the differentiation of LAB species (Justé *et al*., 2008).

The use of specific primers for PCR-based detection of the target organisms in total bacterial DNA extracted from a sample is the fastest culture-independent PCR approach for the genus-, species-, or strain-specific detection of LAB in the cheese matrix (Temmerman *et al*., 2004). The Biodiversity of the bacterial ecosystem in traditional Egyptian Domiati cheese was investigated using culture-independent species-specific PCR with DNA directly extracted from cheese as a template DNA. Species-specific primers confirmed the presence of *Lactobacillus, Enterococcus*, *Lactococcus* and *Staphylococcus* genera representatives.

The main drawback of this approach is that only 'expected' microorganisms will be detected if they are present in the sample and it is much more labor-intensive than approaches that use universal bacterial primers targeting the bacterial community's 16S rRNA genes, followed by PCR amplicon separation (Temmerman *et al*., 2004). As a result, such PCR assays have limited utility in the analysis of complex microbial ecosystems such as artisanal cheeses. Because each bacterial species requires a unique primer pair, such approaches have not been widely used in culture-independent community studies of dairy products. However, it can be a useful approach to detect the presence and unequivocal identification of a targeted species and it can identify even minor LAB community members, which is one of the main drawbacks of other culture-independent approaches (El-Baradei *et al*., 2007).

PCR-denaturing gradient gel electrophoresis (DGGE)

Investigation of microbial diversity and the dynamics of dairy products in culture-independent PCR-DGGE molecular methods uses genomic bacterial DNA and/or RNA extracted directly from the sample, followed by amplification of the variable regions of the 16S gene. If total DNA from the microbiota is used in PCR amplification, the DGGE technique can provide a profile of genetic diversity, whereas total RNA provides a profile of metabolically active microbiota (Rantsiou *et al*., 2008).

DGGE primers are designed to ensure that a GC clamp, or a high melting domain, is inserted into the amplified product prior to gel separation (Sheikha *et al*., 2011). Prior to DGGE analysis, universal primers to the 16S rRNA gene can be used to amplify the segment of any bacteria. This technique, which has become a well-established tool for investigating microbial diversity in many laboratories, has been used by many scientists. PCR-DGGE is commonly used to assess the structure of a microbial community in the absence of cultivation and to determine community dynamics in response to environmental changes (Ercolini, 2004).

The electrophoretic separation of PCR amplicons of equal length in a sequence-specific manner using a polyacrylamide gel containing a denaturing gradient of urea and formamide is the basis of the DGGE principle.
Essentially, PCR-DGGE comprises three steps:

- Extraction of total community DNA from the sample.
- PCR amplification using specific oligonucleotide primers.
- Separation of the amplicons using DGGE.

Although DGGE and TGGE are not quantitative techniques, their application allows for showing and monitoring the spatial/temporal changes that occur during dairy food production and storage (Cocolin *et al*., 2013).

Recently, the PCR DGGE method was developed in order to detect and identify the histaminogenic bacteria present in cheese at the species level. Specific primers were designed based on the hdcA gene sequences available for Gram-positive bacteria and PCR and DGGE were optimized in order to differentiate amplicons that correspond to different histamine producing species (Diaz *et al*., 2016). DGGE consists of an electrophoretic separation of PCR products in a polyacrylamide gel containing a gradient of chemicals (urea and formamide in DGGE). As the DNA molecule migrates through the denaturant gradient, a sequence dependent partial denaturation of the double-strand occurs. PCR-DGGE electrophoresis is carried out at a constant temperature, between 55 and 65°C. TTGE and DGGE are now frequently applied in microbial ecology to compare the compositions of complex microbial communities and to study their dynamics (Diaz *et al*., 2016).

Amplification of a different variable region of the 16S rRNA gene and/or amplification of the same variable region of the 16S gene with a different universal bacterial primer pair might yield different results. Furthermore, different DGGE conditions might result in a different resolution of PCR amplicon separation (Rantsiou and Cocolin, 2006). A study of the bacterial community of Stilton cheese revealed that representatives of the *Leuconostoc* community were detected only by amplification of the V4-V5 region of the 16S rRNA gene while targeting the V3 region failed (Ercolini *et al*., 2003); V6-V8 regions instead of the V3 region (Aponte *et al*., 2008). A significant difference in the DGGE analysis of the cheese *enterococcal* population was observed when different primer pairs were used. This indicates that the selection of appropriate primer pairs is the crucial step for successful DGGE analysis (MoharLorbeg *et al*., 2009). The sensitivity issue of the culture-independent PCR-DGGE method can be enhanced by using group (genus) specific primers, instead of universal bacterial primers and in this way, even a minor community can be detected.

Reverse-transcribed (RT) RNA analyses are performed to reveal the metabolically active microbiota of ripening cheeses. A comparison of the RNA-derived DGGE profile with the DNA-derived DGGE profile indicated a substantially different degree of metabolic activity for the microbial groups detected during the ripening of cheese (Alessandria *et al*., 2016). Therefore, the RT-PCR-DGGE approach might be very useful in studies of long-ripened cheeses since different microbial groups might be active in different ripening periods. Regardless of whether bacteria are viable or nonviable, their DNA is always present in the cheese matrix. Since RNA is less stable than DNA, RNA will degrade more quickly in dead organisms. In addition, it is believed that RNA- based assays are more sensitive than DNA-based assays (Justé *et al*., 2008).

The disadvantages include poor detection of bacteria present in insignificant numbers in a community and incorrect estimation of the bacterial diversity due to the detection of the heteroduplex formed by the heterogeneous rRNA operons. Hong *et al*. (2016) identified *Leu. mesenteroides, Leu. citreum, P. pentosaceus* and *Leu. gelidum* as dominant bacteria during kimchi microflora analysis.

PCR-temporal temperature gradient electrophoresis (TTGE)

PCR-TTGE is a PCR-denaturing gradient gel electrophoresis that allows the separation of 16S ribosomal DNA (rDNA) fragments of the same length using a temperature gradient. The denaturing gradient is obtained by varying the temperature over time without the use of chemicals (Jany and Barbier, 2008). It was designed to detect differences in the 16S rDNA V3 regions of bacteria with low G+C genomes. The TTGE was used to identify various bacterial species found in dairy products, including *Lactobacillus*, *Lactococcus, Leuconostoc, Enterococcus, Pediococcus, Streptococcus* and *Staphylococcus*. TTGE has been used in *in vitro* and in both liquid and solid dairy products to distinguish bacterial species of lactic acid bacteria subspecies, species, or groups of species. However, *Streptococcus thermophilus* was not detectable by TTGE. This is due to the limitations of the approach (Ogier *et al*., 2002).

The weakness of using a DNA-based PCR-TTGE approach to analyze Ragusano cheese from curd to the seventh month of ripening was that there were no changes in the microbiota profile during the ripening period. Such a profile could be explained by the competition of DNA for primers in a PCR reaction. Because of competition during the PCR step, the DNA of dead cells may particularly predominate, masking the DNA corresponding to the growth of minor microbiota during the ripening period. As a result, minor microbiota representatives do not amplify and cannot be detected. The above-mentioned competition would not have occurred if the RNA-based approach had been used because the RNA of dead cells is rapidly degraded.

Single-strand conformation polymorphism-PCR (SSCP-PCR)

Like PCRD/TGGE it is used to separate different DNA fragments of similar lengths, relying on electrophoretic separation due to sequence-dependent variation in PCR product migration speed. In cheese, SSCP has been used to investigate mutations or dynamics among microbial populations at the genus and species levels. Single-stranded DNA folds into tertiary structures under non-denaturing conditions based on its nucleotide sequences and physicochemical environment. This results in variations in electrophoretic mobility in non-denaturing gels (Jany and Barbier, 2008).

The mobility of a single-stranded DNA fragment in a polyacrylamide gel is determined by the DNA conformation, according to the SSCP principle. A typical SSCP profile contains two single-stranded DNA fragments and one double-stranded DNA fragment, though different conformations from the same strand are also conceivable. In the case of cheese, SSCP has not been widely used. Sofu and Ekinc (2016) profiled the community diversity and dynamics of milk and Ezine cheese using PCRSSCP combined with PCRDGGE, revealing the dominance of *L. lactis* and *S. thermophilus* spp.

A recently developed method based on capillary electrophoresis CE SSCP analysis of nuclear ribosomal DNA ITS amplicons (ITS1 and ITS2 conformers) in order to determine the fungal community composition of 36 kinds of cheese, including blue veined, pressed cooked, pressed uncooked, red smear and surface-mold ripened cheeses. The method has proved to be reproducible and sensitive. It can also be considered an effective tool to identify the fungi present in various cheese types and may be of interest to the cheese industry to rapidly describe the composition of cheese fungal communities (Hermet *et al*., 2014).

One disadvantage of the SSCP technique is that the labeled single strand DNA fragments cannot be sequenced to confirm the database-derived species designations. Furthermore, there is currently no theoretical model for predicting the exact conformation of a DNA fragment under various parameters such as mutation, DNA fragment size, G and C content, gel matrix porosity, DNA concentration, ionic strength and pH. In addition, the complexity and variability in the balance of different peaks from one milk sample to the next have been observed (Verdier-Metz *et al*., 2009). SSCP-PCR analysis can be biased due to the coelution of species in some SSCP peaks. As a result, microbial diversity may be overestimated, with only the dominant population detected.

Real-time PCR (qPCR)

Real-time PCR (qPCR) is a molecular technique that is increasingly applied as a rapid and sensitive method for the molecular quantification of bacteria in dairy products. It monitors the amplification of the target DNA in real-time and enables the quantification of a target species. In order to quantify the presence of a certain bacteria by qPCR, the amount of bacterial DNA should be correlated to the amount of bacterial biomass (Edlund, 2007).

A fluorescent probe is used in the qPCR method to monitor the amplification of a target sequence. The two most common methods for detection are the use of DNA-binding fluorescent molecules like SYBR green or the use of a reporter-quencher system like TaqMan® probes. To obtain absolute quantification, the abundance changes of a specific gene are compared to a standard control DNA sequence with a known number of copies. A standard curve can then be used to calculate gene copy numbers (Edlund, 2007). Furthermore, qPCR with SYBR Green and a FAM (6-carboxy-fluorescein)-labeled TaqMan probe specific to *Enterobacters akazakii* has been used to detect *E. sakazakii* in infant formula and quantify *C. tyrobutyricum* spores in dairy products (López-Enriquez *et al*., 2007). In addition to the inherent benefits of PCR, RT PCR has the advantage of being faster than conventional PCR and capable of detecting and quantifying bacteria DNA in the same reaction vessel in real-time (Espy *et al*., 2006).

Real-time PCR detects the PCR product without the need for post-PCR processing such as gel (agarose, polyacrylamide) or capillary electrophoresis, as previously described in PCR fingerprinting methods (DGGE, TTGE, SSCP, RFLP). Furthermore, these methods are considered semi-quantitative, whereas real-time PCR allows for accurate template quantification and identification (Justé *et al*., 2008).

Evaluating the presence and abundance of Enterococcus gilvus in Italian artisan and industrial cheeses, a culture-independent qPCR with a phenylalanyl- tRNA synthase (PheS) as a target gene was optimized. The absolute specificity of a real-time PCR assay was demonstrated by the unequivocal differentiation of Enterococcus gilvus from other LAB species (Zago *et al*., 2009). Furthermore, *Lb. lactis* subsp. *cremoris, Lb. lactis subsp. lactis* and *Leu.* sp. were identified using real-time PCR for the first time. Described a multiplex real-time PCR system for identifying thermophilic LAB such as *Lb. delbrueckii, Lb. helveticus, St. thermophilus* and *Lb.* fermentum in undefined starter cultures of hard-cooked cheeses (Bottari *et al*., 2013).

Ongol *et al*. (2009) quantified *Streptococcus thermophilus* in plain yogurt and yogurt-containing fruits by real-time PCR. The DNA was isolated from the *Streptococcus thermophilus* pure culture and directly from yogurt samples, while a gene sequence encoding 16S rRNA processing protein, rimM, was a target in the PCR reaction. The difference between the enumeration of *Streptococcus thermophilus* by standard plate count (SPC) and quantification by qPCR was 3.96% in favor of SPC, indicating a high correspondence between the two methods. The *Lactococcuslactis ssp. cremoris* ATCC 19257 strain was also successfully quantified by qPCR in milk fermented by mixed cultures. By employing specific primers, the detection limit was 200 CFU of *Lactococcuslactis ssp. cremoris* ATCC 19257 per milliliter of mixed culture (Grattepanche *et al*., 2005). A recent study by Matija *et al*. (2009) demonstrated that real-time PCR based on the amplification of parts of the 16S rRNA gene turned out to be a convenient approach for accurate selective quantification of *Lactobacillus gasseri, Enterococcus faecium* and *Bifidobacterium* infantis present in the probiotic product.

Real-time PCR has recently been used to quantify *thermophilic Lb. delbrueckii subsp. bulgaricus* and *St. thermophilus,* which are commonly used for milk fermentation and cheese ripening (Stachelska and Foligni, 2018). Real-time PCR was used in China to assess the LAB biodiversity in 86 fermented milk products. Among the 705 LAB species identified, the most common were *Lb. delbrueckii subsp. bulgaricus, Lb. helveticus* and *Lb. fermentum.* Cow and yak milk samples contained significantly different microbiota.

Fluorescence *in situ* hybridization (FISH)

FISH with 16S rRNA gene probes is a culture-independent molecular method for microbial identification and physical detection of microorganisms in a food matrix, as well as the distribution of microbial populations in environmental samples. FISH is used in food microbiology to identify bacteria *in situ*, without the need for isolation (Cocolin *et al*., 2007). It is a non-PCR-based molecular technique that uses a fluorescently labeled 16S rRNA bacterial domain probe to observe colonies of microbial cells distributed in a food matrix, such as cheese. FISH uses a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell to detect nucleic acid sequences. Oligonucleotide probes can be designed for each taxonomic level, down to genus and species levels can be designed according to the target region of the 16S rRNA gene (Motter *et al*., 2000).

Basically, the FISH analysis consists of several steps:

(i) Sample preparation and cell fixation, usually by paraformaldehyde.
(ii) Sample immobilization onto microscopic slides.
(iii) Cell treatments to increase the permeability of the probe.
(iv) *In situ* hybridization with fluorescently labeled oligonucleotide probes (Ercolini *et al*., 2003).

FISH was used to investigate the structure and location of bacterial communities in Stilton cheese. For detecting *Lactococcuslactis, Lactobacillus plantarum* and *Leuconostoc pseudomesenteroides*, fluorescently labeled oligonucleotide probes were developed. The combination of these probes and the bacterial probe Eub338 allowed the spatial distribution of different microbial species in the Stilton cheese matrix to be evaluated. FISH was used to examine the microbiota of Feta cheese samples using probes specific for eubacteria, *Streptococcus thermophilus, Lactococcus spp.* and *L. plantarum.*

The FISH method is best suited for mapping a specific group or species rather than mapping total diversity, while there are practical limits to how many oligonucleotide probes can be used simultaneously.

Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a fast and sensitive molecular approach with high resolution and the ability to assess subtle genetic differences between strains, providing a deeper understanding of the structure of microbial communities and dynamics in response to changes in different environmental conditions or studying bacterial populations in their natural habitats. The method combines targeted PCR amplification with restriction enzyme digestion, high-resolution electrophoresis and fluorescent detection. The use of a single fluorescently labeled primer limits the analysis to only the terminal fragment. Because of its simplicity and reliability for bacterial 16S rRNA genes, TRFLP is increasingly being used to analyze microbial communities (Rasolofo *et al*., 2011). Furthermore, it has proven to be a useful method for quickly comparing the temporal changes and relationships of bacterial communities, as well as for identifying the most dominant microbial sequences in dairy samples (Ndoye *et al*., 2011). Fuka and colleagues (2013) used TRFLP and 454 pyrosequencing of tagged 16S rRNA gene amplicons to gain a comprehensive understanding of the bacterial community structure of Croatian raw ewe's milk cheese.

T-RFLP analysis typically consists of five major steps:

1. Finding and purifying DNA from the microbial community.
2. Amplification of the 16S rRNA gene with fluorescently labeled forward and unlabeled reverse primers, followed by restriction enzyme digestion with enzymes that recognize four base pairs.
3. Electrophoresis separation and detection of digested products, with terminal fragments at the 5' end containing a fluorescent label.
4. Data analysis to generate the fragment profile for each sample.
5. Cluster analysis based on the sample profile from the previous step.

T-RFLP has been shown to be sensitive in analyzing the composition of the gut microbiota by amplifying 16S rRNA genes with labeled universal primers ENV1 (5′-6-FAM-AGA GTT TGA TII TGG CTC AG-3′, *E. coli* nr. 8-27) and ENV 2 (5′-CGG ITA CCT TGT TAC GAC TT-3′, *E. coli* nr. 1511-1492) (Sjöberg *et al*., 2013).

This method is useful for generating a comparative bacterial profile of a diverse bacteria sample. Most of the advantages and limitations of this technique are similar to those of RFLP. However, this technique has the unique advantage of being able to give the relative amounts of bacteria flora of a sample containing different bacteria and is suitable for profiling a mixed bacteria culture without prior culturing. Furthermore, the more restriction enzyme used the better the resolution of microbial profiles.

Length heterogeneity PCR (LH-PCR)

LH-PCR is a fingerprinting method that is used to study microbial diversity associated with a specific ecosystem by varying the length of sequences of the 16S rRNA gene or other genes. The length heterogeneity PCR method is similar to the more widely used T-RFLP method. The T-RFLP method identifies PCR fragment length variations based on restriction site variability, whereas LH-PCR analysis distinguishes between organisms based on natural variations in the length of the 16S rRNA sequences. T-RFLP analysis has been used successfully for a wide range of applications, whereas the LH-PCR method has been primarily used to study microbial diversity (Quigley *et al*., 2011).

LH-PCR has a significant advantage over other methods of analysis in that it is quick, simple, efficient, dependable and highly reproducible (Quigley *et al*., 2011). Theoretically, an estimate of the qualitative and quantitative composition of dominant populations within a microbial community is possible. When converting fluorescence data into electropherograms, peaks represent fragments of varying sizes and the areas under the peaks are a measure of the fragments' relative proportions. LH-PCR can produce results in as little as 30-40 minutes.

Furthermore, this method is used to investigate the structure and dynamics of cheese microbiota, as well as to assess the trend of microbial dynamics of LABs throughout the cheese-making process (Santarelli *et al*., 2013). Recently, Lazzi *et al.* (2016) used the same approach to investigate the relationship between the dynamics of growth and lysis of LAB in Grana Padano cheese and their relationship with the formation of volatile flavour compounds during the ripening process.

The disadvantage of LH-PCR was preferential annealing to specific primer pairs or an increase in the incidence of chimeric PCR products as the number of PCR cycles increased and only dominant, active members of the complex community could be amplified. Many rare members, on the other hand, may go undetected (Lazzi *et al*., 2004). The LH-PCR method was used to investigate the biotechnological potential of Lactobacillus, one of the most important LAB genera in dairy products and to demonstrate the difficulties associated with their identification (Martin-Platero *et al*., 2009).

CONCLUSION

Despite the fact that direct compatibility examinations are a simple and interesting method for predicting LAB interaction in food, culture-independent methods are most effective for understanding LAB composition and dynamic changes in LAB microflora. Although culture-independent methods are highly sensitive and reliable, both types of methods have limitations that can be overcome by combining them, thereby providing more information on microbial complexity. Combining culture-dependent and culture-independent methods or a polyphasic approach may be the most effective strategy for studying microbial communities. Multistrain cultures are unquestionably more efficient and consistent than monostrain cultures. Because mixed cultures of starter, probiotic, or adjunct lactic acid bacteria provide greater benefits than single cultures, future research should be guided by compatibility tests to identify the most suitable and beneficial mixed starter bacteria.

Conflict of interest: None.

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