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REVIEW

Selection criteria and tools for malolactic starters development: an update

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Abstract The use of malolactic starter cultures to improve the fermentation process and enhance wine quality and safety is becoming a common winemaking practice, increasingly preferred to spontaneous fermentation. Given its great oenological properties, *Oenococcus oeni* is the species most present in commercial brands of wine lactic acid bacteria (LAB) starters. Stress resistance, technological performances and safety are the key selection criteria to take into account when designing effective malolactic starters. Nowadays, new LAB strains are selected by exploiting advanced technological applications rather than the traditional screening methods based on a trial and error approach. In particular, the progress made in the fields of genetics and molecular biology, as well as in the whole genome sequencing projects, offers new tools to better characterize candidate starter strains. This review aims at providing an updated picture of the technological approaches that should be used to select LAB strains suitable for winemaking. In the near future, the full integration of phenotypic and genetic data will make it possible to rationally develop malolactic cultures that are specific for different types of wine.

Keywords Malolactic starter · Selection criteria · Phenotypic and genetic characterization · Comparative genomics · Transcriptomics

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Introduction

Malolactic fermentation (MLF) is a biological process used worldwide to produce a multitude of superior quality wines. Briefly, it consists of the conversion of L-malic acid into L-lactic acid and CO_2 by lactic acid bacteria (LAB) strains that are well adapted to the wine environment. The direct consequences of this biological reaction on the quality of wine are its microbial stabilization and sensorial improvement, thanks to nutrient depletion and diminished acidity, respectively. Moreover, a number of other desirable effects linked to the enzymatic activity of LAB have been reported, such as a richer bouquet, that would include buttery and nutty attributes, and traits of honey, vanilla, leather, spices, and smoother tannins (Lonvaud-Funel 1999; Bartowsky 2005; Ribéreau-Gayon et al. 2006).

Wine however is a hostile environment for LAB, which have to resist several types of stress, including high ethanol content, low pH, presence of SO_2 and other inhibiting compounds, such as the fatty acids released by yeasts, tannins, and various chemical residues. The species of LAB associated with spontaneous MLF belong to the genera *Oenococcus, Leuconostoc, Lactobacillus* and *Pediococcus,* but *Oenococcus oeni* is the most widespread thanks to its remarkable tolerance of the harsh conditions encountered in wine (Lonvaud-Funel 1999; Bartowsky 2005; Ribéreau-Gayon et al. 2006). Apart from its ability to convert malic acid, *Oenococcus oeni* is considered an ideal species thanks to its many oenological properties, such as low production of acetic acid, presence of enzymatic activities that increase the aroma, and reduced risk of wine spoilage, as the ropy phenotype is very rare (Ribéreau-Gayon et al. 2006). Many ecological and technological investigations however suggest that these positive features are not common to all the individuals of the species. Moreover, the same characteristics

can also be found in strains of other species. Great care is therefore essential when selecting the strains and exploiting them in the winemaking process, so as to drive the MLF as desired.

The aim of this review is to provide an update on the traditional phenotypic and more advanced genotypic tools that can be applied when selecting new LAB strains for use as starter cultures in the wine industry. In particular, this paper highlights how the integration of phenotypic and genotypic approaches is particularly useful for the development of malolactic cultures that are specific for different types of wine.

Selected malolactic bacteria cultures

The use of selected cultures of malolactic bacteria (MB) for MLF instead of native bacteria is becoming a common oenological practice, as it improves this fermentation process and enhances quality and safety of wine. Indeed, spontaneous MLF can increase the risk of stuck/sluggish fermentation, wine spoilage, production of off-flavours and toxic metabolites such as ethyl carbamate and biogenic amines (Lonvaud-Funel 1999).

Currently, winemakers can reduce these risks and improve the success rate of MLF by inoculating the wine with the malolactic starter cultures available on the market.

Table 1 lists a series of malolactic starters produced, currently or in the past, by the most popular oenological brands sold in Northern Italy.

Most of these products contain strains of *O. oeni,* but some contain strains belonging to other LAB species: Viniflora plantarum (CHR Hansen) includes a selected strain of *Lactobacillus plantarum* (Cavin et al. 1993) and Maloferm LG98 (DSM food specialities) consists of *Lactobacillus hilgardii* (Henschke 2003). Apart from the composition and number of strains present, commercial malolactic starters can also differ in form, i.e. liquid, frozen or lyophilised.

Table 1 A selection of malolactic starter cultures marketed in Northern Italy

Company	Culture
AEB group	Biolact Acclimatée [(3)]; Biolact Acclimatée BM [(2)]; Biolact Acclimatée PB1025 [(1)]; Biolact Acclimatée 4R [(4)]; Biolact Fresh [(M)]; Biolact One Fresh [(M)]
CHR Hansen	Viniflora Oenos [(1)]; Viniflora CH11 [(1)]; Viniflora CH16 [(1)]; Viniflora CH35 [(1)]; Viniflora plantarum (*Lactobacillus plantarum*) [(1)] [a]; *Lb. plantarum* LpCHL2 [(1)] [b]
DSM food specialities	Maloferm LG98 [(1)] [b]
EverIntec	Extremo X 03 [(M)]
Laffort	Lactoenos 350 Preac [(1)]; Lactoenos 450 Preac [(1)]; Lactoenos SB3 Instant [(1)]; Lactoenos B16 Standard [(1)]; Microenos HP (*Lb. hilgardii*) [(1)] [c]
Lallemand	Lalvin 31 [(1)]; Lalvin EQ54 [(1)]; Lalvin VP41 [(1)]; Uvapherm Alpha [(1)]; Uvapherm Beta [(1)]; Lalvin PN4 [(1)d]; Lalvin IB (Inobacter) [(1)d]; Lalvin MT01 [(1)d]
Oliver Ogar	Malo Quick [(1)]; Malo-start+ [(M)]
SGBIOTECH	SRW[(1)] [e]
Tebaldi.it	ExperTi oeni [(N)]; ExperTi oeni Alcol [(N)]; ExperTi oeni pH [(N)]
Vason	Amar04 [(1)]

( ) declared number of strains (M = multistrain, N = not declared)

[a] Technical review AWRI No147

[b] Cavin et al. 1993

[c] Mira de Orduña et al. 2000

[d] produced for third parties

[e] liquid culture

Identification, characterization and selection of malolactic starters: the phenotypic and the genotypic approaches

A number of factors should be taken into account when developing bacterial cultures that are specific for different types of wine. Some of these criteria, first reviewed by Henick-Kling in 1995, are listed in Table 2, and include: resistance to ethanol and SO_2, ability to grow at low pH levels, no health hazard for the end consumer, resistance to technological stress (freezing, freeze-drying, hydration and inoculation into wine). Further features are activities of enzymes such as glycosidase that are involved in the production of desirable attributes and aromas. Finally, the selected strains must be phenotypically and genetically stable.

Traditionally, all these characteristics have been analysed using a phenotypic approach, but can be now reassessed in a genotypic perspective. Interestingly, in the same year-1995-in which Henick-Kling described the selection criteria for MB starters, the entire genome of a (simple) living organism-*Haemophylus influenzae*-was sequenced for the first time ever (Fleischmann et al. 1995), an event that marked the beginning of the genomic era.

Since then, many other bacterial genomes have been sequenced, including those of wine-related LAB (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). As regards *O. oeni,* the genomes of strains PSU-1 and AWRI B429 have been completed (Mills et al. 2005; Borneman et al. 2010), that of strain ATCC BAA-1163 is in the draft status, whereas the sequencing of strains KM 334 and KM 383 is in progress (Torriani et al. 2009). Moreover, thousands of sequences relative to traits of industrial importance, e.g.

Table 2 Guidelines for the selection of commercial malolactic starters for oenological applications (adapted from Henick-Kling 1995)

Categories	Property
Stress resistance	Resistance to high levels of ethanol (14% v/v)
	Tolerance to pH 3.0
	Resistance to high SO_2 concentrations
	Resistance to low temperatures
	Bacteriophages resistance, not lysogenic
Technological performances	High malolactic activity
	Ability to perform MLF in different types of wine
	Satisfactory growth in a synthetic medium
	Production of desirable flavours or enhancement of fruity aromas
	Low acetic acid production at the pH of grape juice and wine
	No production of ropy polysaccharides
	No production of off-flavours
	Compatibility with the yeast used for the alcoholic fermentation
	Can be freeze-dried
Safety	No production of biogenic amines
	No production of ethyl carbamate
	Inability to transmit antibiotic resistance genes

those encoding for the pathway of malic acid metabolism, are constantly being added to nucleotide databases.

Thanks to these data, progress in the field of genetic knowledge has been rapid: new diagnostic tools and screening assays have been designed and developed and more information is now available for the correct management of wine production.

The advances made in genomics research can be successfully exploited to replace and/or integrate phenotypic data, so as to characterize LAB strains in greater detail and select the most effective, according to the criteria defined by Henick-Kling (1995). Clearly, a variety of factors should be taken into account when choosing the best approach, such as rapidity, effectiveness and robustness of the data obtained.

The next two sections describe how genotypic and phenotypic approaches can be applied to the processes of strain identification and characterization and used to analyse positive and negative oenological traits.

Strain identification and characterization

The first stage in designing a starter culture for MLF is to establish the precise identity of a novel MB isolate at both genus and species levels. This procedure allows discovering microbial biodiversity and makes it possible to link a given isolate with all the features shared by virtually all the strains of the species it belongs to.

Traditionally, a bacterial isolate is identified through phenotypic methods, e.g. by determining its sugar fermentation pattern, but these procedures are notoriously ambiguous and time-consuming. More recently, nucleic acid-based methods have been developed for this purpose, such as DNA sequencing, species-specific PCRs and PCR-Denaturant Gradient Gel Electrophoresis (Zapparoli et al. 1998; Lopez et al. 2003; Neeley et al. 2005; Renouf et al. 2006). These molecular methods have the advantage of being fast and reliable, and can be considered as an alternative to the phenotypic ones.

The following aspects relative to the identification process deserve special mention. Novel species are constantly being discovered, including several lactobacilli isolated from wine and musts [*Lactobacillus vini* (Rodas et al. 2006), *Lactobacillus bobalius* (Mañes-Lazaro et al. 2008a), *Lactobacillus uvarum* (Mañes-Lazaro et al. 2008b), *Lactobacillus oeni* (Mañes-Lazaro et al. 2009), as well as the spoiling species *Lactobacillus kunkeei* (Edwards et al. 1998) and *Lactobacillus nagelii* (Edwards et al. 2000)]. These examples suggest that a comprehensive research into the biodiversity of MB in specific wines could give rise to unexpected results, and taxonomic identification should always be performed with the greatest care (Felis et al. 2010).

Even when identification is clear, interesting results can emerge from the analysis of intraspecific diversity. In the case of *O. oeni,* the diversity observed between different strains is really remarkable, from both a phenotypic and genotypic viewpoint (Garvie 1967; Beelman et al. 1977, de Las Rivas et al. 2004, Bilhère et al. 2009).

This species for instance is described as being trehalose positive, sucrose negative, but Davis et al. (1988), when analysing the fermentation profile of various *O. oeni* strains, observed that trehalose was unfermented by 2.8% of the collection, while sucrose was used by 45.1%. It is therefore difficult to define species-specific characteristics, and strain-specific traits should be better investigated when dealing with specific applications. When characterizing MB strains, it is essential to define their fermentation profiles, even though this type of assay can prove time-consuming: *O. oeni* in particular is a fastidious species with many nutritional requirements, and some strains also display slow growth rates (Davis et al. 1988; Manca de Nadra et al. 1997; Zapparoli et al. 2004). Moreover, commercial media available for the characterization of such a broad *taxon* as *Lactobacillales* (API 50 CHL by Biomerieux) are unsuitable for this purpose (Pardo et al. 1988), whereas the improved medium proposed by Jensen & Edwards (1991) increases the incubation time, bringing it to 28 days.

Finally, taxonomic procedures could lead to changes in names, although obviously the characteristics of the strains would remain unvaried: the most striking example is *O. oeni* itself, formerly known as *Leuconostoc oenos,* and reclassified in 1995 mainly on the grounds of molecular analyses (Dicks et al. 1995).

Analysis of negative and positive oenological traits

The integration of genotypic and phenotypic approaches is especially useful when analysing traits of oenological importance in MB. In particular, when a phenotype is determined by one or a few more genes, the detection of genetic determinants can convey useful information on the phenotypic properties observed experimentally. Some examples are illustrated below.

Independently of the species, the strains selected as malolactic starters must be safe (Table 2). In this respect, possible hazards to human health are linked to the release of toxic compounds, namely biogenic amines (BAs) and ethyl carbamate.

BAs (e.g. histamine and tyramine) are natural substances produced during the fermentation process by decarboxylation of amino acids. They can be harmful to sensitive individuals, giving rise to headaches, skin rash and other allergic reactions (Lonvaud-Funel 2001). The key role in the production of each BA is played by a specific enzyme, i.e. a decarboxylase, which releases the carboxylic group of an amino acid as CO_2.

Various phenotypic methods based on HPLC protocols or the use of specific media have been developed to detect BA producers. However, in order to apply these techniques, the strains must first be cultured in a synthetic medium or in wine, hence extending by several days the time required to perform the analyses (Bover-Cid & Holzapfel 1999; Torriani et al. 2008). On the other hand, specific PCR-based assays are now available, which can detect the most common BAs using primers designed on the sequence of the gene encoding for their respective decarboxylase (Landete et al. 2007; Torriani et al. 2008). The phenotypic and genotypic approaches give complementary information: since molecular methods are more rapid, they can be used for a preliminary screening for BA-producing strains, so as to reduce the number of candidates for the phenotypic assays.

The same approach can be employed to analyse other negative traits related to the safety and quality of wine, such as the production of ethyl carbamate and the presence of "ropy phenotypes".

Ethyl carbamate is a carcinogenic molecule usually synthesized as a side product of the arginine catabolism, which is regulated by three key enzymes: arginine deiminase, ornithine transcarbamylase and carbamate kinase. Also in this case, the existing phenotypic and genotypic approaches can be combined to give a clearer picture (Mira de Orduña et al. 2000; Divol et al. 2003).

The same is true for the "ropy phenotype", linked to the production of wine-spoiling exopolysaccharides (Lonvaud-Funel 1999; Ibarburu et al. 2007; Walling et al. 2005).

Another interesting, yet often neglected aspect is the antibiotic resistance (AR) that can be observed in wine-related bacteria. Recently, Rojo-Bezares et al. (2006) detected acquired aminoglycoside, erythromycin and tetracycline resistance genes in several strains of *O. oeni, Lactobacillus,* and *Pediococcus* of oenological origin. Although not directly detrimental to human health, since the wine is filtered before consumption, AR may affect the technological performances of MB. Indeed, some of the antibiotics analysed are structurally similar to polyphenols, i.e. the natural antimicrobial compounds present in wine (Ribereau-Gayon et al. 1998). The latter authors suggest that some of the AR gene products, as well as protecting the wine bacteria from antibiotics, may also have other functions.

Desirable oenological traits can also be encoded by one or a few more genes. One of the most interesting enzymatic activities in MB is that of β-glycosidase, which releases the glycosylated precursors of various fruity flavours, thus enhancing the organoleptic properties of wine. This enzyme was also observed to increase the amount of free resveratrol, a nutraceutical molecule mostly found in red wine. There exist both phenotypic and genotypic assays to test LAB for β-glycosidase activity. Once again the two approaches can be combined so as to have a clearer insight into the properties of each strain (Barbagallo et al. 2004; Grimaldi et al. 2005; Spano et al. 2005).

Selected MB can also produce other aromatic compounds such as diacetyl, acetoin, butanediol and acetate through the metabolism of citric acid. Diacetyl is responsible for one of the most evident flavour changes that occur during MLF and confers a "buttery" trait to wine (Bartowsky & Henschke 2004). In this case too, both phenotypic and nucleic acid-based assays have been developed to measure the enzymatic activity of the genes involved in citric acid metabolism (Olguin et al. 2009). Once again, the two approaches are complementary, and together they help define the oenological potential of the selected malolactic strains.

Comparative genomics and transcriptomics: new tools for a more rational approach to strain selection and process management

Some of the most important technological features of bacterial starters, such as the ability to adapt to high acidity

and ethanol levels, can be ascribed to finely tuned mechanisms regulating the physiological state of the cell, which involve a complex network of genes and modulation factors (van de Guchte et al. 2002; Spano & Massa, 2006). Usually, the oenological potential of the MB is directly established by evaluating the growth rate of the strains in the presence of a stress factor, using small-scale wine-making processes under different conditions and finally by trial and error in the actual wineries (Beelman et al. 1977; Zapparoli et al. 2004; Solieri et al. 2010).

In recent years, many efforts have been made to develop rapid molecular methods for the selection of malolactic starters with a strong oenological potential. The resulting methods are essentially of two types: i) physiological assays and expression analysis on a restricted group of genes known to be linked to stress responses (Coucheney et al. 2005; Beltramo et al. 2006), and ii) detection of non-ubiquitous genetic loci linked to stress resistance (Renouf et al. 2008). In both cases the genetic markers observed are a well-defined number of loci selected on the grounds of existing knowledge.

Although these new assays show promise when used to screen MB for complex characteristics, at present they have no clear advantage in respect to the more conventional methods, since they too are laborious and time-consuming. On the other hand, phenotypic and genotypic approaches are complementary and the relationship between the behaviour of the strains in a stressful environment and gene expression patterns or, alternatively, the profile of specific resistance markers, can be exploited in many ways. The link between an observed behaviour (phenotype) and its genetic background is essential to clarify the molecular regulatory mechanisms of microorganisms, and more in general, the biology of bacteria, so as to pave the way to new practical applications.

Whole genome analyses, through comparative genomics and transcriptomics by DNA-microarray, offer novel opportunities for a better characterization of wine LAB and can shed light on the link between complex technological features and their genetic background.

Our group is currently involved in the transcriptome analysis of *O. oeni* strain PSU-1 and the sequencing of another two strains isolated from Italian wines.

The transcriptome has been analyzed using a Combi-Chip with 1,741 target probes, including both the genes and pseudogenes identified in O. *oeni* PSU-1. Five experimental conditions were considered: the standard ones for optimal growth in a medium (pH 4.8); heat shock at 42°C; acidic shock at pH 3.5; ethanol shock at 10% v/v and finally a combined acidic and ethanol shock. The main changes in terms of total number of genes that were modulated were observed in the case of heat shock and the combination of acidic and ethanol shock, while a variation in pH levels seemed to be the condition best tolerated, as expected of an acidophilic bacterium such as *O. oeni* (Felis et al. 2009).

As regards comparative genomics, the preliminary results for strains KM 334 and KM 383, isolated from Chardonnay wine in Franciacorta and from Amarone in Valpolicella, respectively, agree with previous investigations. *Oenococcus oeni* seems indeed to be endowed with a plastic genome (Bon et al. 2009; Borneman et al. 2010) that is presumably so because it lacks the MisMatch Repairing system (Marcobal et al. 2008). Strain-specific regions, possibly linked to the organisms' ability to adapt to its original niche, have been identified (Torriani et al. 2009).

Thanks to these genomics and transcriptomics projects, we can reasonably expect to find new molecular markers for phenotypic traits that can be used to develop reliable screening assays.

Conclusions

Careful screening of wine LAB should take into account as many selection criteria described by Henick-Kling (1995) as possible, using the molecular biology-based techniques developed in recent years. DNA-based molecular methods are fundamental for species identification and can prove very useful for screening both positive and negative traits under the control of one or a few more genes. In these cases, molecular methods are indeed very fast, efficient and reliable. Conversely, the phenotypic approach is very effective for screening technological features expressed by several and mostly unknown genes (complex characteristics), such as resistance to acidic and ethanol stress. Whole genome molecular analyses through comparative genomics and transcriptomics will increase our understanding of regulation mechanisms and help improve MLF; possibly, new molecular markers for the selection of better strains will soon be identified. At present the phenotypic and genotypic approaches are complementary, as mutual validation strengthens the results of the screening procedure and reduces the risk of including false candidates among the selected strains.

Once selected, even the best strains require great care: adequate production, storage and inoculation protocols are essential to guarantee the viability of the selected malolactic starters.

It is also important to monitor the evolution of the malolactic starters after inoculation. The dominance of the inoculated starter over the indigenous LAB should be verified at every stage of MLF, genotyping isolates by means of nucleic acid-based techniques such as PFGE and RAPD-PCR (Zapparoli et al. 2000; Renouf et al. 2008).

Finally, attention should be paid to possible changes in the population of malolactic starters. The genome plasticity of wine LAB highlighted by various authors should be kept in mind so as to prevent loss of performance, and for this reason it is important to schedule a periodical turnover of the strains, replacing the oldest ones.

In the near future, the knowledge gained from both phenotypic and genetic analyses will make it possible to use a more rational approach in the selection and exploitation of better starters in the winemaking industry.

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