Combined use of Wallerstein and *Zygosaccharomyces bailii* modified differential media to isolate yeasts for the controlled reduction of volatile acidity of grape musts and wines

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The level of acetic acid, the main component of volatile acidity, is critical for wine quality. Winemakers have been using a refermentation process to lower the concentration of acetic acid of wines with high volatile acidity, which consists in mixing the acidic wine with freshly crushed grapes or marcs in a proportion of no more than 20-30% (v/v). Though this process implies low costs it harbors the risk of unexpected and detrimental effects on refermented wines. Thus, one challenge to find new solutions for the reduction of excessive volatile acidity is the selection of yeast from refermentation processes of acidic wines to use as starters in a controlled biological process. To this end we set up an isolation protocol with Wallerstein Laboratory Nutrient Agar (WL) to select yeast strains from refermentation processes of acidic wines carried at the winery scale. Among the isolates obtained, 135 were then randomly selected, based on the different colony color pattern and size, and tested for their ability to consume acetic acid in the presence of glucose. For this purpose we used a modified version of a *Zygosaccharomyces bailii* differential medium containing acetic acid and glucose. Characterization of the isolates obtained in this medium by fingerprinting with primer T3B confirmed three *Saccharomyces* strains and one non-*Saccharomyces* strain as predicted by WL and L-Lysine media. Our previous studies revealed that the yeast strains selected by this approach are adequate for the correction of acidic musts and wines with excessive levels of volatile acidity.

Keywords: Volatile acidity; refermentation; acidic wines; winery yeast isolates.

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Introduction

The main component of the volatile acidity of wines is acetic acid. This acid occurs in wines in concentrations ranging from 0.2 to 0.6 g/L, though they may be higher under certain conditions [1]. The Office International de la Vigne et du Vin [2], refers that the maximum acceptable limit for volatile acidity in most wines is 1.2 g/L of acetic acid due to the

associated unpleasant vinegar aroma and acrid taste. Over the years, numerous authors have studied the production of volatile acidity during alcoholic fermentation and in wines [3-8]. Acetic acid, besides its presence in grape musts due to bacterial spoilage of grapes infected with *Botrytis cinerea*, can be formed by apiculate wine yeasts, mainly species of *Hanseniaspora*, anamorph of *Kloeckera* [9, 10], as well as by wine species of the genus *Candida* [11] involved in the early phase of both spontaneous and inoculated fermentations. Acetic acid is a by-product of alcoholic fermentation by *Saccharomyces cerevisiae* under winemaking conditions. It is mainly formed at the beginning of the alcoholic fermentation [12, 13] and may contribute to an increase of volatile acidity in wine. Production of acetic acid by malolactic bacteria has also been reported [14]. High volatile acidity may occur in improperly stabilized wines due to spoilage agents, such as the acetic acid bacteria [15], yeast species of the genera *Dekkera/Brettanomyces* [16-18], and some strains of *Saccharomycodes ludwigii* [19].

However, some yeast species seem to use the acetic acid present at the beginning of alcoholic fermentation, or even in grape musts, via acetyl-CoA in the lipid-producing pathway [8, 20, 21]. In wine-making conditions that lead to high levels of volatile acidity, acetic acid is not sufficiently degraded and remains at the end of fermentation. In wine-making conditions, winemakers have been using a procedure called refermentation to lower the volatile acidity of wines with more than 0.8 g/L acetic acid. In this process the acidic wine is mixed with freshly crushed grapes or marcs (remaining pulp, after draining the newly made wine) in a proportion of no more than 20-30% (v/v). Winemakers use this rather empirical practice to reduce volatile acidity to values in the range of 0.37 g/L of acetic acid. Though this refermentation process implies low costs, it harbors the risk of unexpected final results and detrimental effects on fermentation since the involved yeast flora is largely unknown [22]. A useful strategy to overcome such limitation and contribute to the development of a controlled process for the bio-reduction of volatile acidity might consist in the selection of indigenous strains isolated from acidic wines during refermentation processes.

The aim of this study was to develop a protocol to isolate yeast species from winery refermentation processes with ability to decrease the acetic acid content of grape musts and wines with excessive volatile acidity. To this end we combined the use of Wallerstein medium and a modified version of the differential medium designed for the enumeration of the spoilage yeast *Zygosaccharomyces bailii* in wine [23] in which formic acid was substituted by acetic acid. The selected strains were subsequently characterized by RAPD analysis using primer T3B. The yeast strains selected by this approach can be applied in controlled refermentation processes to correct acidic wines with excessive levels of volatile acidity [24].

Materials and Methods

Refermentations

Samples were collected from four wineries of the Região Demarcada do Douro. In all winery sites namely: Universidade de Trás-os-Montes e Alto Douro (UTAD) – A; Borges (Sociedade dos Vinhos Borges S.A.) – B; Vila Real (Adega Cooperativa de Vila Real) – C and Cumieira (Adega da Cumieira, Caves Santa Marta) – D. Refermentation processes were made with mixtures of must from different grape varieties. From Cumieira winery an additional refermentation was done, using marc (remaining pulp, after draining the newly made wine) - E. The grape-must from Cumieira winery was supplemented with 40 mg/L of sulfur dioxide (SO_2). In all refermentation processes, 1/3 of an improperly stabilized acidic red wine (1.36 g/L acetic acid), provided by the Cumieira winery, was added to 2/3 of must or marc.

Refermentations were conducted at 23 to 28°C and their progress was monitored daily by

densitometry and sugar determination by the DNS method [25]. Immediately after must or marc additions a pump over of 5 min. was carried out. This procedure was repeated during refermentation, in each morning and evening.

Isolation of yeast strains

Samples for the isolation of yeast strains were collected from the acidic red wine, from musts of different origins, after addition of the wine to the musts or marc, and at the end of the refermentation process. Serially diluted samples (10^{-3} to 10^{-6}) were spread onto Wallerstein Laboratory Nutrient Agar (WL) medium (Difco) and YPD medium (glucose 2%, w/v; peptone 1%, w/v; yeast extract 0.5%, w/v and agar 2%, w/v). Both media were supplemented with chloramphenicol (0.5 g/L) to prevent bacterial growth. Colonies were selected from WL medium plates according to their morphology (colony color, size) as described by Pallmann et al. [26]. All the selected isolates were streaked in YPD plates for further purification and frozen at -80°C in YPD broth supplemented with glycerol (30%, v/v).

Selection of yeast strains with ability to consume acetic acid in the presence of glucose was achieved with a differential solid medium adapted from Schuller et al. [23], used for the enumeration of the spoilage yeast *Zygosaccharomyces bailii* in wine, in which formic acid was replaced by acetic acid. This differential mixed-substrate medium was based on a mineral medium supplemented with vitamins and oligoelements [27]. The medium at pH 4.0 contained acetic acid (0.5%, v/v), glucose (0.05%, w/v) and bromocresol green as acid-base indicator (0.005%, w/v), whereas the medium at pH 6.0 contained- acetic acid (0.5%, v/v) and glucose (0.5%, w/v) and bromocresol purple as acid-base indicator (0.005%, w/v).

Isolates from frozen stocks were grown on YPD plates for 48 hours (25°C) to prepare yeast suspensions (OD_{640} nm between 0.7 and 1.0) and drop (10 µl in triplicate) on the surface of the differential mixed-substrate media. Plates were then incubated for 48 to 72 hours at 30°C and the change in color of the medium was evaluated, in comparison to the positive control strain *Z. bailii* ISA 1307 (color change of the acid-base indicator) and the negative control strain *S. cerevisiae* PYCC 4072 (no color change). From the same pre-culture on YPD plates a loop full of cells was transferred to Lysine medium and incubated (25°C for 48-72 hours) for distinction between *Saccharomyces* (no growth) and non-*Saccharomyces* species (growth) [28].

Analytical determinations

Progress of the wine's refermentation was monitored daily by densitometry and sugar consumption by 3, 5-dinitrosalicylic acid (DNS) method [25]. Volatile acidity was determined by distillation using a Cazenave-Ferré followed by titration with phenolphthalein. Analysis of the density, pH, alcohol concentration, SO_2, and titratable acidity were performed according to published methods, outlined in Table 2.1.

Molecular characterization

Yeast genomic DNA of each isolate was extracted as previously described [29]. RAPD PCR analysis was performed with the primer T3B (5'- AGG TCG CGG GTT CGA ATC C-3'), specific for the conserved genes of the conserved tRNA region [30]. Amplification reactions were performed in volumes of 25 µl containing 10x reaction buffer, 25 mM of $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of T3B, 1 U of Taq DNA polymerase (Bioron®). Samples of genomic DNA were diluted (1:10) with sterilized water, and 1 µl (approximately 10 ng) was added to 24 µl of the master mix solution prepared from the above mentioned components. Samples were overlaid with sterile, light mineral oil (Sigma), and amplified in a thermocycler (Perkin-Elmer 9600) as follows: initial denaturation for 10 min at 95°C; denaturation for 30 s at 95°C; annealing for 30 s at 52°C; and extension for 1 min at 72°C. Thirty four cycles were followed by a final extension cycle for 8 min at 72°C. Amplification products were separated by electrophoresis in 1.2% (w/v) agarose gels in 0.5x TBE buffer for 90 min

at 3V/cm. The gels were stained with ethidium bromide and photographed in an UV transiluminator (Vilber-Lourmat).

Results

Isolation of yeast strains on WL medium

As described in material and methods yeast strains were isolated from samples obtained from musts and from the mixture of acidic wine with musts or marc at the beginning and at the end of the refermentation process. Diluted samples were plated onto WL medium. A total of 135 yeast isolates representative of the different types of colonies obtained in this culture medium were selected. As shown in Figure 1–I, colonies were distinguished by their color and size. The predominant color of the colonies was cream (Figure 1–I, letter A1 and Figure 2 and 3) with quite variable sizes. These colonies were isolated from all the wineries and refermentation processes undertaken. Green colonies (Figure 1–I, letters A2 and A3, and Figure 2 and 3) were the second most abundant type, followed by intense green colonies (Figure 1–I, letter B and Figure 2) and cream colonies with a hint of green (Figure 1–I, letter C and Figure 2 and 3). Small cream colonies (Figure 1–I, letter D and Figure 2 and 3) were detected in two samples. WL medium was originally developed for monitoring yeast populations during industrial fermentation processes. As described by Pallmann et al. [26], the yeast isolates could be classified accordingly to their color and size as *Saccharomyces* (green to cream-colored colonies) and non-*Saccharomyces* species commonly present in grape musts/wines namely: *Hanseniaspora uvarum/Kloeckera apiculata* (intense-green colonies); *Zygosaccharomyces bailii* (small-cream colonies); and cream less colonies with a hint of green, probably indicating the presence of *Torulaspora delbrueckii* species. Assuming this classification, the evolution of the yeast species along the refermentation processes was inferred from the percentage of the different types of colonies on WL medium isolated from samples of the four wineries studied. In the refermentation process with Cumieira winery must (Figure 2), the proportion of *Saccharomyces* species increased from 68% (initial must Figure 2) to 93% (at the end of refermentation – Figure 2), while in the refermentation process with marc, in the same winery, the proportion of *Saccharomyces* and non-*Saccharomyces* was maintained from the beginning to the end of refermentation (Figure 2). In the other three refermentation processes with the musts from Borges, UTAD and Vila Real wineries, the *Saccharomyces* species dominate at the end of refermentation (Figure 3), similarly with what occurred in the refermentation process with the must from Cumieira winery.

As previously mentioned, 40 mg/L of SO_2 were added to the grape must at the beginning of refermentation processes at Cumieira winery (with both the must and the marc). This increased considerably the free and total SO_2 of final wines of the Cumieira winery (Table 1, refermented wines D and E). However, no impact was observed on yeast species distribution in different sampling points (must; must + wine at the beginning or end of fermentation, Figure 2) when compared with the refermentations processes from the other wineries (Figure 3). This might be due to the SO_2 inhibition that is less pronounced for yeasts than for bacteria [21].

During refermentation, the initial volatile acidity of 1.36 g/L dropped to values between 0.60 and 0.70 g/L (Table 1). The titratable acidity of the final refermented wines was slightly higher when compared with the titratable acidity of the initial acidic wine, and it varied between the lowest (6.85 g/L) and highest (7.83 g/L) concentration of tartaric acid in the refermented wines from UTAD and Borges wineries, respectively (Table 1, A and B). Sugars were completely consumed after 96 and 168 hours in the refermentation with marc and musts, respectively.

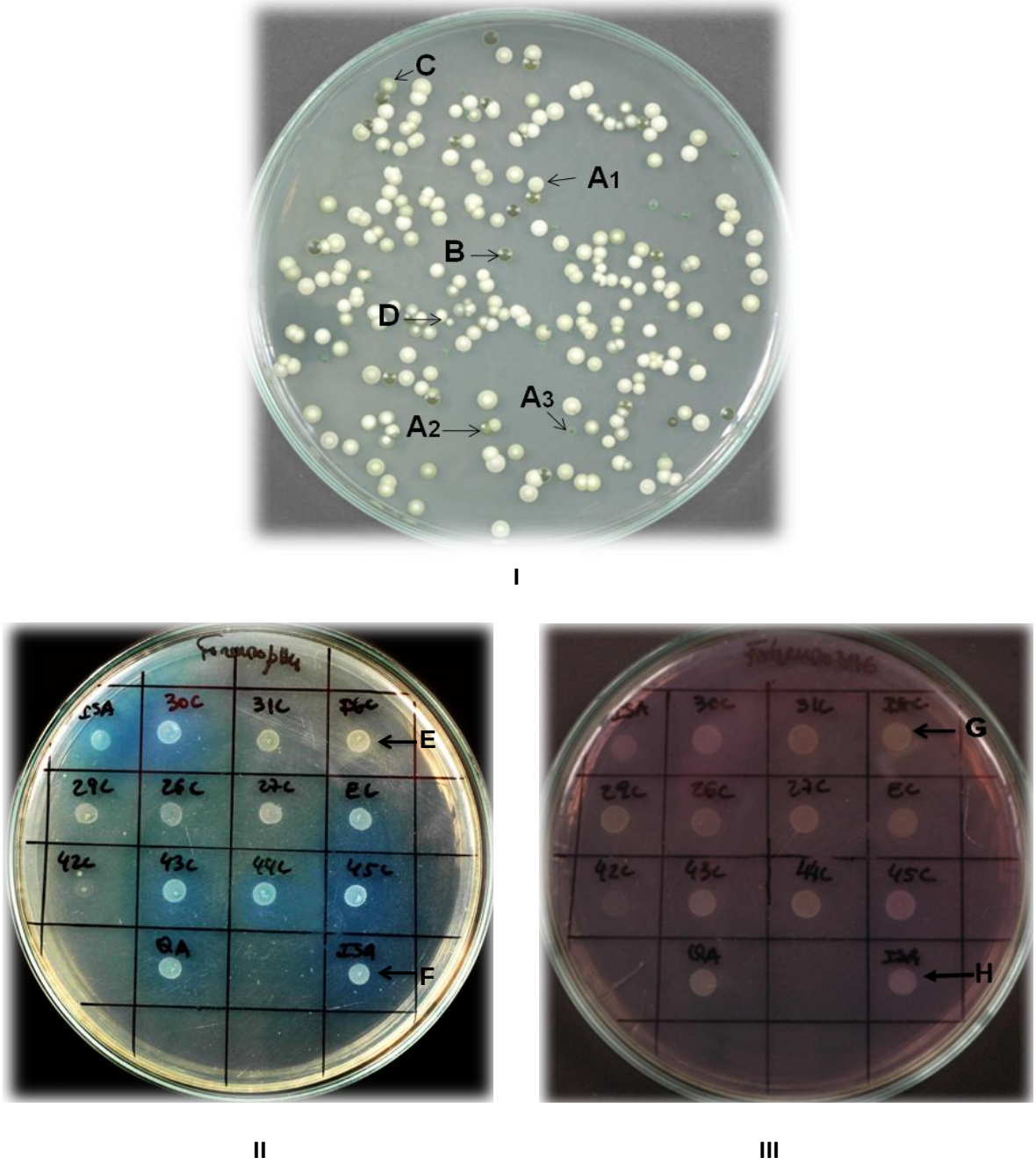

Figure 1. I – Yeast isolates from a refermentation process (dilution of 10-3 cell/mL) in WL medium, showing characteristic colony color and morphologies. According to Pallmann et al. [26]: cream (A1), green (A2) and small green (A3) colonies are probably *S. cerevisiae* isolates; intense green colonies (B) belong to *Hanseniaspora uvarum/Kloeckera apiculata* species; cream colonies with a hint of green (C) to *Torulaspora delbrueckii* and small cream colonies (D) are isolates of *Zygossaccharomyces bailii*. **II -** Growth and color change (due to pH changes) of the differential medium with 0.5% (v/v) acetic acid, 0.05% (w/v) glucose and bromocresol green (0.005% (w/v), at pH 4.0, indicating simultaneous consumption of glucose and acetic acid by the isolated strains 30C, 43C, 44C and 45C. E: *S. cerevisiae* PYCC 4072 (negative control strain); F: *Z. bailii* ISA1307 (positive control strain). **III -** Growth and color change in differential medium containing 0.5% (v/v) acetic acid, 0.5% (w/v) glucose and 0.005% (w/v) bromocresol purple, at pH 6.0. The strain PYCC 4072 (G) shows a negative result with a bright yellow color around the colony H: *Z. bailii* ISA1307 (positive control strain).

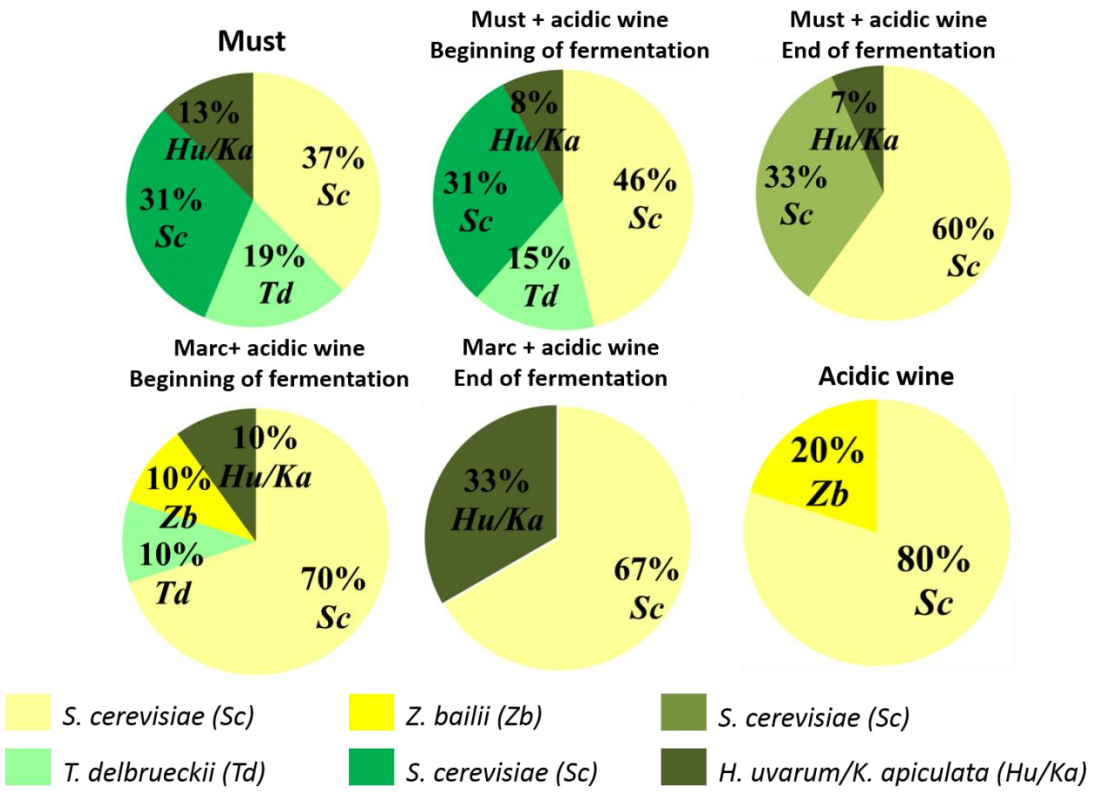

Figure 2. Percentages of the different yeast colonies on WL medium isolated from samples of Cumieira winery and from the refermentation conditions and time points indicated. Colony morphologies are described according to Pallmann et al. [26].

Table 1. Chemical characteristics of the acidic wine, musts and marc used in the refermentation processes and of the final refermented wines after 168 h (refermentation with musts) or 96 h (refermentation with marc).

		Musts and marc					Refermented wines					
Chemical characteristics	**Acidic wine**	**A (must)**	**B (must)**	**C (must)**	**D (must)**	**E (marc)**	**A**	**B**	**C**	**D**	**E**	**Analytical methods***
Titratable acidity (g/l tartaric acid)	6.5	10.85	10.83	10.30	9.69	7.88	6.85	7.83	7.30	7.69	7.57	Titration with bromothymol blue
pH	3.73	3.80	3.70	3.70	3.60	3.55	3.66	3.38	3.51	3.35	3.53	Potentiometer
Density at 20°C	0.9915	1093	1090	1093	1082	1036	0.993	0.994	0.994	0.996	0.998	Densitometry
Free SO_2 (mg/l)	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	0	3.0	4.0	Ripper Method
Total SO_2 (mg/l)	16.71	n.d.	n.d.	n.d.	n.d.	n.d.	11.0	22.5	41.7	110.9	104.6	Ripper Method
Volatile acidity (g/l acetic acid)	1.36	n.d.	n.d.	n.d.	n.d.	n.d.	0.70	0.63	0.63	0.70	0.60	Destillation using a Cazenave-Ferré followed by titration with phenolphthalein
Alcoholic degree (%) ethanol (v/v)	12.2	-	-	-	-	7.2	12.7	12.1	12.7	11.6	11.5	Distillation
Sugar (g/l)	n.d.	215	210	215	192	76	0.0	0.0	0.0	0.0	0.0	Lane-Eynon Method
Estimated alcohol content (%, v/v)	-	12.5	12.0	12.5	12.0	-	-	-	-	-	-	Refractometry

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n.d. – Not determined.
Wineries: A - Universidade de Trás-os-Montes e Alto Douro (UTAD); B - Borges; C - Vila Real; D/E - Cumieira

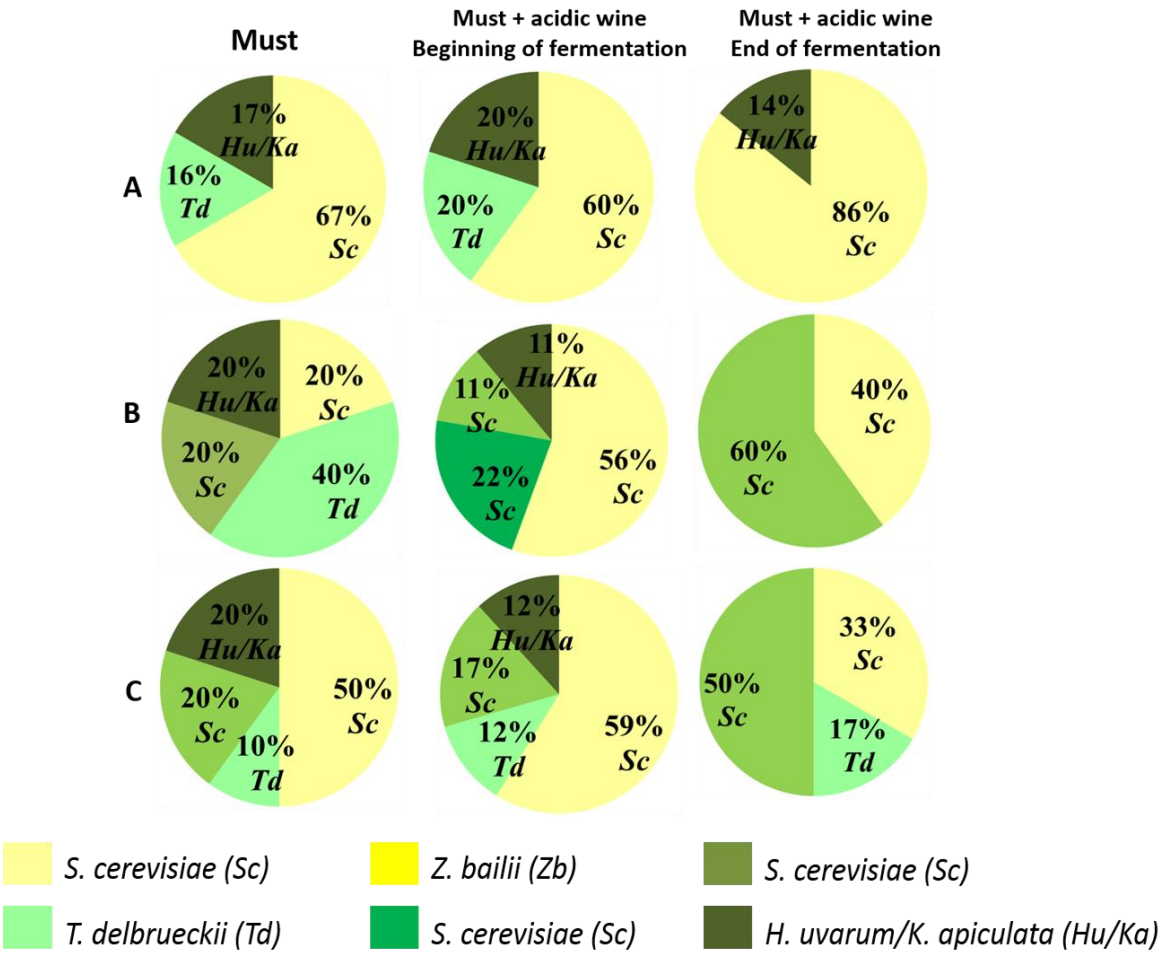

Figure 3. Percentages of the different yeast colonies on WL medium isolated from samples of Borges (A), UTAD (B) and Vila Real (C) wineries and from the refermentation conditions and time points indicated. The acidic wine used is the same represented in Fig.2. Colony morphologies are described according to Pallmann et al. [26].

Selection of yeasts isolates with ability to consume acetic acid in the presence of glucose on a *Zygosaccharomyces bailii* modified differential medium

We then assessed the ability of the 135 yeast isolates to consume acetic acid in the presence of glucose. To this end we used a minimal mineral medium [27] containing glucose and acetic acid (mixed-substrate medium) as the only carbon and energy sources. The pH indicators bromocresol green and bromocresol purple with a transition pH range of 3.8 (yellow/green) – 5.4 (blue) and 5.2 (yellow) – 6.8 (purple), respectively, were also incorporated in the medium at pH 4.0 and 6.0, respectively. Simultaneous consumption of the sugar and the acid was evident by a color change of the medium, in the area surrounding the colony. An isolate exhibiting co-utilization of glucose and acetic acid leads to a higher pH of the culture medium comparatively to another one which displays sequential utilization of glucose and acetic acid. The former isolate will originate a color change from green to blue in the medium with bromocresol green, and a more pronounced purple color in the medium with bromocresol purple [23]. Only four isolates (30C, 43C, 44C and 45C) alkalized the mixed substrate media. This was more evident in the medium with bromocresol green (Figure 1-II) than with bromocresol purple (Figure 1-III), probably due to the higher initial medium pH (6.0). In this second medium, the same isolates showed a slightly purple color (positive result)

while the other strains exhibit a pale yellow color, indicative of acidification and, therefore, of predominant consumption of glucose rather than acetic acid. The four yeast isolates were all collected from the refermentation with marc (Cumieira winery). Isolates 30C, 43C and 45C showed a creamy color in WL medium and were identified as *S. cerevisiae*, considering also that they did not grow in Lysine medium. Isolate 44C was intense green, and hence, according to Pallman et al. [26], a non-*Saccharomyces* species. This classification was in agreement with the strain`s capacity to grow in a medium containing lysine as nitrogen source. Strain 30C was present from the beginning of the refermentation process while the other three strains, 43C, 44C and 45C, were only detected at the end of the process.

The commercial *S. cerevisiae* strains QA23 and EC 1118 were also tested and displayed a change in color to blue (alkaline) in the mixed substrate medium containing bromocresol green after 72 hours of incubation (Figure 1-II) but not in the medium with bromocresol purple. In the medium with bromocresol green (Figure 1-II) *S. cerevisiae* strain PYCC 4072, together with other yeast isolates (26C, 27C, 29C and 42C) did not cause a change in color of the medium. These same strains displayed an identical behavior in the medium with bromocresol purple though their colonies kept a yellowish color, in contrast to 30C, 43C, 44C and 45C which displayed a more pronounced purple color.

Molecular characterization of the isolates 30C, 43C, 45C and 44C

The most widely used typing methods of yeast flora in spontaneous and inoculated fermentation include karyotyping by Pulsed Field Electrophoresis [31], Restriction Fragment Length Polymorphism (RFLPs) analysis of mitochondrial DNA (mtDNA) [32, 33], Randomly Amplified Polymorphic DNA (RAPD) PCR fingerprinting followed by enzymatic restriction of amplified DNA [34], PCR-amplification of interdelta sequence analysis [29, 35] and multi locus sequence typing – MLST [36]. All these methods allow discriminating between species or strains [37]. RAPD fingerprinting with primer T3B, targeting the tDNA regions has been successfully used for species or genera discrimination [30, 38]. This technique was used to characterize the four isolates, which displayed ability to consume simultaneously glucose and acetic acid. Strains 30C, 43C and 45C showed a fragment profile that was similar to the one obtained for the control strain *S. cerevisiae* PYCC 4072 (Figure 4). On the other hand, isolate 44C showed a clearly different RAPD profile and it was previous characterized by being a *Lachancea thermotolerans* species [39].

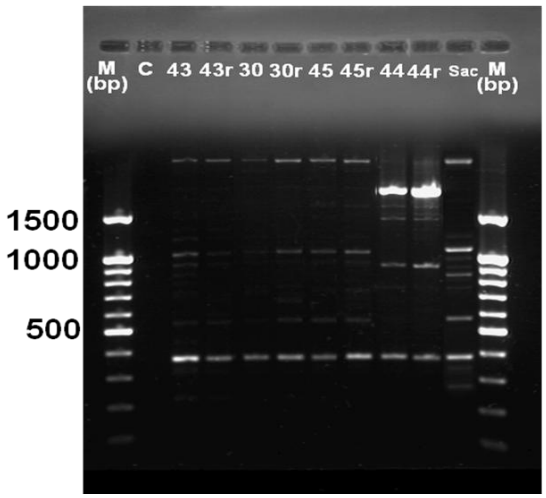

Figure 4. PCR fingerprinting profiles (primer T3B) of isolates 30C, 43C, 44C and 45C with one replica (r). C: control (no DNA); *S. cerevisiae* PYCC 4072 (Sac) and molecular weight patterns (M, bp).

Discussion

The isolation and enumeration of yeasts during alcoholic fermentation of grape must is usually done by plating diluted samples on non-selective media, allowing the growth of all yeast species. In a subsequent step selective or differential culture media can be used to isolate specific species. For instance Lysine (L-lysine) agar [28] is used to selectively enumerate non-*Saccharomyces* species while WL medium is a differential medium that allows the distinction

of yeasts from wine fermentations based on colony color and/or topography [26]. This study describes the combined use of the WL medium (Wallerstein Laboratory Nutrient Agar) and a differential medium to isolate yeast strains able to carry a controlled deacidification process of wines with excessive levels of volatile acidity, which ultimately could substitute the refermentation processes carried empirically by the winemakers and overcome the associated disadvantages. For this purpose refermentations of an acidic wine (1.36 g/L of acetic acid) with musts or marc were performed in four different wineries, and samples harvested along refermentation were plated on WL medium after appropriate dilutions. This medium allowed to discriminate four different species, namely *S. cerevisiae*, *Hanseniaspora/ Kloeckera* species *T. delbrueckii* and *Z. bailii* based on the color and size pattern and to follow their presence along the refermentation processes carried at the different wineries. Cream and green colonies, indicative of the presence of *Saccharomyces* species, were predominant in all samples analyzed, showing the predominance of this species under refermentation conditions. Their high resistance to stress factors such as ethanol [11], low availability of oxygen [40], yeast killer activity [41] and SO_2 antioxidant and antimicrobial properties [21], may have contributed to the observed predominance. Notably species of *Saccharomycodes ludwigii*, *Schizo-saccharomyces pombe*, *Rhodutorula* species, *Metschnikowia pulcherrima*, *Hansenula anomala*, *Pichia membranefaciens* and *Brettanomyces intermedius* also described as being detected in WL medium, were not found in the samples analyzed. However, it must be stressed that this colony type-based classification of the isolates is not conclusive and requires further molecular characterization. Indeed isolate 44C, which displayed an intense green color associated with a flat surface (smooth, opaque and with consistency of butter), classified as a *Hanseniaspora/Kloeckera* species according to Pallmann et al. [26] colony type description, was identified as *Lachancea thermotolerans* [39].

Following the first isolation step in WL medium, 135 yeast isolates randomly selected by their diversity in color and size in WL medium were further tested for their ability to consume acetic acid in the presence of glucose. This capacity would be a desirable characteristic for a controlled refermentation process with must where sugar concentration may be as high as 150 g/L. This specific physiological trait is not generalized among most yeast species due to the glucose catabolic repression phenomena that inhibits the consumption of non-fermentable carbon and energy sources until glucose or other repression inducing sugar is available in the culture medium. Indeed, growth of *S. cerevisiae* PYCC 4072 in a medium containing glucose and acetic acid displays a diauxic growth with consumption of acetic acid only after glucose exhaustion [42, 43]. This behavior is also described for other yeasts species like *Candida utilis* [44], *Torulaspora delbrueckii* [45], and *Dekkera anomala* [46]. In contrast, *Z. bailii* ISA 1307 displays a biphasic growth in a medium containing a mixture of glucose and acetic acid; the first phase is associated with a simultaneous consumption of glucose and acetic acid and the second with the utilization of the remaining acid [47]. However, in chemostat cultures of *S. cerevisiae* grown in mixtures of glucose and acetic acid, cometabolism of the two substrates has been shown [48]. More recently we showed that indigenous *S. cerevisiae* strains, some of which actually selected in the present study, as well as commercial wine strains, are also able to consume acetic acid in the presence of glucose [24, 39].

A differential medium for the detection of the spoilage yeast *Zygosaccharomyces bailii* has been developed by Schuller et al. [23]. The design of this culture medium was based on the ability of yeast species to grow in a mineral medium with glucose and formic acid (mixed-substrate medium) as the only carbon and

energy sources supplemented with an acid-base indicator. By manipulating the concentration of the acid and the sugar it was possible to select conditions where only *Z. bailii* strains give rise to alkalization, associated with a color change of the medium, due to simultaneous consumption of the acid and glucose. Therefore, we sought to explore a modified version of this medium, in which formic acid was substituted by acetic acid, to screen for strains able to metabolize the acetic acid in the presence of glucose. The well-established pattern of acetic acid consumption in the presence of glucose by *Z. bailii* 1307 and *S. cerevisiae* PYCC 4072 led to use the strains as positive and negative controls, respectively. This adaptation of the differential medium developed by Schuller et al. [23] allowed us to select four yeast isolates (30C, 43C, 44C and 45C) with ability to consume acetic acid in the presence of glucose, including *S. cerevisiae* strains. Finally, the approach employed in the present study in combination with molecular methods like PCR fingerprinting, and using type strain DNA fingerprints, retrieved the identification of the four yeast isolates relatedness. Previous studies with the three isolates of *S. cerevisiae* (30C, 43C, and 45C) and the one of *L. thermotolarans* (44C) showed that these strains are adequate to carry a controlled biological deacidification process of musts and wines with high volatile acidity [24, 39].

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