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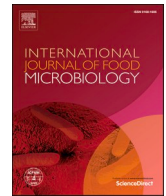
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## Alcoholic fermentation drives the selection of *Oenococcus oeni* strains in wine but not in cider

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### ABSTRACT

*Oenococcus oeni* is the predominant lactic acid bacteria species in wine and cider, where it performs the malolactic fermentation (MLF). The *O. oeni* strains analyzed to date form four major genetic lineages named phylogroups A, B, C and D. Most of the strains isolated from wine, cider, or kombucha belong to phylogroups A, B + C, and D, respectively, although B and C strains were also detected in wine. This study was performed to better understand the distribution of the phylogroups in wine and cider. Their population dynamics were determined by qPCR all through wine and cider productions, and the behavior of the strains was analyzed in synthetic wines and ciders. Phylogroups A, B and C were all represented in grape must and throughout the alcoholic fermentation, but on the transition to MLF, only phylogroup A remained at high levels in all wine productions. In the case of cider, phylogroups A, B and C were detected in stable levels during the process. When they were tested in synthetic wine and cider, all phylogroups performed MLF, but with different survival rates depending on the ethanol content. In this sense, ethanol and fermentation kinetics are the main agent that drives the selection of phylogroup A strains in wine, while B and C strains dominates in cider containing less ethanol.

### 1. Introduction

The environment of wine fermentation is complex, both on account of the chemical profile and the microbial community, which change over time (Beltran et al., 2002). The microbial community involved in spontaneous wine fermentations mainly consists of yeasts and lactic acid bacteria (LAB) that come from the vineyard or are part of the resident microbiota of the cellar (Franquès et al., 2017; Lleixà et al., 2018). *Saccharomyces* yeasts develop in grape must faster than other microorganisms and rapidly reach a population level sufficient to perform the alcoholic fermentation (AF). The conversion of sugar progressively increases the ethanol content and drives a selection of yeasts (Beltran et al., 2002) and LAB species (Renouf et al., 2006). After the completion of AF, LAB perform the malolactic fermentation (MLF), which involves the decarboxylation of L-malic acid into L-lactic acid, related with an

increase in pH, change of the mouthfeel and aromatic properties (Davis et al., 1985; Lonvaud-Funel, 1999; Sánchez et al., 2014). The fermentation of apple juice into cider follows a similar process involving the same microorganisms, but the final ethanol content is much lower and AF and MLF often occurs simultaneously (Cousin et al., 2017).

*O. oeni* is the main bacterial species responsible for MLF, because it is uniquely resistant to the low pH, high ethanol concentration, and other stressors encountered in wine and cider (Bech-Terkilsen et al., 2020; Cousin et al., 2017). The great diversity of *O. oeni* strains is reflected in its different ability to grow in wine, to perform MLF or to modulate wine aroma (Campbell-Sills et al., 2017; Cappelletto et al., 2017; Sánchez et al., 2014). When MLF occurs spontaneously, there are usually several indigenous strains that develop in wine and become more or less abundant from the beginning to the end of the fermentation (González-Arenzana et al., 2012b; Reguant et al., 2005b). Alternatively MLF can be

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induced by inoculation of a selected commercial strain (Sánchez et al., 2014; Torriani et al., 2011).

To date four major phylogroups, named A, B, C and D, have been identified in the *O. oeni* population on the basis of the genetic distances of their genomes (Lorentzen et al., 2019). From these, phylogroup A is by far the most common in wine during spontaneous MLF, and virtually contain all the commercial strains (Bilhère et al., 2009; Bridier et al., 2010; Campbell-Sills et al., 2015; Lorentzen et al., 2019). Phylogroups B and C have been isolated mainly from cider and grape must, but also from wine (Lorentzen et al., 2019; Sternes and Borneman, 2016). Phylogroup D has only been isolated from kombucha, in which the role of *O. oeni* is still unclear (Coton et al., 2017). At the same time the strains appear very specialized to their particular environment. It has been observed that within phylogroup A, some subgroups are related with particular wines, such as the AR and AW subgroups detected in red and white wines, respectively (Breniaux et al., 2018; Campbell-Sills et al., 2017). Nevertheless, the spread of *O. oeni* is thought not to be constrained by geography since the same -or closely related strains- are detected in wines produced in regions far apart from each other (El Khoury et al., 2017).

The aim of this study was to determine why strains isolated from wine and cider usually belong to different phylogroups. We developed qPCR assays to analyze the populations of all four phylogroups and we monitored their population dynamics in wine and cider samples drawn from the start of AF to the end of MLF. We also compared the physiological behavior of phylogroups A, B and C strains under different experimental conditions.

## 2. Materials and methods

### 2.1. Ecological study of *Oenococcus oeni* phylogroups

#### 2.1.1. Sample collection

Red wine samples of five different tanks from different wineries around Bordeaux were collected during the 2015 vintage (Suppl. Table 1). Samples were either from conventional, or organic productions. All wines were produced with the addition of 50 mg/L SO<sub>2</sub> after the harvest. In all cases, each winery inoculated a commercial *S. cerevisiae* strain to perform AF, and MLF occurred spontaneously. Samples were taken during the whole fermentative process. The stage of fermentations was identified by the fermentation duration, decrease in density (AF) or L-malic acid concentration (MLF). Six sampling points were analyzed for each wine as follows: early AF, mid AF, late AF, early MLF, mid MLF and late MLF (Suppl. Table 2). Samples from these sampling points were used for *O. oeni* population quantification.

Separately, two cider fermentations (Suppl. Table 1), with no addition of SO<sub>2</sub>, were followed during the 2016 harvest in the experimental cellar of the French Institute of Cider Production (Le Rheu, France). The musts used are a mix of cider apple varieties mostly used in France, following the traditional blending for cider fermentation. Both AF and MLF were performed spontaneously on 15 L fermenter at 10 °C. In this case, samples were taken at the beginning of the fermentation – after pressing, and once a week for at least two months. These samples were used to monitor the density decrease and *O. oeni* population.

Samples for *O. oeni* population quantification – 5 to 10 mL, were centrifugated at 10,000 rpm for 15 min at 4 °C, and the resulting cell pellet was stored at –20 °C until use.

#### 2.1.2. DNA extraction

Cell pellets were washed twice with TE buffer (Tris-HCl 20 mM, pH 8.0, EDTA 2 mM). DNA isolation was performed with the Wizard Genomic DNA Purification kit (Promega, WI, USA) according to the manufacturer's recommendations – except that lysozyme treatment was extended to 1 h and centrifuge times up to 30 min. The purity of the extracted DNA was tested by Biospec-nano (Shimadzu Biotech, Japan) and quantified on a microplate fluorescence reader (SpectraMax M2,

Molecular Devices, CA, USA) using iQuant (HS kit, GeneCopoeia, MD, USA) or Qubit (ThermoFisher, MA, USA).

#### 2.1.3. Bioinformatic analysis for the design of qPCR primers and probes

226 genome sequences of *O. oeni* strains from phylogroup A (n = 175), phylogroup B (n = 25), phylogroup C (n = 21) and phylogroup D (n = 5) were used in this study. Although the number of genomes of phylogroups B, C and D is much lower than those of phylogroup A, they are also much more divergent (Lorentzen and Lucas, 2019), which ensures the specificity of the primers. Genome sequences were retrieved from Genbank and annotated by MicroScope, where we also used the Pangenome tool to calculate the core genome of the 226 sequences, the set of coding sequences (CDS) present in all genomes (Vallenet et al., 2017; Vallenet et al., 2013). The resulting set of 892 CDSs was trimmed to 723 by removing entries containing fragments or duplicates before alignment with Clustal Omega using a custom Python script (Sievers et al., 2011); (scripts available at <https://github.com/marcgall/Genomics-01>). From the resulting concatenated sequences, all Single Nucleotide Polymorphisms (SNPs) (including indels) were identified (n = 218,180) with another custom script.

To generate target regions for PCR probes, we identified SNPs uniquely belonging to phylogroup A, B, C and D and scanned the core genome for positions with multiple SNPs in close proximity. In conducting this test, an allowance for mismatches was instated for small numbers of strains in a target phylogroup not conforming to the otherwise unique pattern. Candidate regions were inspected in Jalview (Waterhouse et al., 2009). Unique targets were identified for phylogroups B + C, C and D and used to design specific probes, but a subsequent manual analysis of core genome alignments revealed a more specific target for A strains, which was used to design the A probe. Taqman probes and primers were produced by Eurofins Genomics (Ebersberg, Germany) (Suppl. Table 3). Specificity was demonstrated by pairwise alignments of the target regions using BLAST (Altschul et al., 1990) and was tested by qPCR with genomic DNA of a representative strain of *O. oeni* from each phylogroup (A: CRBO VF; B: CRBO S12; C: CRBO 1399; D: UBOCC-A-315001).

#### 2.1.4. qPCR analysis

qPCR reactions were run in duplex (A + C, BC + D) with 0.05 µM of each primer and probe in 20 µL total reaction volume with iQ Supermix (Bio-Rad, CA, USA). All reactions were carried out in triplicate on a Bio-Rad CFX96 Real-Time PCR system with a first step of 10 min at 95 °C and 45 cycles of 15 s at 95 °C, followed by 1 min 5 s at 56 °C. For each probe, a tenfold serial dilution of the designed oligomer standard (10<sup>8</sup> to 1 molecules/mL) was carried out. This standard curve was used for the quantification of the target sequences. The standard curve was added to each qPCR test with all the oligomers and primer set combinations. For calculating the bacterial concentration of phylogroup B, the value for the primer set C was subtracted from the one obtained with the primer set B + C. The raw fluorescence values were imported into R and analyzed with qpcR (Spiess, 2018). In place of the threshold standard curve method, we tested the Cy0 and cpD2 methods to establish more accurate Ct values in qpcR. Both depend upon fitting sigmoidal models to the fluorescence data. The values given by the Bio-Rad instrument were compared with the two methods and the deviation between sample replicates was plotted. The results showed that both Cy0 and cpD2 were superior to the threshold standard curve methods, and the former was selected to calculate the DNA quantities (Guescini et al., 2013). The standard datapoints were inspected to remove outliers from the log-linear regression of the standard curve before calculation of final values.

## 2.2. Physiological characterization of phylogroups A, B, and C

### 2.2.1. Microorganisms and culture conditions

Four *O. oeni* strains of each phylogroup; A, B, and C were used. Strains from each phylogroup were randomly chosen to cover all the

*O. oeni* phylogenetic tree (Lorentzen et al., 2019). Phylogroup A (all wine strains): CRBO 0607, CRBO S28, CRBO 9517, CRBO 0608; phylogroup B: CRBO 9805, CRBO 0502, CRBO 0501 from wine, and CRBO C23 from cider; phylogroup C (all cider strains): CRBO 13120, CRBO 1384, CRBO 1386, CRBO C52. All strains were grown in liquid grape juice medium (1 L): 250 mL of red grape juice (Jafaden, Leclerc, France), 5 g of yeast extract, 1 mL of Tween 80, adjusted to pH 4.8. The medium was sterilized 20 min on an autoclave at 121 °C before use. The cultures were incubated at 25 °C for around 5 days until late exponential phase. Bacterial enumeration was performed by epifluorescence microscopy for inoculation, and by plating in solid grape juice medium (20 g/L agar, supplemented with 100 mg/L of pimaricin) during the experiments.

As AF starter, *S. cerevisiae* Lalvin FC9 (Lallemand Inc., Montréal, Canada) was used, which was maintained in YPD medium (20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone). Yeast viable enumeration for inoculation was performed by microscopy counting with methylene blue, and by plating in solid YPD medium (20 g/L agar) during the experiments.

### 2.2.2. Growth curve

Tubes containing 20 mL of grape juice liquid medium were inoculated with each *O. oeni* strain for an approximated population of  $5 \times 10^4$  cell/mL. Tubes were incubated statically at 25 °C. Population dynamics was followed by plating. Each strain was studied in duplicate. The growth rate of each strain was calculated considering the exponential growth phase (from day 1 to day 3).

### 2.2.3. Ethanol tolerance in red grape must

For the ethanol tolerance assays a natural Merlot must was used. The basic parameters of the must were 235 g/L of reducing sugars, 2.51 g/L of L-malic acid, 112 mg/L of yeast assimilable nitrogen, and pH 3.48. Prior to inoculation, the must was adjusted to 200 mg/L of YAN (yeast assimilable nitrogen) with THIAZOTE® (Laffort, Bordeaux, France), and pasteurized 30 min at 80 °C. Before inoculation, sterility was confirmed by plating on YPD and grape juice solid media.

Each strain was grown in grape juice medium and was progressively adapted to the grape must containing 6 % (vol/vol) of ethanol. After, cells were inoculated for a population of  $10^5$  cell/mL in eight tubes containing 20 mL of fresh Merlot must with 6 % of ethanol. Ethanol concentration was increased a 2 % daily in some tubes, maintaining others without addition as follows: after 24 h, six tubes increased their ethanol content to 8 %, maintaining two without addition; after two days, 4 tubes increased the ethanol content to 10 %, maintaining 4 without addition - two with 6 % and two with 8 %, etc. Ethanol concentration was increased until 14 % in the fourth day after inoculation. Tubes were incubated statically at 25 °C and studied in duplicate.

### 2.2.4. Fermentation performance

**2.2.4.1. MLF performance in wine-like medium.** Each *O. oeni* strain was inoculated in wine-like medium (WLM) for a population of approximately  $10^7$  cell/mL. WLM was prepared according to Balmaseda et al. (2021), substituting cas-amino acids by yeast extract. Bacterial population and L-malic acid concentration was monitored 2, 7, and 14 days after inoculation. L-Malic Acid Assay Kit (Megazyme, Wicklow, Ireland) was used for L-malic acid quantification.

**2.2.4.2. Wine fermentation.** 20 mL-screwed vials were filled with 14 mL of Merlot grape must (Section 2.2.3) inoculated with  $2 \times 10^6$  cell/mL of *S. cerevisiae* and around  $5 \times 10^4$  cell/mL of each *O. oeni* strain. Vials (Thermo Fisher Scientific, Bordeaux, France) were closed with screw cap-magnetic (Agilent Technologies, hdsp cap 18 mm PTFE/sil 100 pk, Les Ulis, France) with a magnetic stirring bar. Caps were perforated by two hypodermic needles: one for allowing CO<sub>2</sub> release (0.8 × 38 mm, Terumo, Tokyo, Japan), and the other (0.6 × 80 mm, B. Braun,

Melsungen, Germany) connected to a 2 mL syringe (B. Braun, Melsungen, Germany) for allowing sampling. Fermentations were carried out at 25 °C in agitation at 150 rpm, and in triplicate.

AF progression was monitored by weight loss, at least once a day by a precision balance with automatic weight recording (LabX system, Mettler Toledo, Viroflay, France). AF was considered as finished using the local polynomial regression model described in Peltier et al. (2018). Once the AF was finished, the remaining volume was transferred to a 10 mL syringe (BD, Franklin Lakes, USA) coupled with a hypodermic needle (0.8 × 38 mm, Terumo, Tokyo, Japan) and incubated statically at 20 °C.

Bacterial population was monitored by plating each 2–3 days during AF and regularly after AF. L-malic acid concentration was quantified after AF and periodically when bacterial population was  $>10^5$  CFU/mL. pH was determined at the end of AF and once the experiment was finished.

**2.2.4.3. Cider fermentation.** For cider fermentation a commercial apple juice (Jafaden, Leclerc, France) with the following characteristics was used: 94 g/L of reducing sugars, 101 mg/L of YAN, 5.31 g/L of L-malic acid, and pH 3.51. Similarly, to Merlot must, apple juice was adjusted to 200 mg/L of YAN with THIAZOTE® (Laffort, Bordeaux, France), and pasteurized 30 min at 80 °C. Before inoculating sterility was confirmed by plating on YPD and grape juice solid media.

Fermentation conditions were analogous to wine fermentation. Briefly,  $2 \times 10^6$  cell/mL of *S. cerevisiae* and around  $5 \times 10^4$  cell/mL of each *O. oeni* strain in 20 mL vials containing 14 mL of apple juice. Once the AF was finished, the remaining volume was transferred to a 10 mL syringe coupled with a hypodermic needle and incubated statically at 20 °C. Fermentations were carried out in triplicate.

Besides, another set of cider fermentation was carried out, under the same conditions, in the same apple juice with doubled sugar concentration (200 g/L of reducing sugars) by adding 50 g/L of D-glucose and 50 g/L of D-fructose.

## 3. Results and discussion

### 3.1. Ecology of *Oenococcus oeni* phylogroups in wine and cider

#### 3.1.1. Design of the qPCR analysis

The monitoring of specific bacterial strains is easily achievable in laboratory culture media inoculated with selected strains, but it is a challenge in natural environments where many other strains may be present and make detection of the targeted strain difficult. qPCR assays are usually species-specific, rather than targeting a particular bacterial strain, due to the difficulty in designing strain-specific primers. Indeed, few works exploit strain-specific qPCR assays, such as those designed for detecting pathogenic *Escherichia coli* strains or phylogroups (Smati et al., 2013), probiotic *Lactiplantibacillus plantarum* strains (Kim et al., 2021; Zhao et al., 2022), or a plant-growth promoting *Azospirillum brasilense* strain (Stets et al., 2015). To monitor the *O. oeni* phylogroups A-D in wine and cider samples, we used a genomic database of *O. oeni* strains and designed qPCR primers targeting the phylogroup A (175 strains), B (25 strains), C (21 strains) and D (5 strains). The core genomes of the 226 strains were aligned; all group-specific SNPs and indels were detected ( $n = 218,180$ ). A custom script was used to filter and report the positions of all SNPs specific to a given phylogroup, along with the number of mismatches in the ingroup and outgroup. The output was screened by looking for  $\geq 3$  phylogroup-specific SNPs inside a 20-bp window to allow the size of a Taqman probe.

197 SNPs and 3 prospective regions were found for phylogroup A, 95 SNPs and 2 regions were found for phylogroup C and 357 SNPs and 3 regions were found for phylogroup D. 116 SNPs and 1 prospective region were found for phylogroup B, but the number of mismatches (mainly due to sharing SNPs with phylogroup C) was unacceptable; instead, a combined BC region was found, which allowed the B population to be

calculated by subtracting the C probe results from BC. Thus, in the absence of a unique sequence for phylogroup B, it was possible to estimate phylogroup B population assuming that the population detected by the primer set B + C minus the population detected by the primer set C is equal to phylogroup B population. Probes were designed with Primer3 (Untergasser et al., 2012) to fit the unique SNPs and primers were designed for ~100 bp regions around the probe target sequence (Suppl. Table 3). Primers were tested with extracted *O. oeni* DNA from four strains, one from each phylogroup, verifying their sensitivity with each designed probe (Fig. 1), and specificity with extracted DNA of strains from each phylogroup (Suppl. Fig. 1). The sensitivity of each primer set to the designed probes was confirmed under the studied conditions (Fig. 1). An absolute quantification (rather than relative) was performed by using the probes for each phylogroup in known quantities (10 to  $10^7$  molecules/mL) in each qPCR assay. Specificity of each primer set was also evaluated with the available DNA of phylogroups A, B, and C in the lab (Suppl. Fig. 1). Primers A showed a concentration dependent response to the DNA of *O. oeni* phylogroup A. Besides, amplification was observed with DNA of the other phylogroups in a non-concentration dependent (Fig. 1). This amplification was observed in high PCR amplification cycles (Ct > 36), not interfering with the qPCR quantification. Primers BC and C produced a clear concentration dependent response to the target DNA (Fig. 1). The dose dependent response of the designed primer sets in the studied range allowed the qPCR quantification the target sequences.

### 3.1.2. *O. oeni* population dynamics during wine fermentation.

The qPCR assays were used to analyze samples of five tanks of wine collected at different stages of fermentation, starting from grape must up to the end of MLF. Phylogroup D was not present in any sample (Fig. 2). This is not surprising as it has only been detected in kombucha (Lorentzen et al., 2019). In contrast, phylogroups A, B, and C were present in all five tanks (Fig. 2). While previous studies had reported A and B strains in many wines from diverse countries, phylogroup C strains had only been reported in Australian wines (Campbell-Sills et al., 2017; El Khoury et al., 2017). Here, not only the phylogroup C was detected for the first time in French wine productions, but it was detected in all the tanks. However, it was exclusively associated with grape must and AF, and generally absent during MLF – it was only detected in early MLF stage in wine 1 and 5 (Fig. 2). A similar situation was found for phylogroup B, which was present in all five tanks from grape must to the end of AF, but not during MLF. Interestingly, phylogroup B was more abundant than C at all fermentations stages. It is probably for this reason that phylogroup B strains have been quite often isolated during wine-making, while phylogroup C strains have almost never been identified (Borneman et al., 2012; Franquès et al., 2018; Lorentzen et al., 2019). Phylogroup A was the only one detected at all stages of fermentations, and the only phylogroup during MLF. Therefore it is normal that the vast majority of *O. oeni* described to date belong to phylogroup A, since *O. oeni* strains are usually isolated from samples collected during MLF (Franquès et al., 2017; González-Arenzana et al., 2012a; Leixà et al., 2018; Portillo et al., 2016).

Previous studies have shown that the total LAB population typically decreases to levels as low as 100 CFU/mL during AF because most species that were initially present on the grape surface do not tolerate ethanol (Lonvaud-Funel, 1999). In the five tanks analyzed here, the total population of *O. oeni* was significantly higher (about  $10^3$ – $10^4$  cell/mL, Fig. 2) throughout the AF, although it slightly decreased towards the end of the fermentation. This situation is not exceptional because it happens that the LAB develop during AF in certain types of wine, in particular those produced without sulfites (Andorrà et al., 2008). It is also possible that this difference is due to the use of qPCR, which can detect dead cells or cells in a viable but non-culturable state, unlike plate counts.

The population levels of the three phylogroups A, B and C ranged from  $10^2$  to  $10^5$  cell/mL throughout AF in the 5 wines (Fig. 2). The populations were different depending on the wine, but generally stable

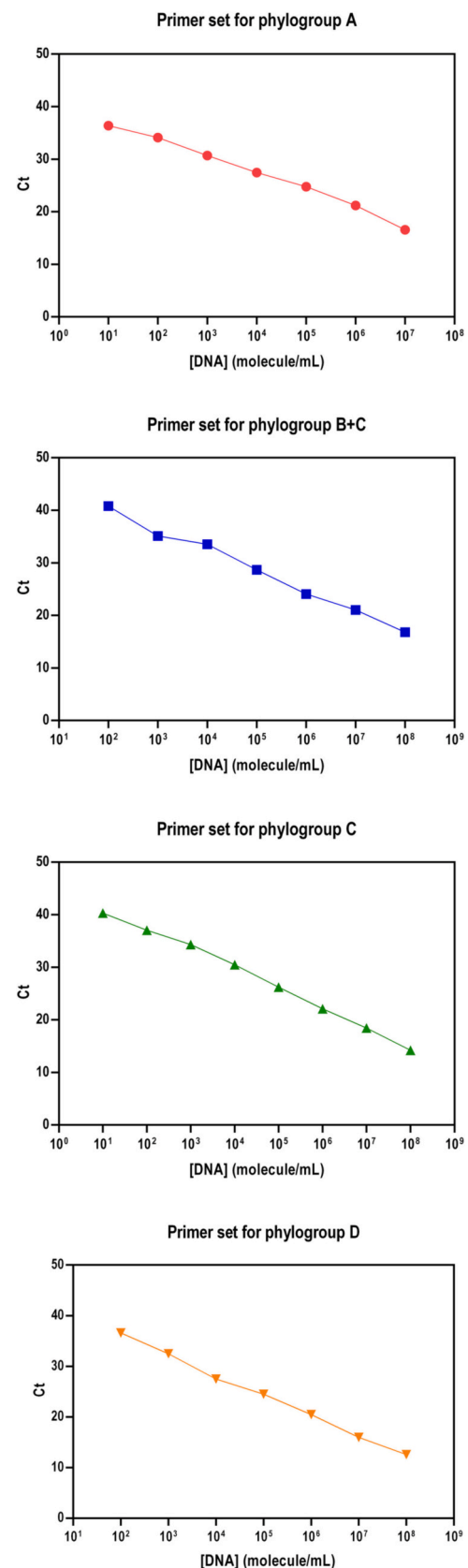


Fig. 1. Sensitivity of PCR primer sets targeting phylogroups A, B + C, C, and D. The selected primer sets were tested in qPCR assays using the probes designed for *O. oeni* strains of phylogroup A (●), B (■), C (▲), and D (▼). Values are the mean of triplicates.

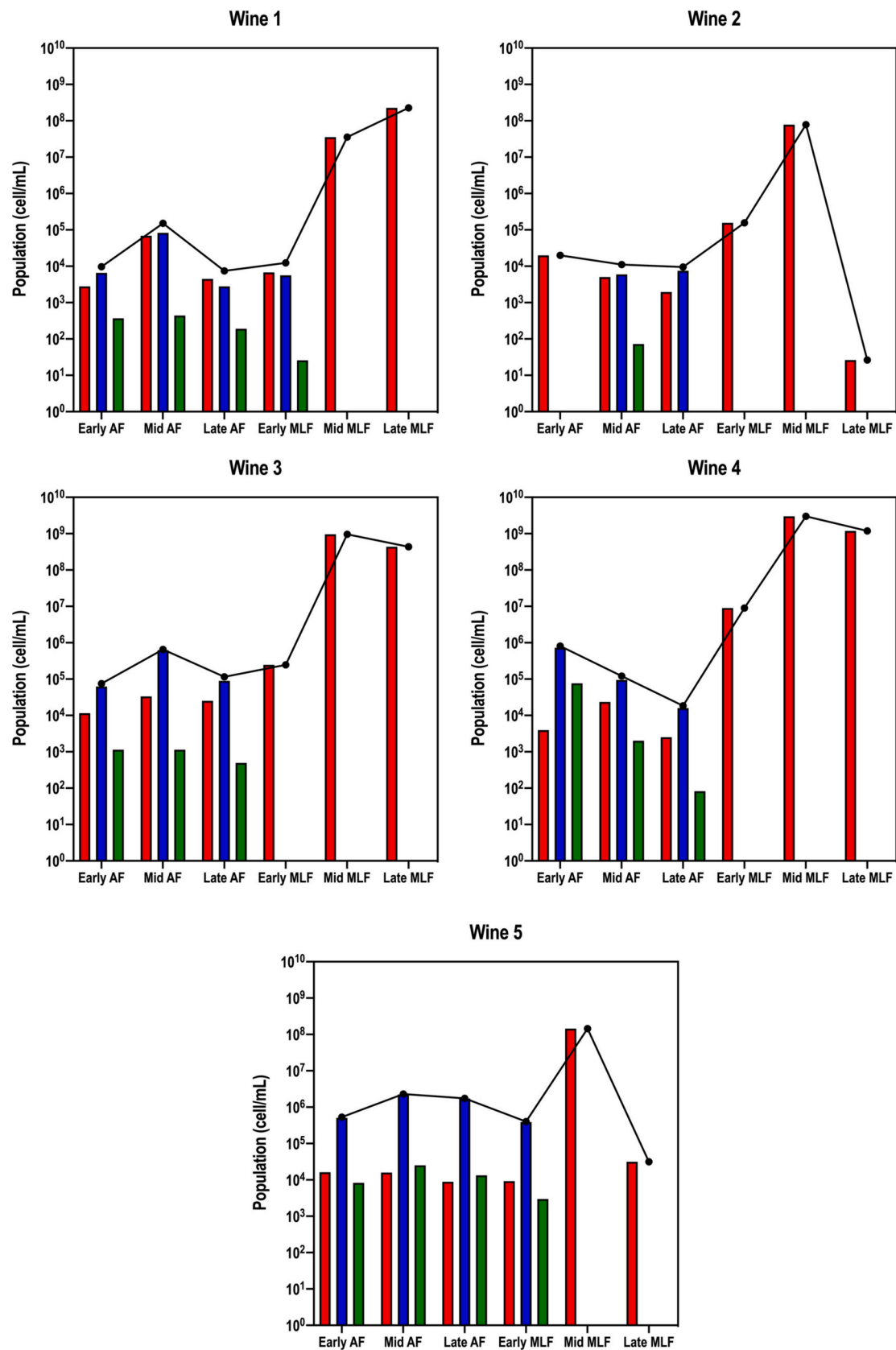


Fig. 2. Population dynamics of *O. oeni* phylogroups during the fermentations of the studied five wines. The population of phylogroups A (■), B (■), and C (■) and total *O. oeni* population (—●—) were determined by qPCR at different fermentation stages. Each value is the mean of triplicates. *O. oeni* phylogroup D was not detected in any sample.

in each wine. Only the population of phylogroup C seemed to decrease with AF in several wines. It should be noted that the population of the three phylogroups was not equal. Phylogroup B was the most abundant in almost all samples until the end of AF, sometimes 10 times or more higher than A and C (Fig. 2).

In general, after the end of AF and early MLF, phylogroups B and C completely disappeared, while phylogroup A increased up to  $10^8$ – $10^9$  cells/mL in all wines and was the only phylogroup present until the end of MLF (Fig. 2).

The cumulative inhibitory effects of ethanol and acidity that occurs during AF is known to cause a selection of LAB species, with *O. oeni* being almost the only species that is still detectable at the end of AF (Lonvaud-Funel, 1999). Our result show that there is also a selection of *O. oeni* phylogroups during AF. Phylogroup A strains are the best adapted to the increase of ethanol, while phylogroups B and C seem to be more inhibited by ethanol like most other LAB species.

### 3.1.3. Population dynamics in organic and conventional wines

The five wines analyzed in this study were produced by using either conventional or organic practices (Suppl. Table 1). Organic wine production differs from conventional one notably in the vineyard, where vine protection is carried out without synthetic pesticides, which may have a significant impact on the microbiota that develop at the surface of grapes (Börlin et al., 2020). Windholtz et al. (2021) described a different initial yeast population in grape must with different vine treatments. Regarding to *O. oeni* population, Piao et al. (2015) found differences in organic and conventional samples during wine fermentation, but surprisingly no *O. oeni* was detected in the organic wines and MLF was not completed. In contrast, we found that the population of *O. oeni* phylogroups was similar in conventional and organic wines, (Fig. 2). Both types of samples displayed the same pattern of diversity during AF, and phylogroup A was the only one detected after early MLF stage.

### 3.1.4. *O. oeni* population dynamics during cider fermentation

*O. oeni* is the main bacterium responsible for MLF in wine and cider, but while phylogroup A strains predominate in wine (El Khoury et al., 2017), only phylogroups B and C strains were isolated from cider to date (Bridier et al., 2010). To better understand this distribution, we have analyzed the populations of the three phylogroups throughout the 10 and 13 weeks of two cider fermentations. Phylogroup D was also investigated, but it was not detected in any sample. The total *O. oeni* population increased from  $10^3$  -  $10^6$  cell/mL at the start of AF, to  $10^6$  -  $10^7$  cell/mL at the end of AF, and the three phylogroups A, B and C were present all through both cider productions (Fig. 3). However, the populations of the three phylogroups were very different. In almost all the samples the populations of phylogroups B and C were larger than those of phylogroup A. Phylogroup B and C populations increased continuously throughout the AF while that of phylogroup A remained relatively stable or declined slightly. By the end of AF, the populations of phylogroups B and C were 100 to 10,000 time greater than phylogroup A. When comparing phylogroups B and C, although they evolved similarly all through the fermentation, the population of phylogroup B was usually larger than that of phylogroup C, especially in one of the two cider productions (Fig. 3).

These results explain why it is mainly strains of phylogroup B, which were isolated during cider production, and sometimes strains of phylogroup C, but never of phylogroup A. It is remarkable that the behavior of the three phylogroups is so different during wine and cider production. The two beverages have quite similar physicochemical properties – fermented beverages with low pH and moderate to high ethanol concentration -, but with differences that must be responsible for the better development of *O. oeni* strains from one phylogroup or another one. The ethanol content may be particularly important as it is double in wine compared to cider, and known to contribute to the selection of microorganisms and LAB species in wine (Davis et al., 1985; Makarova and Koonin, 2007).

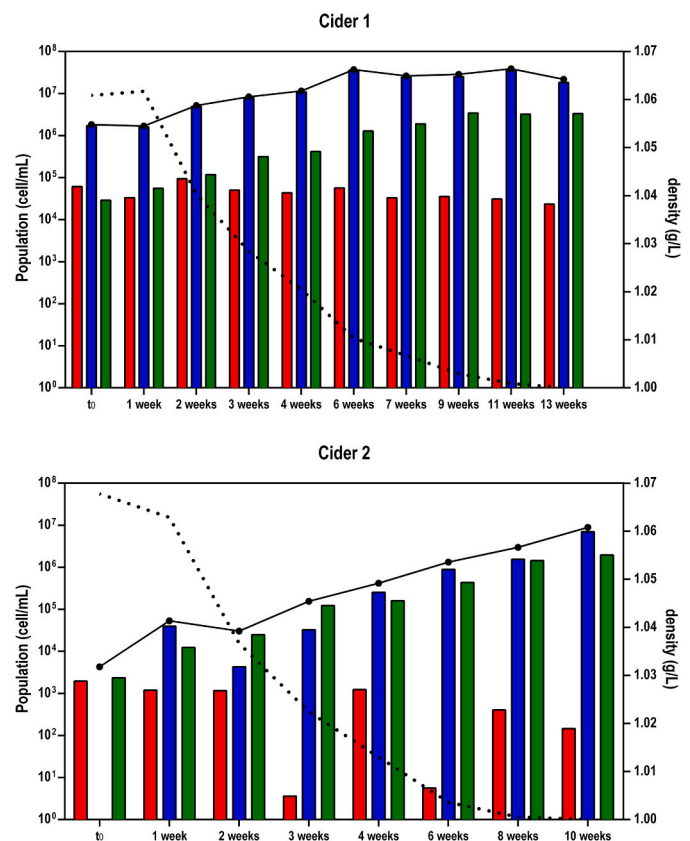


Fig. 3. Population dynamics of *O. oeni* phylogroups during the fermentation of two ciders. The population of phylogroups A (■), B (■), and C (■) and total *O. oeni* population (●) were determined by qPCR. Each value is the mean of triplicates. The density (dotted line) represents the progress of the AF.  $t_0$  is the start of the fermentative process. *O. oeni* phylogroup D was not detected in any sample.

## 3.2. Physiological characterization of phylogroup A, B, and C strains

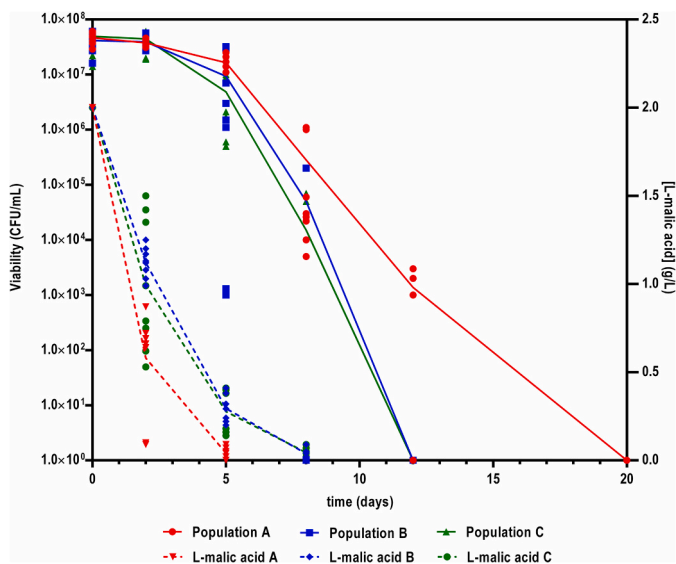
### 3.2.1. Capacity to perform MLF

To determine the physiological differences between *O. oeni* strains, which give them better adaptation to wine or cider, we have analyzed the growth and metabolism of four strains from each phylogroup (A, B, and C) under various conditions. First, we have determined if they can perform MLF, because the conversion of L-malic acid to L-lactic acid confers adaptation to acidic environments by regulating the intracellular pH and/or indirectly producing ATP (Salema et al., 1996).

The 12 strains of each three phylogroups were inoculated to  $5 \times 10^7$  cells/mL in a synthetic wine-like medium (WLM) and their MLF performance was evaluated. Fig. 4 shows that all the strains could achieve MLF. Phylogroup A strains were the most effective and fully consumed L-malic acid in just five days, compared to eight days for phylogroup B and C strains. All the strains kept a good viability during the few days of the MLF, but they then rapidly declined, the strains of phylogroup A being significantly more resistant than those of the two other groups. These results confirm that the strains of the three phylogroups possess the enzymatic machinery required to perform MLF and that they can all carry complete MLF when they are inoculated at a high population. However, it is possible that if they had been inoculated at lower populations, not all of them would have been able to grow sufficiently to achieve MLF, as previously demonstrated for other strains (Reguant et al., 2005a).

### 3.2.2. Growth in grape juice without ethanol

We have analyzed the growth of the 12 strains following inoculation



**Fig. 4.** MLF performance of *O. oeni* strains in WLM. Four strains of each phylogroup A (●), B (■) and C (▲) were inoculated in a WLM adjusted to 12 % ethanol and pH 3.4 and monitored by plate counts for 20 days. L-malic acid was also determined. All experiments were done in duplicates. Values are the mean of duplicates. Lines connect the mean of the replica of each sampling point.

in a grape juice medium in the absence of ethanol. All the strains showed quite similar growth kinetics (Fig. 5, Table 1). Only the time required to reach the maximum population was significantly longer for the strains of phylogroup A, but without significantly changing their growth curve compared to strains of the other two phylogroups. The growth rate ( $\mu$ ) was not significantly different between the three phylogroups, but it differed significantly between strains. The highest growth rates were measured for phylogroup B and C strains (CRBO C23, CRBO C52), and the lowest for two strains of phylogroup A (CRBO S18 and CRBO 0607). We concluded that the predominance of phylogroup A strains during winemaking is not due to better adaptation to grape must.

**3.2.3. Ethanol tolerance in red grape must**

To compare the ethanol tolerance of phylogroup A, B and C strains, they were tested in a Merlot grape must containing 6, 8, 10, 12 or 14 %

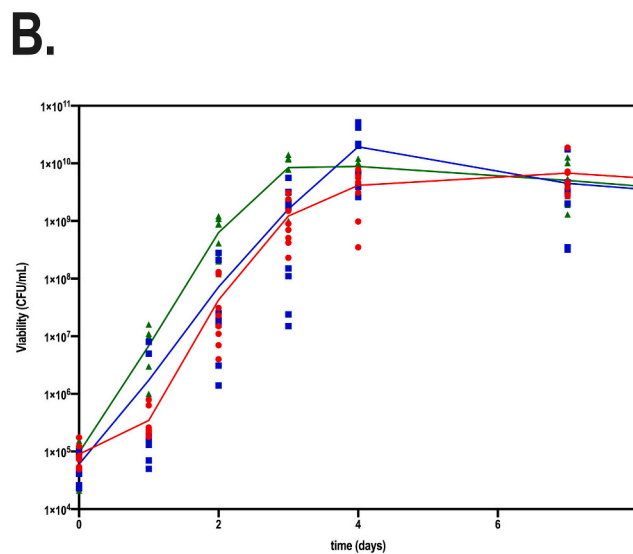
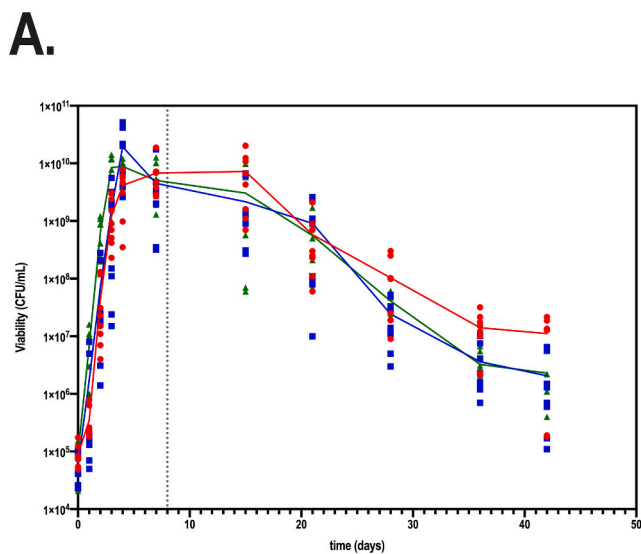
**Table 1**

Growth parameters of *O. oeni* strains in red grape juice medium. Values are the means of duplicates  $\pm$  SD. Bolded values represent the mean of the four strains of each phylogroup.

Phylogroup	Average	Growth rate, $\mu$ (days <sup>-1</sup> )	Maximal population (CFU/mL)	Time for maximal population (days)	
Phylogroup A	Average	<b>1.16 <math>\pm</math> 0.35</b>	<b>1.08 <math>\times</math> 10<sup>10</sup> <math>\pm</math> 6.06 <math>\times</math> 10<sup>9</sup></b>	<b>9.6 <math>\pm</math> 4.6<sup>B</sup></b>	
	CRBO 0607	0.95 $\pm$ 0.09	1.3 $\times$ 10 <sup>10</sup> $\pm$ 8.2 $\times$ 10 <sup>9</sup> ab	7 $\pm$ 0 ab	
	CRBO S28	0.74 $\pm$ 0.08	4.7 $\times$ 10 <sup>9</sup> $\pm$ 0.0 a	5.5 $\pm$ 2.1 ab	
	CRBO 9517	1.55 $\pm$ 0.01	1.6 $\times$ 10 <sup>10</sup> $\pm$ 6.7 $\times$ 10 <sup>9</sup> ab	15 $\pm$ 0 c	
	CRBO 0608	1.41 $\pm$ 0.01	9.8 $\times$ 10 <sup>9</sup> $\pm$ 3.7 $\times$ 10 <sup>9</sup> ab	11 $\pm$ 5.7 bc	
	Phylogroup B	Average	<b>1.53 <math>\pm</math> 0.29</b>	<b>1.97 <math>\times</math> 10<sup>10</sup> <math>\pm</math> 1.82 <math>\times</math> 10<sup>10</sup></b>	<b>4.4 <math>\pm</math> 1.1<sup>A</sup></b>
		CRBO 9805	1.7 $\pm$ 0.04 de	6.9 $\times$ 10 <sup>9</sup> $\pm$ 2.8 $\times$ 10 <sup>8</sup> ab	4 $\pm$ 0 ab
CRBO 0502		1.41 $\pm$ 0.02	4.3 $\times$ 10 <sup>9</sup> $\pm$ 5.7 $\times$ 10 <sup>8</sup> a	5.5 $\pm$ 2.1 ab	
CRBO 0501		1.16 $\pm$ 0.04 abc	2.1 $\times$ 10 <sup>10</sup> $\pm$ 1.1 $\times$ 10 <sup>9</sup> ab	4 $\pm$ 0 ab	
CRBO C23		1.85 $\pm$ 0.04 e	4.7 $\times$ 10 <sup>10</sup> $\pm$ 7 $\times$ 10 <sup>9</sup> c	4 $\pm$ 0 ab	
Phylogroup C		Average	<b>1.40 <math>\pm</math> 0.32</b>	<b>1.01 <math>\times</math> 10<sup>10</sup> <math>\pm</math> 2.84 <math>\times</math> 10<sup>9</sup></b>	<b>3.5 <math>\pm</math> 0.54<sup>A</sup></b>
		CRBO 13120	1.56 $\pm$ 0.26 cde	8.7 $\times$ 10 <sup>9</sup> $\pm$ 1.6 $\times$ 10 <sup>9</sup> ab	4 $\pm$ 0 ab
	CRBO 1384	0.98 $\pm$ 0.06 ab	1.2 $\times$ 10 <sup>10</sup> $\pm$ 3.5 $\times$ 10 <sup>8</sup> ab	3 $\pm$ 0 a	
	CRBO 1386	1.36 $\pm$ 0.12 bcd	6.8 $\times$ 10 <sup>9</sup> $\pm$ 9.9 $\times$ 10 <sup>8</sup> ab	4 $\pm$ 0 ab	
	CRBO C52	1.71 $\pm$ 0.17 de	1.3 $\times$ 10 <sup>10</sup> $\pm$ 1.4 $\times$ 10 <sup>9</sup> ab	3 $\pm$ 0 a	

a-e Values are significantly different at  $p \leq 0.05$  according to a Tukey HSD post-hoc comparison test considering the value for each strain. A-B Values are significantly different at  $p \leq 0.05$  according to a Tukey HSD post-hoc comparison test considering the replicas of all four strains of each phylogroup as a group. No letter means no significant differences.

ethanol. To mimic the progressive increase of ethanol that occurs during AF, and allow bacteria to adapt to high ethanol content, they were initially inoculated in the must containing only 6 % ethanol and its



**Fig. 5.** Growth kinetics of *O. oeni* strains in red grape juice medium. A: Four *O. oeni* strains of each phylogroup A (●), B (■) and C (▲) were inoculated to  $5 \times 10^4$  cell/mL and monitored by plate counts for 42 days. The vertical dotted line represents the exponential phase of growth. B: Focus on the first seven days of the exponential phase of growth. All cultures and determinations were performed in duplicates. The red, blue, and green lines connect the mean of the replica at each sampling point.



concentration was progressively increased by 2 % every 24 h, up to the final expected concentration. All the strains developed well in the presence of 6 % ethanol, although different lag phases were observed depending on the phylogroup (Fig. 6). Phylogroup A strains restarted growth after a lag phase of only four days, whereas it was seven and nine days for phylogroup B and C strains, respectively. The difference between the three phylogroups was even more important at higher ethanol concentrations. Phylogroup A strains were the only one able to develop at 8 and 10 % ethanol and to maintain a high viability at 12 % ethanol (Fig. 5). Phylogroup B and C strain populations decreased whichever the ethanol content (from 8 to 14 %), and more and more with increasing concentrations. It is therefore the high ethanol content of wine which favors the selection of phylogroup A strains in wine. Strains of phylogroups B and C can grow in grape must and during the first days of AF, but they cannot maintain a high viability at the end of AF.

### 3.3. Growth and MLF performance during wine production

To compare their growth during wine production, each of the 12 strains was inoculated into a sterile grape must at a low population ( $\sim 5 \times 10^4$  cells/mL) mimicking a natural LAB population, and a yeast strain was added to start AF. The fermentation lasted for nine days and reached the same ethanol content in all the experiments (data not shown). Bacterial populations and residual L-malic acid content were determined throughout AF and for 100 days after the end of AF (Fig. 7). During the nine days of AF, a general viability loss was observed, especially for phylogroups B and C (Fig. 7.A). Phylogroup A strains were the most resistant, losing less than one log in population during this period. It is worth noting that three of them restarted the growth during AF and the fourth one immediately after the end of AF. All these strains reached maximal populations of about  $10^8$  CFU/mL, which they maintained until the end of the experiment, although some variations were noticeable between them (Fig. 7.A). Phylogroup B and C strains were much more sensitive to AF. A near complete loss of viability was noticed for all the phylogroup C strains, and two phylogroup B strains. Strain CRBO 0501 (phylogroup B) lost only one log in population during AF and resumed growth immediately afterwards. In this sense, it behaved similarly to phylogroup A strains. However, it grew much slowly, barely reaching  $10^6$  CFU/mL in 100 days after AF. A second strain of phylogroup B (CRBO 0502) survived at a level of 10–100 CFU/mL during AF and then started growing, but with even more difficulty than the previous strain, reaching only  $10^4$  CFU/mL at the end of the experiment. None of the other phylogroup B and C strains could develop significantly after their almost complete loss of viability during AF.

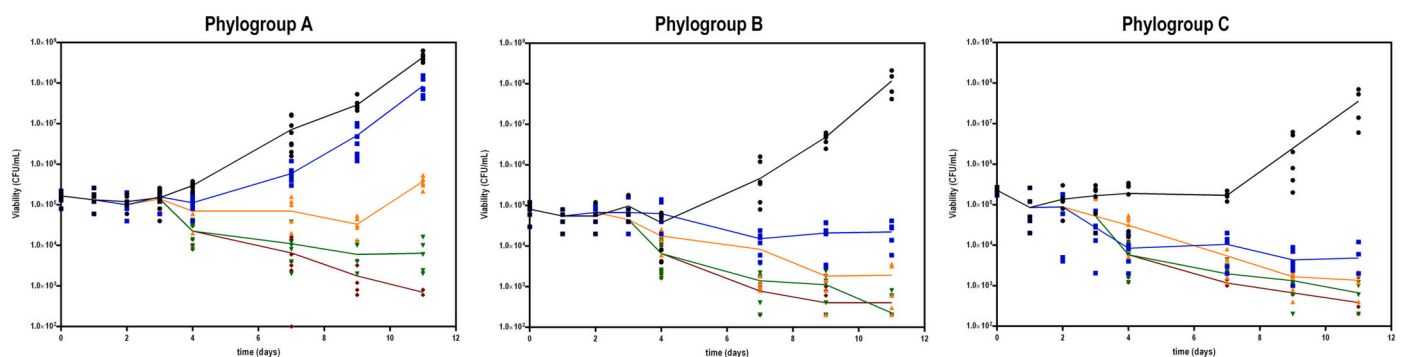
These results agree with those observed in grape juice medium supplemented with ethanol (Fig. 6). Phylogroup A strains are by far the most resistant to the increased ethanol content that occurs during AF

and those with the fastest growth during/after AF. Therefore, it is not surprising that they were the only strains capable of performing MLF (Fig. 7B). They could do MLF because they reached the population level that is considered as the threshold above which the degradation of malic acid becomes detectable ( $10^6$  CFU/mL). Higher populations cause acceleration of MLF (Reguant et al., 2005a), as is clearly the case for two phylogroup A strains (CRBO 0608 and CRBO S28: MLF complete in 21 and 34 days after inoculation), compared to the other two (CRBO 0607 and CRBO 9517: MLF complete in >75 days). The two strains of phylogroup B, which developed after AF, did not reach a sufficient population.

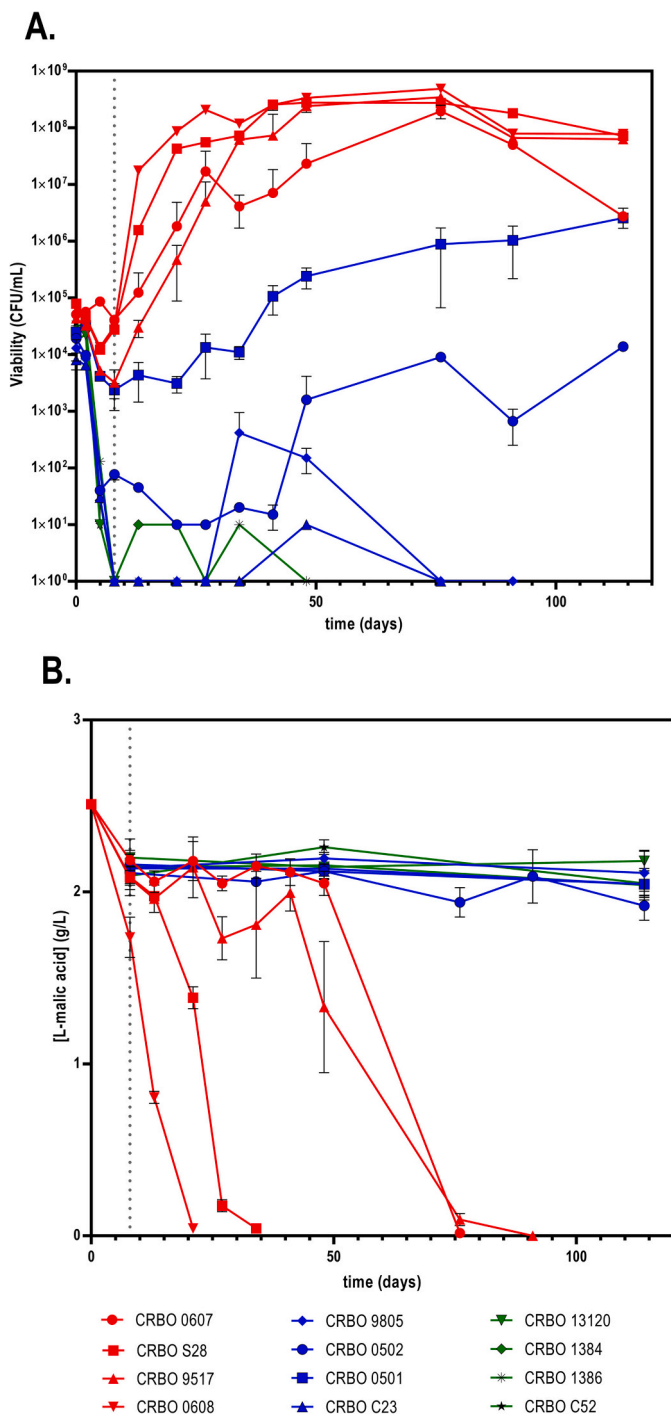
According to these very different behaviors of phylogroup A, B and C strains, it is easy to explain their population variations during wine production (Fig. 2). Phylogroup A strains are usually the only ones detectable when MLF starts, because they resist better to the increase of ethanol during AF, and they develop quickly during or after AF. Some strains of phylogroup B could also perform MLF and were sometimes isolated from wines (Lorentzen et al., 2019), but in most situations they are not detected after AF because they do not survive and develop in wine when ethanol concentration is high.

### 3.4. Growth and MLF performance during cider production

In order to investigate the population variations of phylogroups A, B and C strains during cider production (Fig. 3), we used the same strategy as above, by inoculating the bacteria in an apple juice along with a yeast strain to perform AF (Fig. 8). In all experiments, AF completion took only five days. Phylogroup A and B strains showed a similar behavior. They started growing immediately after inoculation, during and after AF, and reached a maximum population of approx.  $10^9$  CFU/mL after 12 days (Fig. 8.A1). Their populations were stable up to day 30 and then they started to decrease. In contrast, phylogroup C strains declined of about one log during AF and started growing just at the end of AF. They reached the same maximal population as phylogroup A and B strains, but in 30 days instead of 12, and their population was quite stable until the end of the experiment (62 days). All strains completed MLF, but more or less rapidly depending on their population level (Fig. 8.A2). It was surprising that phylogroup A strains grew similarly to B strains and better than C strains, as this is inconsistent with the population levels determined during cider production (Fig. 3). However, under our experimental conditions, AF was performed much faster than in real cider productions (5 days compared to 60–80 days). It is likely that phylogroup C strains do not adapt well to a fast increase in ethanol. This is consistent with results obtained in grape must supplemented with ethanol (Fig. 6). In contrast, the strains of phylogroup A resisted to ethanol, which allowed them to develop as well as those of phylogroup B under our experimental conditions. Perhaps in real cider productions,



**Fig. 6.** Ethanol tolerance of *O. oeni* strains. Four strains of each phylogroup A, B, and C were inoculated in a red grape must supplemented with 6 % of ethanol, progressively increased up to 14 %, and monitored for 11 days by plate counts. Medium was maintained at 6 % ethanol (●), increased to 8 % (■), 10 % (▲), 12 % (▼), up to 14 % (◆), increasing progressively a 2 % each day until reaching 14 %. All the cultures were performed in duplicates. Values are the mean of duplicates. Lines connect the mean of the replica of each sampling point.



**Fig. 7.** Growth and MLF performance of *O. oeni* strains in wine. A: Bacterial viability during fermentation. B: L-malic acid concentration during fermentation. Four strains of each phylogroup A (■), B (■) and C (■) were inoculated in a red grape must along with a yeast strain to perform AF (potential alcohol of 13.33 % vol/vol). Bacterial populations were monitored by plate counts during and after AF up to 110 days. L-malic acid was also determined. All the analyses were done in triplicates. Values are the mean  $\pm$  SD of the triplicates. Lines connect the mean of the replica of each sampling point. The vertical dotted line points the end of alcoholic fermentation.

they are disadvantaged by the longer AF, so their populations remain lower than that of B or C strains (Fig. 3).

To determine if the different bacterial behaviors in wine and cider derived from the different ethanol contents or from other physico-chemical parameters, we repeated the experiment using the same

apple juice supplemented in sugar to double its concentration and reach a similar concentration as the Merlot grape must.

In this more sugared apple juice, AF was complete in nine days and the final ethanol content was the double (Fig. 8.B1). Phylogroup A strains were the most resistant and started growing during AF as previously noticed in wine. Interestingly one of the four strains grew very slowly, reaching the maximum population after 50 days compared to 20 for the other three strains. The phylogroup B and C strains declined considerably during AF, but not to the same extent as previously observed in wine. All the four phylogroup B strains restarted growing after AF and were able to initiate MLF (Fig. 8.B2). Phylogroup C strains were more sensitive to the high ethanol content, but at least two of the four strains restarted growing and reached a sufficient population to initiate MLF. If we consider that phylogroup B and C strains were probably disadvantaged by the rapidity of AF compared to normal cider production conditions, it is remarkable that most of them were still able to develop in the presence of a high ethanol content.

Therefore, it is not only the high ethanol content of wine that selects phylogroup A strains in wine, but also the kinetics of AF. In addition, there must be other physico-chemical parameters or microbial interaction phenomena that disadvantage phylogroup A strains in cider since they seem to be able to develop in cider as efficiently as the other strains under these experimental conditions.

#### 4. Conclusion

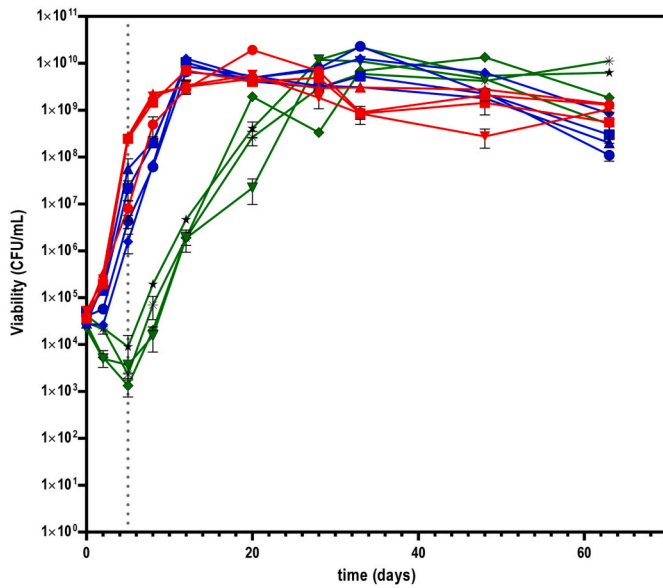
We developed a qPCR assay which allowed us to monitor the population dynamics of *O. oeni* phylogroups in wine and cider. Its utilization with samples collected during real wine productions showed that significant populations of the three phylogroups A, B, and C are always present from the grape must to the end of AF. This is the first time that phylogroup C strains were detected in Bordeaux wines, and interestingly, they were present in all the wines. However, its population was always lower than those of phylogroups A and B strains, which explains why they are rarely isolated from wine. Following AF, only phylogroup A strains survived and developed in all the analyzed wines. Phylogroup B and C strains disappeared totally after early MLF, thus explaining why it is almost always phylogroup A strains the ones isolated during wine MLF. The qPCR assay also showed for the first time that phylogroup A strains are present in cider along with phylogroups B and C strains. Phylogroup A strains were never isolated from cider in previous studies because their population are always lower than those of B and C strains.

By testing the strains separately, we found that strains of the three phylogroups can perform MLF. Still, phylogroup A strains are the most frequent during MLF because they are better able to resist the fast increase of ethanol occurring during wine AF, and because of some other parameters found in wine that inhibit the development of phylogroup B and C strains. This was demonstrated using double-sugared cider, in which most phylogroup B and C strains could develop and start MLF, even if the ethanol content was similar to wine. In contrast, phylogroup A strains did not develop as efficiently as B and C strains during real cider production, although they performed the best under experimental conditions. It seems that in real cider fermentation there could be some physicochemical parameters or microbial interactions that inhibit phylogroup A development when growing in a complex microbial environment, not observed in isolated strains' fermentations.

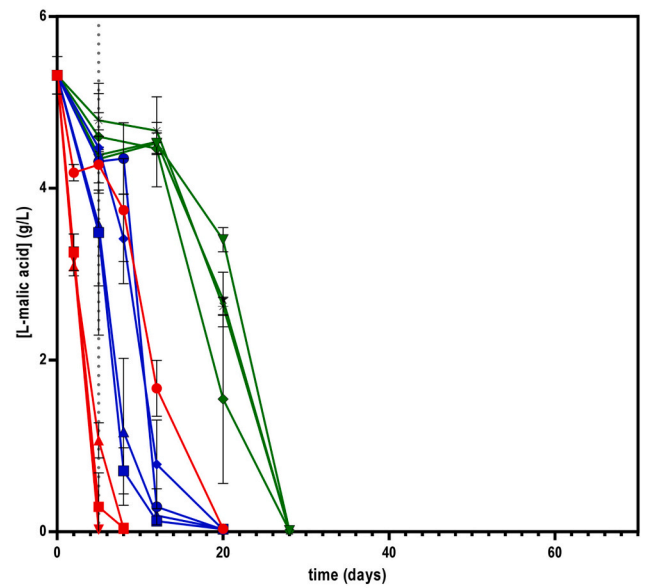
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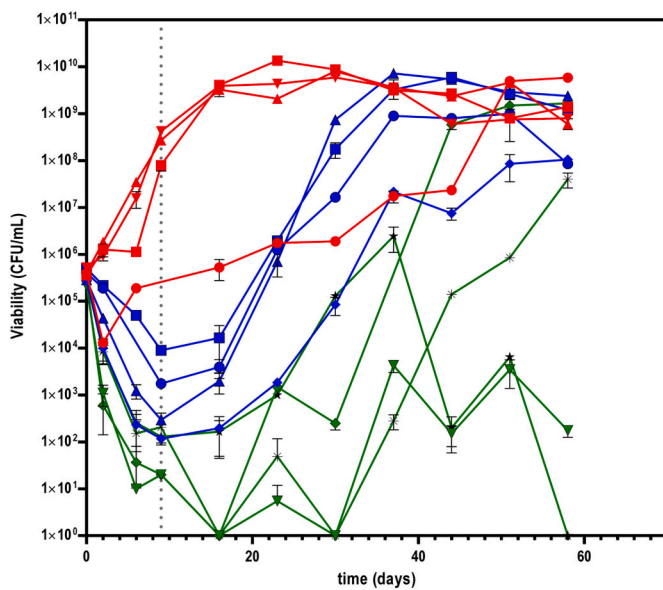
A1.



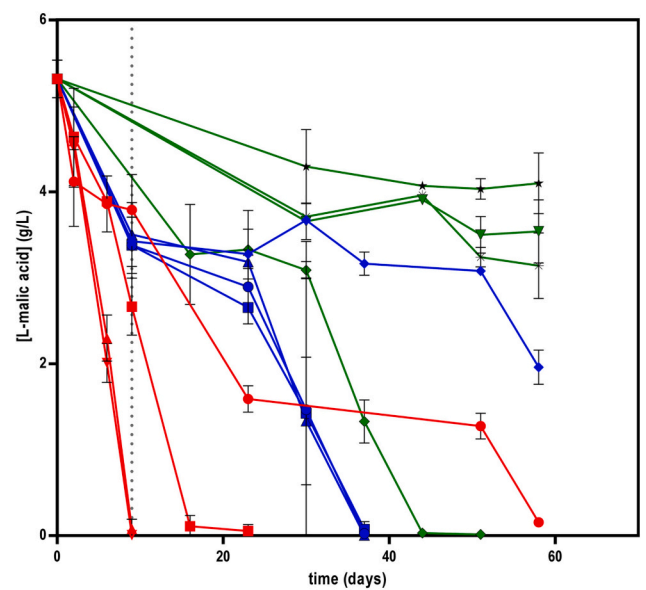
A2.



B1.



B2.



- CRBO 0607      ● CRBO 9805      ● CRBO 13120
- CRBO S28      ● CRBO 0502      ● CRBO 1384
- ▲ CRBO 9517      ■ CRBO 0501      ● CRBO 1386
- ▼ CRBO 0608      ▲ CRBO C23      ● CRBO C52

- CRBO 0607      ● CRBO 9805      ● CRBO 13120
- CRBO S28      ● CRBO 0502      ● CRBO 1384
- ▲ CRBO 9517      ■ CRBO 0501      ● CRBO 1386
- ▼ CRBO 0608      ▲ CRBO C23      ● CRBO C52

**Fig. 8.** Growth and MLF performance of *O. oeni* strains in cider. A: Four strains of each phylogroup A (■), B (●) and C (▲) were inoculated in an apple juice along with a yeast strain to perform AF (potential alcohol of 5.6 % vol/vol). B: The same experiment was performed in the same apple juice supplemented with 100 g/L of glucose + fructose (potential alcohol of 10.75 % vol/vol). Bacterial populations were monitored by plate counts up to 60 days (A1, B1). L-malic acid was also determined (A2, B2). All the analyses were done in triplicates. Values are the mean ± SD of the triplicates. Lines connect the mean of the replica of each sampling point. The vertical dotted line points the end of alcoholic fermentation.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2023.110276>.

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