Competition between Spontaneous and Commercial Yeasts in Winemaking: Study of Possible Factors Involved

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Due to the importance of carrying out safe fermentations and obtaining wines with similar and adequate characteristics, the use of starter cultures is very common in Enology. The present report includes the studies carried out in order to know the use of starter cultures and their implantation as well as different factors that determine the competition between wild and commercial inoculated yeasts. For that purpose, the following works took place: (i) determination of implantation levels of commercial preparations during alcoholic fermentation by molecular techniques and study of yeasts biodiversity in the process. It was observed that only 40 % of cases had an efficient yeasting. Results agreed with the ones found in co-fermentations at laboratory scale (ii) characterization of the most competitive dry and wild yeasts regarding to vitality, intracellular carbohydrate content and oenological characteristics. Results showed that in general, physiological conditions of spontaneous strains were better (iii) analysis of amino acids consumption from must for both kind of yeasts, being observed that in the majority of cases, the wild ones assimilated lower quantity of nitrogen compounds.

Keywords wild strains; commercial yeasts, winemaking

1. Introduction

Traditionally, alcoholic fermentations have been carried out spontaneously by wild yeasts present in the grape musts; nowadays, however, in view of the growing interest in wine quality and flavour, the trend in winemaking has changed, producers preferring to control the must biota by selected starter usage. Consequently, a unique strain, known and controlled, carries out the fermentation.

Despite its interesting advantages, there is controversy about starter usage. Some scientists think that selected strain inoculation significantly suppresses wild yeast development [1] and leads to a loss of wine aroma complexity. On the other hand, other authors state that using starters favours the wild *Saccharomyces* strains development, as they may inhibit non-*Saccharomyces* growth [2]. Some studies show that commercial strains compete with wild strains but do not inhibit them completely until several days after the process has started. [3,4]. Nevertheless, the inoculation of musts using selected *Saccharomyces* strains does not ensure their dominance at the end of fermentation.

In any case, the necessity of ensuring the success of fermentation has led to a progressive increase in the use of starters, as they guarantee product uniformity, despite causing the loss of some complexity in the final product [5]. Starters, however, would not display any of their advantages if the cells did not present proper physiological conditions for fast growth. The time necessary to start the fermentation depends on cell activity and factors such as storage conditions, rehydration stage or characteristics of the must [6]. The rehydration stage, in which the active dry yeasts recover the water lost in their industrial production, is essential to obtain functional cells able to ferment the musts efficiently.

Success in commercial yeast implantation depends mainly on factors such as the dose of inoculum used, rehydration conditions or the must temperature when the starter is inoculated. Other factors, however, directly influence cell viability and therefore their capacity to compete with natural must biota. These are principally: intracellular content of reserve carbohydrates, nitrogen and vitamin content in musts, presence of survival factors and fermentation temperature.

Little information can be found about starters' implantation percentages for industrial fermentations and whether there are competence or synergy phenomena between commercial and wild yeasts. This research therefore seeks to discover the behaviour of commercial strains during winemaking and to evaluate the impact of certain factors on the dominance of these starters, such as enological practices, strain vitality, stress tolerance and nitrogen requirements.

2. Materials and Methods

2.1 Sampling and yeast isolation

Samples were obtained from 41 vats directly inoculated with 27 different commercial strains from 18 wineries in La Mancha region (Spain). They were collected at two different stages of fermentation: beginning (density : 1070–1080 g/mL), and middle of the process (density: 1040 g/mL), All of them were growth in YPD medium (1% yeast extract, 2% peptone, 2% glucose) during 48 h at 28 ºC. Colonies from countable plates were replicated on lysine agar to select

those belonging to the genus *Saccharomyce*s. After comparison with lysine agar plates, 20 *Saccharomyces* isolates were randomly selected from each YPD agar plate, thus yielding a total of 1640 pure cultures.

2.2 Genetic characterisation

Yeasts identified as *Saccharomyces* were characterized to the strain level using restriction analysis of mitochondrial DNA [7]. DNA (10 µL) was digested with *HinfI* endonuclease (Boehringer Mannheim). This technique was also applied to each commercial strain used as a starter for their use as markers.

Non-*Saccharomyces* yeasts were identified by PCR–RFLP (polymerase chain reaction/restriction fragment length polymorphism) with ITS primers amplifying variable and intergene regions (ITS1 and ITS4) of the 5.8S rDNA gene. For species identification of yeasts, amplification products were digested with restriction enzymes *HinfI*, *CofI* and *HaeIII* (Boehringer Mannheim GmbH).

All the amplification products and the restriction fragments obtained in both techniques were separated by electrophoresis on 1.5% agarose gel with added ethidium bromide (0.5 µg/mL). Gels were visualised in a UV transilluminator.

2.3 Co-fermentations

In order to determine whether the behaviour of wild yeasts versus commercial ones was the same as observed in winery fermentation tanks, co-fermentations were performed at laboratory scale. Wild strains accounting for over 30% of all strains isolated in a sample were considered dominant strains and were isolated. A total of 15 wild yeasts and 11 commercial strains were used. Assays were carried out by co-inoculating 10^6 cfu/mL of each wild yeast with 10^6 cfu/mL of the commercial yeast with which it co-existed in the winery vat.

A commercial white must was inoculated with chosen strains, and fermentation was carried out in anaerobic conditions at 28ºC until constant weight. Sampling was done during the exponential growth phase and when fermentation was complete. The implantation of the yeasts inoculated was studied by restriction analysis of the mt-DNA, following the method explained in 2.2

2.4 Evaluation of yeast vitality

An indirect technique was employed to evaluate the physiological activity of the yeasts. It was based on the measurement of variations in impedance in a 0.2% KOH solution, caused by absorption of the CO_2 produced during fermentation [8], with a µTrac 4200 impedance analyser (SY-LAB Instruments). A test tube containing 5 mL YNB (Difco™ Yeast Nitrogen Base) supplemented with sugars and acids was inoculated with 10^7 cells/mL of each yeast preculture and incubated at 20 ºC. In all cases, detection time was taken as the time by which impedance variation reached 20%.

In order to examine the effect of co-fermentation on fermentative activity, tests were repeated by inoculating each wild strain together with its corresponding commercial counterpart at a concentration of $0.5*10^7$ cells/mL in each case. For data normalisation purposes, a reference strain (W27) was used in all tests.

2.5 Trehalose and glycogen intracellular content

The 35 studied strains were cultured on YPD medium for six days at 28ºC, and shaken at 160 rpm. Cells were harvested and dried at 105 ºC to constant weight [9]. Trehalose and glycogen levels were measured by enzymatic digestion [10].

2.6 Amino acid consumption

Consumption of 20 amino acids was analysed by high performance liquid chromatography (HPLC) after derivatisation with DEEMM (diethyl ethoxymethylenemalonate). Amino acids were derivatised following [11]. Three wild *Saccharomyces* strains (W16, W34 and W35) and their two commercial counterparts (C5 and C11) were used [4]. These wild strains were selected according to their domination in both winery and laboratory fermentations. Amino acid uptake was analysed in single fermentation and mixed cultures. In addition, amino acid necessities were evaluated in two wine strains in their both fresh and dry format.

3 . Results and discussion

3.1 Implantation of starters in fermentation tanks

After isolating and obtaining yeasts pure cultures, it was observed that, as expected, most of the non-*Saccharomyces* yeasts appeared at the beginning of fermentation. All of them were identified by PCR-RFLP, displaying different

species of *Candida*, *Pichia*, *Hanseniaspora* and *Kluyveromyces* in the first stages, whereas *Zygosaccharomyces fermentati* and *Toruslaspora sp* were able to survive until the middle or even the end of the process.

It can be observed that the commercial yeasts displayed predominance between 80% and 100% in almost 40% of the vats, indicating effective yeasting. In 38% of them, however, less than 50% of genetic profiles matched those of the yeast used, which in some cases had been wholly displaced by wild strains (Fig. 1). This has also been reported by other authors [2, 12]; nevertheless, other studies found the commercial yeasts were responsible for the fermentation although they did not suppress significant development of natural strains during the first stages [7, 13].

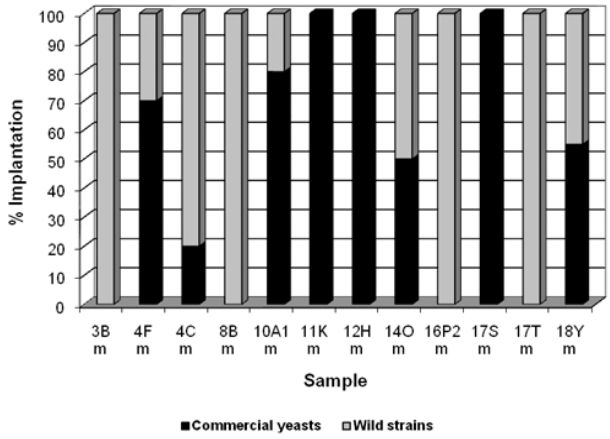

Figure 1. Biodiversity of *Saccharomyces* strains in inoculated vats in the middle of fermentation

The implantation of commercial strains was generally better in white musts than in red ones, probably owing to the wine-making process itself, where maceration with grape skins increases the quantity of yeasts in the must competing with the starter at the beginning of the fermentation.

The study of *Saccharomyces* strain biodiversity showed a different population dynamic and biota for each fermentation: some commercial yeasts competed with one or several dominant wild strains, which were isolated over 30%; in other musts, the inoculated yeast was completely displaced by only one wild strain at mid-fermentation; for some fermentations a wide variety of wild yeasts was found, none of them dominating the medium.

In those cases in which the commercial yeast was unable to predominate in the fermenting must inadequate practices of rehydration and inoculation of the strains, or certain physical-chemical characteristics of the must, which proved highly stressful for the yeasts, may be the cause. This is the case of using lower doses of inoculum than the ones recommended by the manufacturer, low assimilable nitrogen content in the must, or excessively low inoculation temperatures. These factors led to non-optimal physiological conditions of the starter for competing with the wild biota, causing its growth inhibition by other strains better adapted to the enological environment. Nevertheless, ineffective starter implantation was also observed in some fermentation processes despite the use of correct winemaking practices.

Against this backdrop, it was decided to test the ability of the wild yeasts isolated from fermenting vats (over 30% presence) [4] to compete with commercial strains at laboratory scale and to study certain properties of the yeast used. Fifteen co-fermentations were carried out following the methodology explained in material and methods (section 2. 3).

A total of 421 *Saccharomyces* isolates were obtained and characterised by mtDNA analysis. The comparison of the commercial and wild strain genetic profiles allowed us to discover their implantation percentages. The wild strain dominance in both winery vats and laboratory assays is reported in Table 1.

It may be observed that three wild strains (W16, W31, W33) which completely dominated the industrial fermentation were also predominant under the controlled conditions in the lab; nine of them (W17, W18, W19, W22, W26, W28, W30, W32, W34, isolated over 30% in the vat, had an effective implantation in co-fermentations and finally only three (W27, W29, W35) were unable to displace the commercial yeast again. In some instances, the same starter was displaced by different wild strains, showing a priori, better physiological conditions than the commercial ones. Implantation percentages obtained in the lab are in agreement with the ones observed in wineries during vinification processes.

Table 1. Comparison of the implantation percentages displayed by wild yeasts in both fermentation vats and the mixed cultures at laboratory scale

Co-fermentation	Implantation		Co-fermentation	Implantation	
	Winery	Lab		Winery	Lab
M2 (C11-W16)	100	100	M15 (C13-W29)	30	50
M3 (C11-W17)	40	100	M16 (C7-W30)	60	90
M4 (C6-W18)	50	100	M17 (C9-W31)	80	100
M5 (C6-W19)	40	100	M18 (C5-W32)	60	100
M8 (C2-W22)	30	100	M19 (C4-W33)	80	100
M12 (C10-W26)	50	100	M20 (C5-W34)	40	100
M13 (C12-W27)	40	50	M21 (C5-W35)	30	40
M14 (C14-W28)	30	100			

3.2. Competence of wild and commercial strains: some factors involved

3.2.1. Vitality and reserve carbohydrate content

Vitality is described as the measurement of physiological activity and may be an important variable than can explain the low imposition of some of the starters studied. There are many factors affecting yeast vitality such as dehydration [14], storage [15], and rehydration [16] in the case of active dry wine yeast, as well as cell integrity or physiological characteristics [17]. Detection times ranged from 0.67 to 1.80 hours and from 0.70 to 1.39 hours for commercial and wild strains, respectively. Generally wild yeasts exhibited significantly higher vitality (lower detection time) than commercial ones in identical conditions. Figure 2 shows the detection time of wild and commercial yeasts in both pure and co-inoculated cultures.

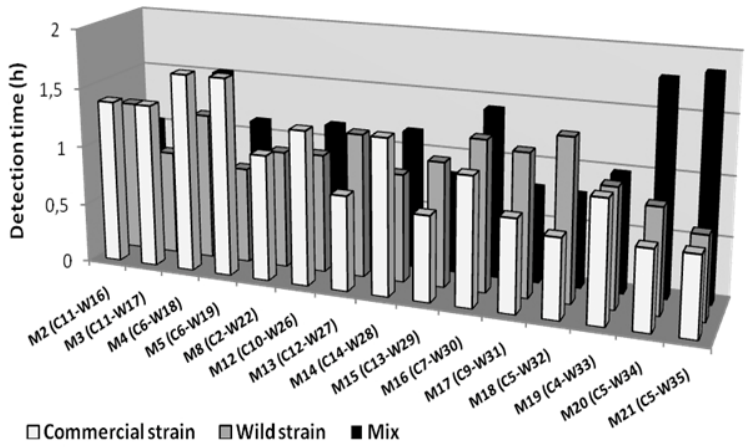

Figure 2. Vitality of commercial and wild yeast strains, and their co-fermentations, expressed as detection time (hours)

On the other hand, it was interesting to observe the effect of co-fermentations in the process kinetics: vitality was determined when both yeasts were inoculated at the same concentration. Some mixed cultures showed shorter detection times, proving synergy among the cells. In other samples, however, antagonism was seen, leading to a slower process compared with the fermentations where the pure cultures were inoculated. In the remaining samples the competence of both strains led to detection times halfway between the single fermentations.

The study of other enological characteristics revealed the adaptation of both commercial and wild yeasts to the fermenting musts, as they proved resistant to SO_2, high ºBrix values and the presence of killer toxin.

Technological processes like winemaking itself or desiccation are highly stressful for yeasts, affecting their fermentative activity. The cell survival in those conditions is closely related to the intracellular content of reserve carbohydrates (trehalose and glycogen). The stress resistance of both commercial and wild yeasts was evaluated by studying the capacity to accumulate these carbohydrates. Statistical analysis revealed significant inter-strain differences among the strains in the production of these two sugars, results which accord with data shown by [18].

Figure 3 shows the intracellular content of trehalose (a) and glycogen (b) in the studied strains; it may be observed that commercial strains accumulated higher concentrations of both sugars. This fact may be explained by the industrial production process in which the starters are pushed to accumulate reserve carbohydrates to face the desiccation stage. Despite having higher stress protection, however, they displayed lower vitality. Therefore, the starter's low competence should be attributed to different factors.

The function of these two carbohydrates in cellular viability has been studied [19], finding that trehalose and glycogen start to accumulate after nitrogen depletion [20] and they help the cell to survive longer during the stationary phase. However, the highest growth rate is correlated with the lowest trehalose concentration [21].

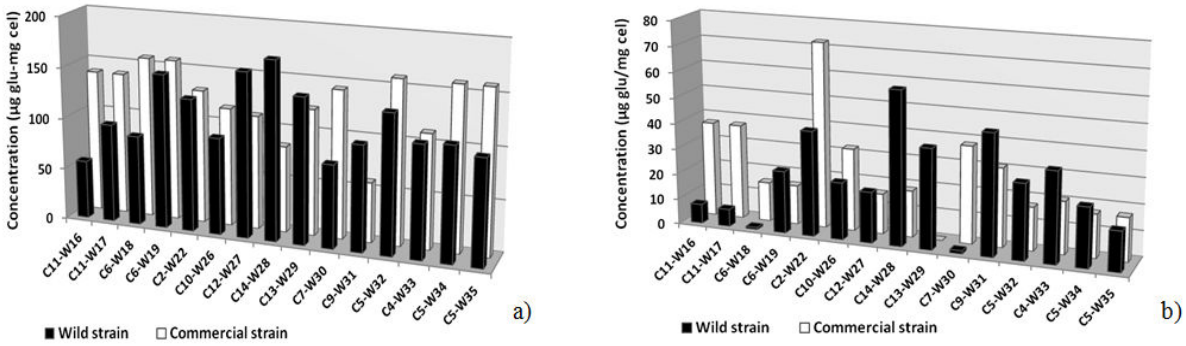

Figure 3. Intracellular concentration (µg/mg cel) of trehalose (a) and glycogen (b) in commercial and wild strains.

3.2.2 Nitrogen consumption

Nitrogen content in musts is a decisive variable for the yeast's metabolism and for this reason the addition of exogenous nitrogen sources is common practice in wineries. Nevertheless, it is still possible not to reach optimal levels for cellular activity so the yeast fails to dominate the fermentation. Therefore it is interesting to ascertain the strains' nitrogen requirements and include this parameter in the selection process of starters. Selecting yeasts with low nitrogen necessities would be a good alternative to nitrogen addition to the musts, which may be excessive and lead to sensorial deviations in the wine.

The next step in this research work was to study the amino acid consumption of wild and commercial *S.cerevisiae* strains, in pure and mixed cultures, throughout the fermentation of a synthetic must with high ammonium concentration. In addition, it was observed how industrial drying affects the amino acid requirements of two yeasts. Three wild strains were chosen from those which proved dominant in both winery and lab, together with their two commercial counterparts. Single fermentations and co-fermentations were carried out, sampling being performed in the exponential phase and at the end of the stationary stage, in order to quantify the amino acids metabolised by high-performance liquid chromatography.

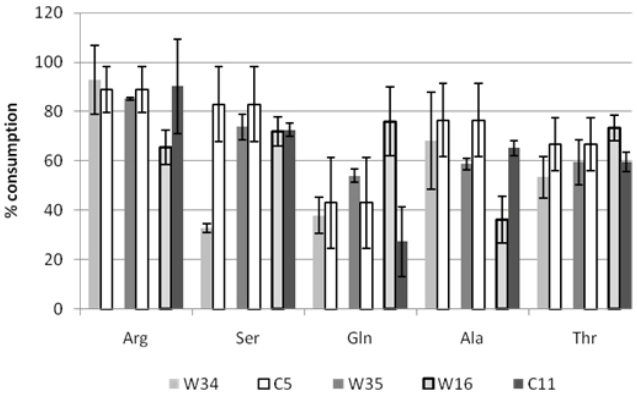

Figure 4. Comparison of the amino acid uptake of each couple of yeast inoculated. Only five of the most consumed amino acids are shown

The results show that nitrogen consumption is strain-dependent, differences being more quantitative than qualitative, as also reported by [22] and [23]. Figure 4 shows the uptake of some amino acids analysed in the strains inoculated. The

variance analysis only showed statistically significant differences in the uptake of seven out of the twenty amino acids analysed. The most consumed ones were, in all cases, arginine, phenylalanine, serine, leucine, lysine and methionine. Commercial strains were observed to have higher requirements of fifteen amino acids, which makes them less competitive in fermenting musts with low nitrogen content.

On the other hand, the analysis of the nitrogen uptake in the three co-fermentations carried out did not show any clear trend, finding synergic, antagonist and competitive behaviours. For example, in one of the co-fermentations eleven amino acids were consumed in lower quantity than in single fermentations.

The ratio between the uptake percentage of each amino acid and its initial concentration shows the yeast preference for the amino acid assimilation. Cysteine, tyrosine, glycine and alanine were consumed in similar proportion to arginine or glutamic acid in spite of being in much lower concentration, therefore showing a great affinity for them.

The fact that wild yeasts showed lower nitrogen requirements led us to study the amino acid metabolism of two *S.cerevisiae* strains (1 and 2) in their fresh and dry formats. Principal component analysis and its plane representation allowed to separate fresh from dry yeasts by a lower uptake of valine, glutamic acid, leucine, glutamine, aspartic acid, isoleucine, tryptophan, threonine and cysteine. Therefore, it should be noted that the industrial drying of yeast strains may increase some amino acid requirements.

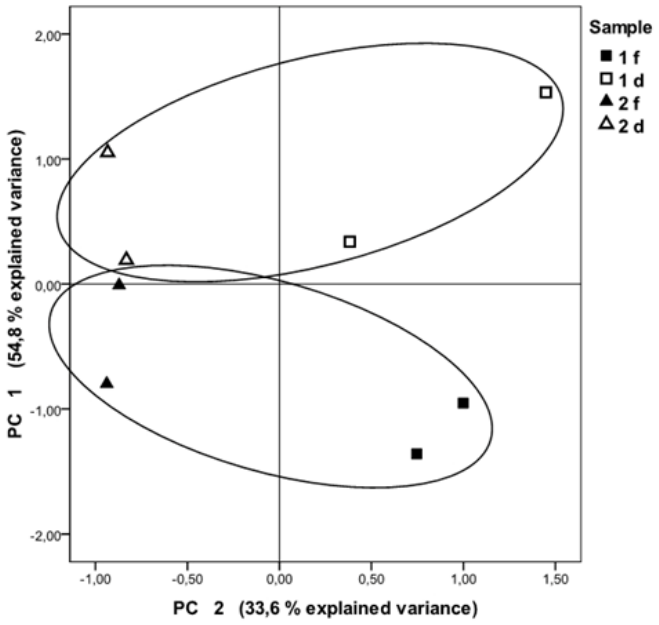

Figure 5. Representation of Principal Component Analysis based on amino acid consumption of fresh and dry formats of strains 1 and 2.

4. Conclusions

The general conclusions of the present work show that the inoculation of tanks with dried yeasts does not assure its implantation during fermentation process, probably due to lower doses than recommended, thermal shock, osmotic stress or long rehydration times, although sometimes, the yeasting is also inefficient in spite of an adequate manipulation of the commercial yeast. On the other hand, co-fermentations at laboratory scale verified results found in fermentation tanks, even increasing the predominance of wild yeasts. It could be explained by vitality values, since the 70 % of spontaneous yeasts present higher vitality when they are inoculated at same proportion than commercial strains. In spite of having higher intracellular trehalose content commercial yeasts displayed lower vitality and implantation percentages. Regarding to nitrogen consumption, the utilisation of amino acid shows that the requirements are strain dependent so the selection of yeast with low nutritional requeriments is advisable for fermenting poor musts. In general, commercial preparations consume more amino acids than wild and fresh strain.

In any case, the use of starters in enology is advisable as they assure fast and secure fermentations despite of coexisting with wild yeasts. This fact might be favourable since wild biota may contribute to enhance wine complexity and produce different wines.

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