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review

Methods for Culture-Independent Identification of Lactic Acid Bacteria in Dairy Products

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Summary

Culture-independent molecular tools have been introduced into food microbiology during the last ten years. Most of them are based on the amplification of a bulk bacterial DNA extracted directly from a sample, the targeting of a selected gene, or a variable region of the selected gene. Many studies have explored indigenous lactic acid bacteria in dairy products by culture-independent molecular approaches. It is well known that indigenous microbiota significantly contribute to the uniqueness of artisanal cheeses. However, there is no molecular method that can provide complete qualitative and quantitative insight into the microbiota associated with a certain ecosystem. Therefore, a combination of molecular approaches should be applied to get a more objective picture of the microbiota. This paper aims to present the most widely used culture-independent molecular tools for identifying lactic acid bacteria in dairy products.

Key words: culture-independent identification, molecular methods, lactic acid bacteria, dairy products

Introduction

The dairy microbial ecosystem is very complex and responsible for the broad diversity of tastes, aromas, and textures of dairy products. 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. Cheese processing is largely based on fermentation by lactic acid bacteria (LAB), which are added as starter cultures or are adventitiously present in the biotope and selected during the fermentation process (*1*). The microbiota of dairy products are made up of starter and non-starter lactic acid bacteria, other bacteria, yeasts, and filamentous fungi that form secondary microbiota, which play a significant role during cheese ripening. Starter and secondary bacteria modify the physical and chemical properties of cheese and largely influence its aroma, taste and textural characteristics (*2*,*3*).

Molecular methods provide an outstanding tool for detection, identification and characterization of microorganisms found in environmental samples, foods and other complex ecosystems. In the last 10 years the application of culture-independent molecular methods based on isolation of total microbial DNA from a sample without prior cultivation, and amplification of the 16S rRNA gene by the polymerase chain reaction (PCR) method, have been introduced into food microbiology. It should be mentioned that molecular methods can be applied as culture-independent or culture-dependent, which exclusively depends on whether bacterial DNA was extracted directly from a sample, or from bacterial colonies grown

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on culture media. It is well known that conventional culturing does not cover the entire microbial diversity of complex environments (*4–7*). Typical sensorial qualities of traditional cheeses depend on many factors, including traditional cheese making practices, feeding of dairy animals, and the diversity and dynamics of the microbial community. The qualitative and quantitative composition of the cheese microbial community and its activity during ripening play an important role in the development of sensorial characteristics (*8*).

Studies of the diversity and dynamics of dairy ecosystem microbiota increasingly rely on culture-independent methods based on the direct analysis of DNA (or RNA) without any cultivating step. Basically, these methods include total bacterial DNA and/or RNA extraction directly from the sample and amplification by PCR. There are numerous combinations of primers, gene(s), and/or variable regions of the 16S rRNA gene already published that have been widely used in microbial community studies. The PCR amplicons can be separated by gel or capillary electrophoresis or hybridized to specific probes (*2,9*). The aim of this paper is to present and summarise the most frequently used culture-independent molecular approaches applied for the identification of lactic acid bacteria associated with dairy products.

Culture-Independent Polymerase Chain Reaction (PCR)

PCR is a fundamental aspect of molecular biology, and many molecular methods based on PCR have been developed to study microbial communities. In PCR, DNA serves as a template for PCR amplification of genetic targets with universal, genus- or species-specific primers to amplify target sequences of a given population. Basically, PCR consists of three major steps: denaturation of DNA 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 (*10*). It must also be underlined that optimization of PCR conditions for each step is inevitable in every experiment. Very often, many experimental trials must be performed to set up optimal PCR conditions (*11–16*).

An efficient direct extraction of total bacterial DNA from a sample is a prerequisite for a successful culture-independent molecular approach based on PCR. The total bacterial DNA extraction and purification can be performed by many protocols described in detail by other authors (*13–16*). 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 with the amplicons of all LAB present in the sample. Additionally, some genotypes may remain undetected due to low species abundance in the sample, low species availability due to insufficient homogenization of the cheese matrix, or incomplete cell lysis that prevents the release of nucleic acid or inhibits PCR amplification (*2,11,13*). Furthermore, a crucial step for molecular identification of microbial communities is also the selection of a gene or a genetic marker that can be used for amplification in PCR to differentiate a wide variety of organisms. At present, the bacterial 16S ribosomal RNA operon, encompassing 16S rRNA and 23S rRNA genes, is most frequently used as a molecular marker in microbial ecology studies (*11*). However, the 16S rRNA gene shows discrimination pitfalls in the identification of closely related species. Therefore, the other target genes, such as the elongation factor *Tu* gene, *rpo*B gene, *rpo*A gene, the DNA recombinase gene (*rec*A) and *phe*S gene, have also been exploited for the differentiation of LAB species (*9,11,12*).

The fastest culture-independent PCR approach for the genus-, species- or strain-specific detection of LAB in the cheese matrix is the use of specific primers for PCR-based detection of the target organisms in the total bacterial DNA extracted from a sample. The main disadvantage of such an approach is that only 'expected' microorganisms will be detected if they are present in the sample (*17*). Therefore, such PCR assays are of limited value in the analysis of complex microbial ecosystems like artisanal cheeses. Such approaches have not been widely used in culture-independent community studies of dairy products since a specific primer pair is required for every bacterial species. However, it can be a helpful approach for confirming the presence and unequivocal identification of a targeted species (*1,18*). Biodiversity of the bacterial ecosystem in traditional Egyptian Domiati cheese was explored by employing culture-independent species-specific PCR using DNA directly extracted from cheese as a template DNA. Thirty-one species-specific primers were used to confirm the presence of bacterial species in the cheese. Many representatives of genera *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Staphylococcus* were confirmed by species-specific PCR (*18*). However, such culture-independent PCR approaches are much more labour-intensive than approaches using universal bacterial primers targeting 16S rRNA genes of the bacterial community, followed by separation of PCR amplicons. Nevertheless, such approach can identify even minor LAB community members, which is one of the main drawbacks of other culture-independent approaches.

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) and PCR-Temporal Temperature Gradient Electrophoresis (TTGE)

To investigate microbial diversity and the dynamics of dairy products by culture-independent PCR-DGGE/TTGE molecular methods, genomic bacterial DNA and/or RNA must be extracted directly from the sample, followed by amplification of the variable regions of the 16S gene. If the total DNA of the microbiota is used in PCR amplification, the DGGE technique can provide a profile of the genetic diversity, while if total RNA is used, the profile indicates metabolically active microbiota (*14,15*). PCR-DGGE was introduced into microbial ecology by Muyzer *et al.* (*19*). Many scientists have been using this technique, which has become a well-established tool for investigating microbial diversity in many laboratories. PCR-DGGE is usually employed to assess the structure of a microbial community without cultivation, and to determine the community dynamics in response to environmental variations (*20,21*).

The principle of DGGE relies on 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. Essentially, PCR-DGGE comprises three steps: (*i*) extraction of total community DNA from the sample; (*ii*) PCR amplification using specific oligonucleotide primers; and (*iii*) separation of the amplicons using DGGE. For this purpose, a reproducible and efficient method of total DNA extraction is indispensable and must be evaluated and optimized depending on the nature of the sample (*9*,*13*,*22*). In the subsequent PCR step, multiple PCR primer sets with different resolutions can be used. The use of universal primers allows any microbial community to be detected. However, it must be indicated that in ecosystems with a high diversity only the dominant microbiota will be detected. In order to focus on specific subpopulations, genus-specific PCR primers should be used. In most PCR-DGGE applications on bacteria, universal or specific primers target the 16S rRNA gene. However, an insufficient number of polymorphic regions or the presence of multiple rRNA operons in a single strain may complicate the interpretation of DGGE data. Therefore, single-copy housekeeping genes such as *rpo*B are being evaluated as alternative target genes for community analysis using DGGE (*5*,*23*).

Separation of the resulting PCR amplicons is performed by DGGE or TTGE electrophoresis. The fingerprint of a microbial community in DGGE or TTGE gel can be identified by comparing the migration position of a band to that of a molecular ladder of representative species and by sequencing. Sequencing of bands should be applied because closely related species might co-migrate and take the same position in the gel. Therefore, identification based just on comparison with the reference ladder does not absolutely guarantee an unequivocal identification of bacterial species (*21*). In both TTGE and DGGE, DNA fragments of the same length but with different sequences are separated, based on decreased electrophoretic mobility of partially melted double-stranded DNA molecules. For PCR-DGGE, the denaturing conditions rely on the use of chemical denaturants (formamide and urea) incorporated into acrylamide gel as a linear denaturing gradient. PCR-DGGE electrophoresis is carried out at constant temperature, between 55 and 65 °C. For PCR-TTGE, the denaturing gradient is obtained by varying the temperature over time without chemicals (*2*,*21*). TTGE and DGGE are now frequently applied in microbial ecology to compare the compositions of complex microbial communities and to study their dynamics (*1*,*24*). Diversity and dynamics of microbial communities of dairy ecosystem, revealed by a culture-independent approach through PCR-DGGE/TTGE, has been studied by many authors (*14*,*18*,*25–36*).

In several studies, it has been indicated that microbiota of different cheese varieties are specific and unique since they are influenced by the technology of cheese manufacturing. Moreover, the same type of cheese produced at different locality is also characterised by a different microbiota composition. Furthermore, there is no universal molecular approach that could reveal representatives of all microbiota characteristic of any cheese. Recent studies only confirm the need for a polyphasic approach to investigate microbial diversity and dynamics of artisanal cheeses and highlight the different capabilities of the methods employed to detect LAB species (*9*,*37*). 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 (*20*,*38*). A study of the bacterial community of Stilton cheese revealed that representatives of *Leuconostoc* community were detected only by amplification of the V4-V5 region of the 16S rRNA gene, while targeting the V3 region failed (*29*). Similar results were obtained in a study of diversity and dynamics of Provolone del Monaco cheese, in which higher microbial diversity was obtained by amplifying the V6-V8 regions instead of the V3 region (*37*). A significant difference in the DGGE analysis of 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 (*39*). Thorough detection of species in a mixed microbial community is another drawback of the DGGE method. Namely, species that constitute a low percentage of the population are not readily detectable (*19*). Very often, individual members from a mixed population cannot be identified by PCR-DGGE when their concentration is lower than 10^4 CFU/g (*40*,*41*). However, this may not be true when other primers are applied in the PCR amplification step (*38*). The detection limit in DGGE analysis depends on the species and perhaps even on the strain considered. Nevertheless, 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 (*6*,*42*). A limit of detection for TTGE was observed when the minority species accounted for 1:100 or less of the total DNA concentration. Detection of species by TTGE may be limited either by low DNA concentration or by the presence of high concentration of competing DNA. Competition for PCR primers, by DNA of the dominant species in a total extracted DNA, is a limiting factor for TTGE sensitivity (*43*,*44*).

In order to reveal metabolically active microbiota of artisanal cheeses, some authors have performed analyses on reverse-transcribed (RT) RNA. By combining RT-PCR-DGGE (RNA-based) and PCR-DGGE (DNA-based), it is possible to differentiate metabolically active (RNA-derived) microbiota from the total diversity (DNA-derived) of microbiota (*2*,*14*,*15*,*28*). 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 ripening of artisanal Sicilian cheese (*28*). 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

(*11*). A drawback of DNA-based approaches was observed by Licitra *et al.* (*32*) in the PCR-TTGE analysis of Ragusano cheese from curd stretching to the 7th month of ripening. PCR-TTGE did not exhibit any changes in the microbiota profile during the ripening period. The explanation for such a profile may be the competition of DNA for primers in PCR reaction. Namely, the DNA of dead cells can be predominant, masking by competition during the PCR step the DNA corresponding to the growth of minor microbiota during the ripening period. Therefore, the minor microbiota representatives will not amplify and they cannot be detected. As the RNA of dead cells is rapidly degraded, the competition indicated above would not have occurred if the RNA-based approach was applied (*32*).

Single-Strand Conformation Polymorphism-PCR (SSCP-PCR)

SSCP-PCR is a molecular technique using either an acrylamide gel-based or a capillary-based automated sequencer for the separation of denatured (single-stranded) PCR products. Under nondenaturing conditions, single--stranded DNA folds into tertiary structures according to their nucleotide sequences and their physicochemical environment. This causes differences in electrophoretic mobility in nondenaturing gels (*2*). Because of the use of universal primers, SSCP, like the other culture-independent molecular methods, can be applied without any *a priori* information on the species to give a more objective view of the microbial community (*8*). PCR products must be heat-denatured and cooled in order to adopt a single strand conformation that is sequence-dependent. PCR products that have a similar size can be separated on the basis of their sequences and detected by a laser using a genetic analyzer. To improve the separation of numerous sequences, several primer pairs targeting a different variable region of the 16S rRNA gene can be used (*45,46*). After PCR-DGGE/TTGE, SSCP-PCR is the most frequently used method to study complex microbial communities of artisanal cheeses (*2*). It has been used to analyze microbial communities of Salers cheese made from raw milk by amplifying the V4 region of the 18S rRNA gene (*47*) and by amplifying the V2 and V3 regions of the 16S rRNA gene (*8,46*), and to analyze artisanal Saint--Nectaire cheese and raw milk samples by amplifying the V3 region of the 16S rRNA gene (*48,49*), and soft red smear cheese (*45*). SSCP-PCR analysis can be biased due to coelution of species in some SSCP peaks. Therefore, microbial diversity may be underestimated and only the dominant population detected (*49,50*).

Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) with 16S rRNA gene probes is a culture-independent molecular method enabling microbial identification and physical detection of microorganisms in a food matrix. It also provides information about the distribution of microbial populations in environmental samples. In food microbiology, FISH is used for the identification of bacteria *in situ*, without the need of isolation (*51*). It is 'non-PCR--based' molecular technique that uses a fluorescently labelled 16S rRNA bacterial domain probe to allow observation of colonies of microbial cells distributed in a food matrix such as cheese. FISH detects nucleic acid sequences by a fluorescently labelled probe that hybridizes specifically to its complementary target sequence within the intact cell. Oligonucleotide probes, for each taxonomic level down to genus-specific and species-specific, can be designed according to the target region of 16S rRNA gene (*52,53*). Basically, FISH analysis consists of several steps: (*i*) sample preparation and cell fixation, usually by paraformaldehyde; (*ii*) sample immobilisation onto microscopic slides; (*iii*) cell treatments to increase permeability of the probe; and (*iv*) *in situ* hybridisation with fluorescently labelled oligonucleotide probes. After hybridisation, slides are observed by epifluorescent microscopy (*4,54*). FISH was used to study bacterial community structure and location in Stilton cheese. Fluorescently labelled oligonucleotide probes were developed to detect *Lactococcus lactis*, *Lactobacillus plantarum* and *Leuconostoc pseudomesenteroides*. The combined use of these probes and the bacterial probe Eub338 allowed the assessment of spatial distribution of different microbial species in the Stilton cheese matrix (*53*). Microbiota of Feta cheese samples were also analyzed by FISH using probes specific for eubacteria, *Streptococcus thermophilus*, *Lactococcus* spp. and *L. plantarum* (*14*).

The FISH method is most suitable when targeting a specific group or species, rather than aiming to map total diversity, as there are practical limits to how many oligonucleotide probes can be used simultaneously (*55*).

Real Time PCR (qPCR)

Real-time PCR (qPCR) is a molecular technique that is increasingly applied as a rapid and sensitive method for molecular quantification of bacteria in dairy products. It monitors amplification of the target DNA in real--time and enables quantification of a target species (*56,57*). 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 (*58*). The qPCR method uses a fluorescent probe to monitor the amplification of a target sequence. The two most common methods for detection are DNA-binding fluorescent molecules, such as SYBR green, or the use of a reporter-quencher system, such as TaqMan® probes. To obtain absolute quantification, the changes in abundance of a specific gene are compared to a standard control DNA sequence with a copy of known numbers. The gene copy numbers can then be calculated from a standard curve (*59,60*). Real--time PCR enables detection of the PCR product, avoiding the need for a post-PCR processing like gel (agarose, polyacrilamide) or capillary electrophoresis, as already described in PCR fingerprinting-based methods (DGGE, TTGE, SSCP, RFLP). Moreover, these methods are considered as semi-quantitative, while real-time PCR allows identification and accurate template quantification (*11, 61,62*).

Culture-independent qPCR using a phenylalanyl--tRNA synthase (*Phe*S) as a target gene was optimised to evaluate the presence and abundance of *Enterococcus gilvus* in Italian artisan and industrial cheeses. Unequivocal distinguishing of *Enterococcus gilvus* from other LAB

species was achieved, demonstrating the absolute specificity of a real-time PCR assay (*56*). Ongol *et al.* (*63*) quantified *Streptococcus thermophilus* in plain yoghurt and yoghurt containing fruits by real-time PCR. The DNA was isolated from the *Streptococcus thermophilus* pure culture and directly from yoghurt samples, while a gene sequence encoding 16S rRNA processing protein, *rim*M, was a target in the PCR reaction. The difference between enumeration of *Streptococcus thermophilus* by standard plate count (SPC) and quantification by qPCR was 3.96 % in favour of SPC, indicating a high correspondence between the two methods. The *Lactococcus lactis* 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 *Lactococcus lactis* ssp. *cremoris* ATCC 19257 per millilitre of mixed culture (*62*). A recent study by Bogovič Matijašić *et al.* (*64*) 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.

In addition to DNA-based real-time PCR approaches, Monnet *et al.* (*65*) developed a method for extraction of RNA from cheese without culturing, and optimised real-time reverse transcription PCR for *Lactococcus lactis*. RNA-based real-time PCR could provide valuable data about the quantitative and metabolic status of the target. However, as RNA is less stable than DNA, an efficient and reproducible extraction of RNA still remains a challenge. Nevertheless, the development of culture-independent real-time PCR offers advantages such as sensitivity, accuracy and the possibility of robotic automation (*66*).

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

The T-RFLP technique is a culture-independent, rapid, sensitive and reproducible method for assessing the diversity of complex microbial communities based on variation in the 16S rRNA gene. The analysis can be used in researching microbial community structure and dynamics in response to changes in different environmental conditions or to study bacterial populations in their natural habitats (*67*). T-RFLP analysis allows fingerprinting of a microbial community by analyzing the polymorphism of a certain gene. Analysis is based on the restriction endonuclease digestion of fluorescently end-labelled PCR products, the 16S rRNA gene. The method provides distinct profiles (fingerprints), depending on the species composition of the microbial communities of the samples (*68*).

The typical T-RFLP analysis is comprised of five major steps: (*i*) isolation and purification of DNA from the microbial community; (*ii*) amplification of the 16S rRNA gene, using fluorescently labelled forward and unlabelled reverse primers, followed by restriction enzyme digestion with enzymes that have 4 base pair recognition sites; (*iii*) separation and detection of the digested products by electrophoresis, where the terminal fragments at the 5´ end will contain a fluorescent label; (*iv*) analysis of data to generate the fragment profile for each sample; (*v*) clustering analysis based on the profile of the sample from the previous step (*67*).

T-RFLP analysis was used to characterize the members of the complex bacterial flora present on the surface of three Tilsit-type cheeses during the eight-week ripening period (*69*). Individual strains of the defined smear starters yielded characteristic terminal restriction fragments (TRFs) using two different restriction enzymes (*Hae* III and *Cfo* I). Most of the starter strains reached the maximum level after 2 to 4 weeks, but were not observed at week 8. An exception was the *Corynebacterium* species, which remained as the dominant bacterial genus on the surface of the fully ripened cheese (*69*). The application of T-RFLP analysis to hard-type cheeses (Gouda-type and Maasdam) and yoghurt was reported by Rademaker *et al.* (*70*). T-RFLP analysis enabled simultaneous characterization of bacterial population structure and dynamics between long time spans, such as in cheese ripening and short time spans, such as in yoghurt production. T-RFLP analysis can be suitable for performing semi-quantitative analyses of simple microbial ecosystems, such as defined simple dairy starter cultures. Analysis of complex ecosystems with high microbial diversity means differences in cell lysis resistance, genome size or G+C content, which can lead to differential amplification and, as a result, T-RFLP analysis can overestimate the number of species present in samples of unknown composition (*71*).

Length Heterogeneity PCR (LH-PCR)

Length heterogeneity PCR analysis is similar to the more commonly used T-RFLP method. The difference between these two methods is that the T-RFLP method identifies PCR fragment length variations based on restriction site variability, whereas LH-PCR analysis distinguishes different organisms based on natural variations in the length of the 16S rRNA sequences. T-RFLP analysis has been used successfully for a variety of applications, while LH-PCR method has been limited more to studies of microbial diversity (*72*).

The major advantage of LH-PCR over other methods of analysis is that it is efficient, reliable and highly reproducible. It is theoretically possible to obtain an estimate of both qualitative and quantitative composition of dominant populations within a microbial community. When converting fluorescence data into electropherograms, peaks represent fragments of different sizes and the areas under the peaks are a measure of the relative proportions of the fragments. Results can be obtained rapidly by LH-PCR, in as little as 30–40 min. Like other PCR-based techniques, LH-PCR has particular biases, such as preferential annealing to particular primer pairs or an incidence of chimeric PCR products with increasing numbers of PCR cycles. Also, only dominant, active members of the complex community may be amplified and therefore many rare members may not be detected (*73*).

The LH-PCR method was used to study the biotechnological potential of one of the most important LAB genera in dairy products, *Lactobacillus*, and to demonstrate the difficulties inherent in their identification (*74*).

Predominant *Lactobacillus* species detected by LH-PCR were *L. plantarum* and *L. paraplantarum*, followed by *L. curvatus* in Quesailla Arochena cheese, and *L. plantarum* followed by *L. curvatus/L. corynformis* in Torta Arochena cheese. LH-PCR detected a low number of *L. paracasei* in Torta Arochena, while *L. curvatus* as well as *L. paracasei*, could not be distinguished by TTGE analysis (*74*). Population fingerprints obtained by LH-PCR were sufficiently characteristic to differentiate dominant LAB species within whey starter cultures for Grana Padano cheese and to evaluate the main microbial differences among various starters (*73*). Community fingerprinting by LH-PCR was used to determine the typical microbial composition of Grana Padano cheese whey starter. Dominant species were *L. helveticus*, *L. delbrueckii* ssp. *lactis/bulgaricus* and *Streptococcus thermophilus* (*75*). In order to better understand the microbial ecology of Parmigiano-Reggiano cheese, LH-PCR analysis was used for the first time to monitor microbial dynamics from the whole and lysed cells in fermented food during production and during 24 months of ripening (*76*). Recovering DNA from lysed cells can be an indicator of complex microbial dynamics during subsequent stages of cheese making.

Further Challenges and Perspectives in Culture-Independent Microbiota Studies

Culture-independent molecular approaches still fail to completely identify microbiota. Therefore, it is worthwhile to devote effort to improving the detection limits of culture-independent methods (*2*). The study of gene expression and translation into proteins within natural environments are two emerging fields in microbial ecology that hold special promise in the study of bacterial function. Moreover, massive parallel sequencing, metagenomics and metatranscriptomics will allow us to study microbial diversity more deeply (*77*). The approach of combining 16S rRNA gene profiles and the profiles of functional genes may enable the structure of microbiota to be related to the function in the ecosystem. Functional diversity, which is closely related to the complexity of the cheese microbiota, plays a crucial role in flavour compound development during cheese ripening. Therefore, structure–function studies should provide new insights into the role of complex cheese microbial community (*9,78*).

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