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# CLASSIC VERSUS MODERN TOOLS TO STUDY MICROBIAL POPULATION DYNAMICS DURING FOOD FERMENTATION PROCESSES

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***Abstract***

*For a better understanding of microbial processes and dynamics of microbial population in fermented food is essential the taxonomical definition of their content. It is difficult to estimate true microbial diversity due to inability to cultivate most of the viable bacteria or to evaluate stressed cells. The most appropriate approach it seems to be the integration of phenotypic and genotyping data, while the molecular methods alone are not enough to establish distinct boundaries among phyllogenetically related species. It is important for identification of microbial strains to connect physiological, morphological and biochemical features as well as the aspects of its genetic profile. The most common genotypic and phenotypic methods are reviewed in this paper, highlighting on the suitable techniques which can be used to differentiate among microbial strains.*

***Key words****: molecular techniques, food fermentation, genotyping, phenotyping.*

## INTRODUCTION

Fermentation is a metabolic process in which an organism converts a carbohydrate, such as starch or sugar, into an alcohol or an acid. For example, the yeast performs fermentation to obtain energy by converting sugar into alcohol. Lactic bacteria perform fermentation, transforming carbohydrates into lactic acid. This process is used to produce wine, beer, yogurt and other products.

Fermentation is a natural process. People have been fermenting to produce products like wine, honey, cheese and beer long before the biochemical process is understood. In the 1850s Louis Pasteur became the first scientist to study fermentation when it was shown to be caused by living cells.

The most important fermentations involved in food production are alcoholic fermentation, lactic fermentation and acetic fermentation. In the alcoholic fermentation yeast and certain bacteria perform the fermentation of carbohydrates in which pyruvic acid is broken into ethanol and carbon dioxide; this process is specific to bread, wine or beer production.

In the lactic fermentation, the lactose is converted through pyruvic acid in lactic acid. This type of fermentation is used for the production of cheese and dairy products. Acetic fermentation is another type of fermentation and is produced by acetic bacteria, and as an intermediate product results acetic acid.

By the acetic fermentation of the wine we get the vinegar. Acetic fermentation is also used to conserve pickles. Although it is considered fermentation, it is carried out in the presence of oxygen.

During these fermentations, which are conducted on natural sources of carbohydrates (grapes, milk, cereals etc.), the microbial biodiversity and levels are in a continuous changing.

To get the best final product it is important to conduct an optimal fermentation process, in which the microorganisms involved are an important factor.

To characterize the fermentation microbial biodiversity the approach is complex and should be taken into account phenotypic and genotypic methods and to establish correlation between the results of these methods (Girafa et al., 2004; Cocolin et al., 2013; Donelli et al., 2013; Matei et al., 2018).

In the following will be presented both the phenotypic and genotypic tools useful in characterizing the microbial diversity and levels.

## MATERIALS AND METHODS

Online information research was conducted by the use of different database collections and on-searching engines (Google Academic, Web of Knowledge, PubMed and ScienceDirect). The information has been structured according to the approach used in the characterization of the microbial diversity.

## RESULTS AND DISCUSSIONS

Phenotypic studies have been broadly used during years, this is why the presented data will mainly focus on the molecular tools used in the characterization of microbial biodiversity during food fermentations.

### (1) Phenotypic methods

Phenotypic characterization of microbial strains is based on data supplied by all the typing methods not based on DNA or RNA, including chemotaxonomic methods that are able to give information on chemical constituents of microbial cells. Thus, the classical phenotypic tests are important sources of taxa, from species up to genus and family. In many cases the set of all the morphological, physiological and biochemical features of a strains allows the recognition of taxa. These phenotypic characteristics in specific microbial groups, such as lactobacilli and bifidobacteria, are not enough to completely describe or differentiate taxa and must be performed in addition to genotypic analysis (Tannock, 1999; Mastromarino et al., 2002)

The morphological investigation of a microorganism both by light and electron microscopy provides information on cell shape, flagella and inclusion bodies while color, dimension and form of its colonies are detected macroscopically on a suitable agar plate. Physiological data useful for classification purposes include growth temperature, pH value, salt concentration and oxygen requirement whereas biochemical features of interest include enzymatic activity, gas production and compound metabolism (Yang et al., 2010; Nomura et al., 1999).

For rapid phenotypic characterisation in practice are used API stripes, which are test kits for identification of Gram positive and Gram negative bacteria and yeast produced by Biomerieux company. The system offers a large and robust database now accessible through the Internet-based APIWEB™ service. According to the most common protocols, carbohydrate fermentation analysis for lactobacilli is carried out using API 50 CH, a research strip that enables the study of the metabolism of 49 carbohydrates and is able to identify *Lactobacillus* species within 48 hours. However, some epidemiological studies have reported shortcomings in the use of this methodology due to the possibility of identifying lactobacilli belonging to different species as the same microorganism (Vasquez et al., 2002), thus fermentative profile seems to be inaccurate method for identification and classification of *Lactobacillus* species which therefore needs to be performed additional genotypic analysis (Pavlova et al., 2002).

FAME (fatty acid methyl esters) analysis (Miller, 1982) has been successful used since fatty acids are the major constituent of lipids and lipopolysaccharides in microbial cells and have been used for taxonomic purposes. In fact, more than 300 different chemical structures of fatty acids and their variability in chain length, double-bond position and substituent groups has been very useful for the characterization of bacterial taxa (Suzuki et al., 1993). This is a cheap and rapid method with high degree of automation that was recently used to investigate the diversity of 94 *L. reuteri* isolates (Hilmi Hanan et al., 2007).

### (2) Genotypic methods

The application of molecular biology methods has greatly improved the bacterial identification and classification, by genotyping directed toward to DNA or RNA molecules.

The currently available molecular-based typing methods are mainly based on restriction analysis of the bacterial DNA, polymerase chain reaction (PCR) amplification of specific targets and identification of DNA sequence polymorphisms. Table 1 presents the use of different molecular tools in the characterization of microbial dynamic during fermentation.

**Random Amplification of Polimorphic DNA (RAPD-PCR)** is a PCR method based on segments of DNA that are amplified randomly, for the identification of the genetic variation;

by the use of a single arbitrary primer in a PCR reaction is resulting the amplification of many DNA products. This procedure detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermocylic amplification. RAPD-PCR method was used to differentiate between probiotic *Lactobacillus* in wine (Plessas et al., 2017), identifying *Oenocococcus oeni*, *Leuconostoc mesenteroides* in wine (Ruiz et al., 2008; Lucena-Padros et al., 2014); genotypes have been found in olive fermentations of *L. pentosus, L. paracollinoides, L. rapi, Pediococcus* sp., *Staphylococcus* sp., *Candida thaimueangensis, S. cerevisiae, Hanseniaspora* sp. (Lucena-Padros et al., 2014), bio-typing of *Lactobacillus sakei, L.paracasei, L.curvatus, L. plantarum, L. fermentum* in traditional fermented sausage (Tremonte et al., 2017; Pisacone et al., 2015), characterization of *L. brevis, L. plantarum, L. pentosus, L. fermentum* in eggplant (Sesena et al., 2005), characterization of *Weissella* sp., *Pediococcus* sp, *Lactococcus* sp., *Lactobacillus* in Mexican fermented beverage (Väkeväinen et al., 2018), rapid identification of *L. casei, L paracasei, L. plantarum, L. rhamnosus, L. helveticus, L. fermentum, L. brevis, Streptococcus thermophilus, Enterococcus faecalis, Lactococcus lactis* in dairy (Rossetti et al., 2005) and identification of *L. plantarum, L. sanfranciscensis, Leuconostoc mesenteroides, L. fermentum, Weissella cibaria, L. pentosus, L. brevis, L. paraplantarum* in sourdough fermentation (Rizzello et al., 2014).Limitations of the method are: mismatches between the primer and the template may result in the total absence of PCR product, RAPD-PCR is an enzymatic reaction, therefore, the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome.

**PCR-denaturing gradient gel electrophoresis (PCR-DGGE)** is the most worldwide used molecular method, being introduced approximately 25 years ago. These techniques consist of amplification of the genes encoding the 16S rRNA from the matrix containing different bacterial populations, followed by separation of the DNA fragments on gel electrophoresis, molecules with different number of pair base will migrate on different position generating a patterns which can provide a preliminary view of predominant species. PCR-DGGE has been a useful method for identification of *Lactobacillus sp., Yarrowia lipolytica, Debaryomyces hansenii, Rhodotorula mucilaginosa*, *Candida stellata, S.cerevisiae, L. curvatus, L. plantarum, S. xylosus* in Ciauscolo, a traditional Italian salami (Silvestri et al., 2007; Aquilanti et al., 2007), identification and characterization of *Lactobacillus sanfranciscensis, Candida milleri, Sacharomyces cerevisiae,* in sourdough (Palla et al., 2017), for detection of *Aspergillus niger, Botrytis cinerea, Hanseniaspora uvarum, S. cerevisiae, C. stelatta, Leuconostoc mesenteroides, O. oeni, S. cerevisiae* (Andorra et al., 2010; Perez-Martin et al., 2014; Portillo et al., 2016), identification of *Leuconostoc mesenteroides, Tetragenococcus halophylus*, *Enterococcus farcium, Enterococcus faecium, B. subtilis, B. licheniformis, Mucor plumbeus*, *Aspergillus oryzae, Debaromyces hansenii,* in soybean paste (Kim et al., 2009; Do Ham et al., 2012) and characterization of *L. sakei, L. paracasei, L. curvatus, L. plantarum*, *L. fermentum, S. xylosus, S. saprophyticus, S. pasteuri, S. epidermis, S. simulans, S. equorum* in traditional sausage (Pisacane et al., 2015; Fonseca et al., 2013). This method requires long time to be performed, works well only with short fragments less than 600 bp, thus limiting phylogenetic characterization, results difficult to reproduce between gels and laboratories.

**Real-Time qPCR** method is using primers pair specific for a desired targeted sequence and internal probe, labelled with fluorescent dye, with each amplification cycle, the fluorescence intensity is increasing, which is collected by the instrument system. The cycle number at which an amplification plot crosses this threshold fluorescence level is called the „Ct" or threshold cycle. This Ct value can be directly correlated to the starting target concentration of

the sample. This method has been used for the detection of *Streptococcus thermophilus* and *Lactococcus lactis* in dairy (Pega et al., 2017), detection of *A. niger, Botrytis cinerea,*

**Table 1. Method used to describe the dynamic of microbial population in different food matrix**

| Method/Matrix | Microorganism | References |
|---|---|---|
| **PCR-DGGE** | | |
| Italian salami | *Lactobacillus* sp., *Yarrowia lipolytica, Debaryomyces hansenii, Rhodotorula mucilaginosa*, *Candida stellata, S. cerevisiae, L. curvatus, L. plantarum, S. xylosus* | Plessas et al., 2017 |
| Sourdough | *Lactobacillus sanfranciscensis, Candida milleri*, *Sacharomyces cerevisiae* | Aquilanti et al., 2007<br>Palla et al., 2017 |
| Wine | *Aspergillus niger, Botrytis cinirea*, *Hanseniaspora uvarum, S. cerevisiae, C. stelatta* | Andorra et al., 2010 |
| Cocoa bean | *L. plantarum, L. fermentum, Acetobacter pasteurianus* | Lefeber et al., 2011 |
| Sorghum | *Lactococcus lactis, Weissella cibaria, L. curvatus*, *Enterobacter* sp. | Madoroba et al., 2011 |
| Sausage | *L. sakei, L. plantarum, Weissella hellenica, Leuconostoc mesenteroides* | Tremonte et al., 2017 |
| Cassava dough | *L. plantarum, L. fermentum, L. pentosus, L. case, L. acidophilus* | Oguntoyinbo et al., 2010 |
| Shenqu | *Pediococcus acidilactis, Rhizopus* sp., *Aspergillus oryzae, Enterobacter* sp., *Klebsiella* sp., *Erwinia* sp., *Pantoea vagan* | Lin et al., 2017 |
| Wine | *Gluconobacter* sp., *Acetobacter* sp., *Gluconoacetobacter* sp., *Bifidobacterium* sp., *Hanseniaspora* sp., *Sacharomyces* sp., *Candida* sp. | Portillo et al., 2016 |
| Soybean paste | *Leuconostoc mesenteroides, Tetragenococcus halophylus, Enterococcus farcium, Enterococcus faecium, B. subtilis, B. licheniformis, Mucor plumbeus* | Kim et al., 2009<br>Do Ham et al., 2012 |
| Traditional sausage | *Aspergillus oryzae, Debaromyces hansenii, L. sakei, L. paracasei, L. curvatus, L. plantarum*, *L. fermentum, S. xylosus, S. saprophyticus, S. pasteuri, S. epidermis, S. simulans, S. equorum* | Pisacane et al., 2015<br>Fonseca et al., 2013 |
| Wine | *Leuconostoc mesenteroides, O. oeni, S. cerevisiae* | Perez-Martin et al., 2014 |
| Chinese liquor | *Methanocorpusculum* sp., *Methanobrevibacter* sp., *L. acetotolerans, L. alimentarius, Clostridium kluyveri, Clostridium sartagoforme, Methanobacterium* sp., *Methanoculleus* sp. | Ding et al., 2015<br>Zheng et al., 2013 |
| Alcohol fermentation | *Rhizopus orizae, R. microsporus*, *Absidia corymbifera, Amylomyces sp., S. cerevisiae, Pichia anomala, Candida tropicalis, Clavispora lusitanie, Pediococcus pentosaceus, L. plantarum, L. brevis, Weisella confuse, B. subtilis, Acetobacter orientalis, A. pasteurianus* | Thanh et al., 2008 |
| Olive fermentation | *L. plantarum, Marinilactibacillus* sp., *Propionibacterium olivae, Alkalibacterium* sp., *Halolactobacillus* sp., *Pediococcus acidilactici* | Lucena-Padros et al., 2015 |
| Leek fermentation | *Leuconostoc mesenteroides, L. sakei, L. plantarum*, *L. brevis, L. parabrevis* | Wouters et al., 2013 |
| Palm wine | *S. cerevisiae, S. ludwigii, Zygosacharomyces bailii, Hanseniospora uvarum, Candida parasilopsis, C. fermentati, Pichia fermentans* | Stringini et al., 2009 |
| **RAPD-PCR** | | |
| Feta cheese | *Lactobacillus* sp. | Plessas et al., 2017 |
| Wine fermentation | *Oenococcus oeni, Leuconostoc mesenteroides* | Ruiz et al., 2008 |
| Olive fermentation | *L. pentosus, L. paracollinoides*, *L. rapi, Pediococcus* sp., *Staphylococcus* sp., *Candida thaimueangensis, S. cerevisiae, Hanseniospora* sp. | Lucena-Padros et al., 2014 |
| Fermented sausage | *L. sakei, L. plantarum, Weisella hellenica*, *Leuconostoc mesenteroides, S. xylosus, S.saprophyticus, S. pasteuri, S. epidermis, S. simulans, S. equorum* | Tremonte et al., 2017<br>Pisacone et al., 2015 |
| Eggplant | *L. brevis, L. plantarum, L. pentosus, L. fermentum* | Sesena et al., 2005 |
| Fermented beverage | *Weissella* sp., *Pediococcus* sp., *Lactococcus* sp., *Lactobacillus* sp. | Väkeväinen et al., 2018 |
| Italian sausage | *Staphylococcus xylosus* | Iacumin et al., 2006 |
| Dairy | *L. casei, L. paracasei, L. plantarum, L. rhamnosus, L. helveticus, L. fermentatum, L. brevis, S. thermophilus, Lactococcus lactis* | Rossetti et al., 2005 |
| Sourdough fermentation | *L. plantarum, L. sanfranciscensis, L. fermentum*, *Leuconostoc mesenteroides, Weissella cibaria, L. pentosus, L. brevis, L. paraplantarum* | Rizzelo et al., 2014 |
| **qPCR** | | |
| Dairy | *Streptococcus thermophilus, Lactococcus lactis* | Pega et al., 2017 |
| Wine | *A. niger, Botrytis cinerea, Hanseniospora uvarum, S. cerevisiae, Candida stellata* | Andorra et al., 2010 |
| Cocoa bean | *L. sakei, L. plantarum, Weissella hellenica*, *Leuconstoc mesenteroides* | Schewendiman et al., 2017 |
| Sourdough | *L. curvatus, L. brevis, L. pontis, Weissella* sp., *Pediococcus pentosaceus, L. plantarum, S. cerevisiae* | Michel et al., 2016<br>Lin et al., 2014<br>Sienwerts et al., 2018 |
| Wine | *Gluconobacter* sp., *Acetobacter* sp., *Gluconoacetobacter* sp., *Bifidobacterium* sp., *Hanseniaspora* sp., *Sacharomyces* sp., *Candida* sp. | Portillo et al., 2016<br>Andorra et al., 2011 |
| Spanish sausage | *Staphylococcus equorum, L. sakei* | Fonseca et al., 2013 |
| Cheese milk | *Propionibacterium freudenreichii, P. thoenii, P. jensenii*, *P. acidipropionici* | Turgay et al., 2018 |

*Tabel 1 (Continued)*

| | | |
|---|---|---|
| White cheese | *Saccharomyces cerevisiae, Enterococcus* sp., *L. brevis, L. curvatus* | Kadiroglu et al., 2014<br>Ladero et al., 2010 |
| Fish sauce | *Virgibacillus halodentrificans, Tetragenococcus halophilus* | Udomsil et al., 2016 |
| **Sau-PCR** | | |
| Wine fermentation | *Saccharomyces cerevisiae* | Perrone et al., 2013 |
| Sausages | *Staphylococcus xylosus* | Iacumin et al., 2006 |
| **T-RFLP** | | |
| Wine fermentation | *S. cerevisiae, H. uvarum, Pichia minuta, Sacharomycodes ludwigii, Candida zemplinina* | Sun and Liu, 2014 |
| **PFGE** | | |
| Wine fermentation | *S. cerevisiae, H. uvarum, Pichia minuta, Sacharomycodes ludwigii, Candida zemplinina* | Sun and Liu, 2014 |
| Youghurt | *L. delbrueckii, S. thermophilus* | Rademaker et al.,2006 |
| **Cells-qPCR** | | |
| Wine | *B. bruxellensis, S. cerevisiae, Z. bailii, L. plantarum, Oenococcus oeni, A. aceti, Gluconobacter oxydans* | Soares-Santos et al.,2017, 2018 |
| **ARDRA–ITS RFLP** | | |
| Sourdough | *L. sanfranciscensis, Candida milleri, S. cerevisiae* | Palla et al., 2017 |
| Cocoa fermentation | *B. subtilis, B. pumilus, B. sphaericus, B. cereus*, *B. thuringiensis, B. fusiformis* | Ouattara et al., 2011 |
| **FISH** | | |
| Olive | *L. plantarum, L. paraplantarum, L. pentosus* | Ercolini et al., 2006 |
| Wine | *S. cerevisiae, Hanseniospora guilliermondii* | Andorra et al., 2011 |
| **MS – PCR** | | |
| Food spoilage | *L. plantarum, L. paraplantarum, L. pentosus* | Dakal et al., 2018 |
| **PCR-RFLP** | | |
| Food spoilage | *L. plantarum, L. paraplantarum, L. pentosus* | Dakal et al., 2018 |
| **PMA – qPCR** | | |
| Wine | *S. cerevisiae, B. bruxellensis, O. oeni, L. plantarum, Acetobacter paseurianus* | Rizzotti et al., 2015 |
| **Box** | | |
| Sourdough | *L. curvatus, L. brevis, L. pontis, Weissella* sp., *Pediococcus pentosaceus* | Michel et al., 2016 |
| **Box – PCR** | | |
| Enzyme food | *Bacillus coagulans, L. plantarum, L. oris, S. epidermis* | Zhu et al., 2014 |
| **Flow cytometry** | | |
| Wine | *S. cerevisiae, B. bruxellensis, Candida vini, L. plantarum, L. casei, L. brevis, O. oeni, Acetobacter* sp., *Gluconobacter* sp., *Gluconoacetobacter* sp., *S. cerevisiae, Hanseniospora guilliermondii* | Longin et al., 2017<br>Andorra et al., 2011 |
| **Nested – PCR** | | |
| Shenqu | *Pediococcus acidilactici, Rhizopus* sp., *Aspergillus oryzae, Enterobacter* sp., *Klebsiella* sp., *Erwinia* sp., *Pantoea vagan* | Lin et al., 2017 |
| Soybean paste | *Leuconostoc mesenteriodes, Tetragenococcus halophylus, E. faecium, B. subtilis, B. licheniformis, Mucor plumbeus, A. oryzae* | Kim et al., 2009 |
| **Rep – PCR** | | |
| Italian sausage | *Staphylococcus xylosus* | Iacumin et al., 2006 |
| Cocoa bean | *S. cerevisiae, Candida ethanolica, L. fermentum, L. plantarum, Acetobacter pasteurianus, Acetobacter syzygii, Hanseniospora uvarum, Pichia manshurica* | Visintin et al., 2016 |

*Hanseniaspora uvarum, S. cerevisiae, Candida stellata* in wine fermentation (Andorra et al., 2010; Andorra et al., 2011; Portillo et al., 2016), *L. plantarum* and *L. fermentum* in cocoa bean fermentation (Schwendimanet al., 2015), *L curvatus, L. brevis, L. pontis, Weissella* sp., *Pediococcus pentosaceus* in sourdough (Michel et al., 2016; Sienwerts et al., 2018), *Staphylococcus equorum, L. sakei* in Spanish sausage Chorizo (Fonseca et al., 2013), *Propioni bacterium freudenreichii, P. thoenii, P. jensenii*, *P. acidipropionici* in cheese milk (Turgay et al., 2018; Kadiroglu et al., 2014), *Virgibacillus halodenitrificans, Tetragenococcus halophilus* in fish sauce (Udomsil et al., 2016).

**Sau-PCR** technique is based on the digestion of genomic DNA with the restriction endonuclease Sau3AI and subsequent amplification with primers whose core sequence is based on the Sau3AI recognition site. This method has been used for investigation of the dominance behaviour of *Saccharomyces cerevisiae* strains during wine fermentation (Perrone et al., 2013) and

characterization of *Staphylococcus xylosus* isolated from naturally fermented Italian sausages (Iacumin et al., 2006)

**Terminal-Restriction Fragment Length Polymorphism (T-RFLP)** is a method that analyzes variation among 16s rRNA genes from different bacteria, being based on the restriction endonuclease digestion of fluorescent end-labeled PCR products. Restriction fragments are separated by gel electrophoresis and the fluorescence signal is quantified. Distinct patterns are obtained as each fragment represents each species present. This method has been used in investigation of yeasts species: *Saccharomyces* sp., *Hanseniospora uvarum, Pichia minuta, Saccharomycodes ludwigii, Candida zemplinina* in wine fermentation (Sun and Liu, 2014) and for assesement of *L. delbrueckii, S. thermophiles* in youghurt (Rademaker et al., 2006).

**Pulsed-Field Gel Electrophoresis (PFGE)** is a highly discriminative molecular typing technique that is used in worldwide. PFGE is based upon the variable migration of large DNA restriction fragments in an electrical field of alternating polarity. By comparing the DNA fingerprints of two isolates, it can be investigated if they belong to the same strain or if they are genetically unrelated. According to Ruiz et al., 2008, PFGE method has been used to study intraspecific genetic diversity of *Oenococcus oeni* and *Leuconostoc mesenteroides* from malolactic fermentation of Cencibel wines. Oguntoyinbo et al., 2010, studied dynamics of *L. plantarum, l. fermentum, L. pentosus, L. casei, L. acidophilus* during the spontaneous fermentation of cassava dough.

**Cells-qPCR** is a quantitative PCR assay and has been developed for rapid detection and quantification of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) from grape must and wine that does not require DNA extraction. This method is robust, reliable, fast and specific method to detect and quantify different bacteria and yeasts, like *B. bruxellensis, S. cerevisiae, L. plantarum, Oenococcus oeni, Acetobacter aceti, Gluconobacter oxydans, Zygosaccharomyces bailii*, overcoming the presence of inhibitors like polyphenols and ethanol (Soares-Santos et al., 2017; Soares-Santos et al., 2018).

**Amplified Ribosomal DNA Restriction Analysis (ARDRA**) is a tool to study microbial diversity that relies on DNA polymorphism. Fragments of 16S rDNA gene, obtained by applying either universal or genus-specific primer sets, are amplified and digested by restriction endonucleases, followed by separation of the resulting fragments on high-density agarose or acrylamide gels. The emerging profiles are then used either to cluster the community into genotypic groups or for strain typing. ARDRA method has been used to describe *Lactobacillus sanfranciscensis, Candida milleri, S. cerevisiae*, in sourdough (Palla et al., 2017), *B. subtilis, B. pumilus, B. sphaericus, B. cereus, B. thuringiensis, B. fusiformis* has been isolated and identified from cocoa fermentation (Ouattara et al., 2011).

**Flow Cytometry (FCM**) is a rapid and sensitive technique that measures each cell size. FCM technique is based on sorting of the stained cells through a process called hydrodynamic focusing in a narrow steam, the cells are then hit with a laser beam and florescence emitted is detected by several photomultipliers. This method has been used to quantify pathogen, spoilage microorganisms and microorganisms of interest such as *S. cerevisiae, B. bruxellensis, Candida vini, L. plantarum, L. casei, L. brevis, O. oeni*, *Acetobacter* sp., *Gluconobacter* sp., *Gluconoacetobacter* sp. from wine (Longin et al., 2017; Andorra et al., 2011).

**The Fluorescence in Situ Hybridization (FISH)** with rRNA targeted oligonucleotide probes has been developed over the last years, a number of variants of this basic technique have been described until now. Microbial cells are treated with appropriate chemical fixative and then immobilized onto microscopic slides. Probes used are 15-20 nucleotides in length and are labeled covalently at the 5'-end with a florescent dye. After hybridization and washing, specifically stained cells are observed by epiflourescence microscopy. This method

has been used for detection of *L. plantarum, L. paraplantarum, L. pentosus, L. acidophilus, L. brevis, L. casei, L. curvatus, L. fermentum, L. paracasei, L. reuteri, L. rhamnosus* in natural fermentation of olives (Ercolini et al., 2006), Analysis of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* during wine fermentation (Andorra et al., 2011).

## CONCLUSIONS

Phenotypic and genotypic analysis can contribute to characterize any microbe at species and strain level; this can be obtained by the combination of different identification and classification procedures and then to discrimination by molecular techniques.

The most used method is PCR-DGGE, followed by q-PCR and RAPD-PCR among genotyping methods, being a very useful tool for detection of probiotic bacteria, spoilage bacteria and pathogens bacteria during fermentation processes, the other molecular methods being less used.

It is necessary to expand possibilities to investigate microbial diversity within natural populations by analysing less conserved genes. Culture-independent methods can not completely avoid biases from estimating microbial diversity introduced by maceration and blending of the food sample, dilution of the homogenate, plating of dilution onto agar media and isolation and identification of colonies.

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