Chapter

Biodiversity of *Saccharomyces cerevisiae* Yeasts in Spontaneous Alcoholic Fermentations: Typical Cellar or Zone Strains?

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Abstract

Spontaneous fermentation is the most traditional way and a low-intervention method for conducting alcoholic fermentation in wineries, giving rise to the most complex wine profiles. However, inoculation with single culture inocula of *Saccharomyces cerevisiae* strains has become widespread in the modern wine industry. Nevertheless, some authors have pointed out that the use of the same yeasts in all the winegrowing regions of the world can cause a loss of typicity and have a standardizing effect on the wines. For this reason, many wineries and regions are carrying out programs of isolation and selection of yeasts that are typical of their vineyards/wineries. The aim of this work was to study the ecology of spontaneous fermentations in 11 wineries from all over the Rioja qualified designation of origin (Spain) during 3–4 consecutive years in order to establish the existence of typical strains belonging to wineries, sub-zones, or regional ecosystems. The results obtained showed a great diversity of strains of *Saccharomyces cerevisiae* in each fermentation studied. These strains were different each year in each winery, and hardly any common strains were detected between neighboring wineries, which would indicate that there are no representative strains from the winery or the area.

Keywords: Rioja qualified designation of origin, alcoholic fermentation, *saccharomyces cerevisiae*, diversity, ecology, typical strains

1. Introduction

Spontaneous fermentation is the most traditional way and a low-intervention method for conducting alcoholic fermentation in wineries, giving rise to the most complex wine profiles. This complexity develops because of the large number of different yeast species involved (*Saccharomyces* spp. and non-*Saccharomyces*) [1]. However, the presence of unknown microbiota makes it a risky and unpredictable practice. For this reason, the inoculation with single culture inocula of *Saccharomyces cerevisiae* has become widespread in the modern wine industry to reduce the risk of wine spoilage. Nevertheless, some authors have pointed out that the use of the same

yeasts in all the winegrowing regions of the world can cause a loss of typicity and have a negative effect on the biodiversity of natural yeasts present in the wineries [2].

S. cerevisiae is the predominant yeast species in alcoholic fermentation and the main element responsible for the characteristics of the wines. Many surveys carried out with spontaneous fermentations in different wine-producing regions have demonstrated that there is high genetic diversity within this species in each vinification [3]. However, in most cases, only a small number of strains of *S. cerevisiae* are dominant, mainly in the tumultuous and final fermentation stages, representing a high percentage out of the total number of strains identified.

Earlier studies have shown that some strains of *S. cerevisiae* have been isolated in several consecutive years in the same winery, which is why some authors have suggested the term "winery effect" [4], and also some strains of these species were detected in different wineries of the same wine-producing area, suggesting that they were representative of a specific enological ecosystem [5, 6]. Knight et al. [7] even found specific genotypes from a particular region. These findings suggest that specific native *S. cerevisiae* strains could be associated with a *terroir* and have an influence on terroir-associated wine characteristics [8].These authors found a correlation between genotypic and phenotypic groups and the geographical origin of the strains, supporting the concept that there can be a microbial aspect to *terroir*.

Nowadays, many wineries and regions are carrying out strain selection programs with yeasts isolated from their vineyard/winery ecosystems, based on the idea that these yeasts are better adapted to their musts, which have characteristics determined by the grape varieties and the *terroir* [9, 10]. Thus, the use of these typical strains as starter yeasts could provide wines with distinctive characteristics of a particular winery or region. For this reason, studying the existence of strains which are specific to one winery or enological area is very interesting for the wine industry [11].

Rioja is a wine region in Spain with qualified designation of origin status. It is subdivided into three sub-zones: Rioja Alta, Rioja Oriental, and Rioja Alavesa. Rioja Alta is located on the western edge of the region and at higher elevations with an Atlantic climate. Rioja Oriental, the eastern section, is strongly influenced by a Mediterranean climate which makes this area the warmest and driest part of the region. Rioja Alavesa, with a similar climate to Rioja Alta, produces different wines due to the relatively poor condition of the soil. Each sub-zone has its own character, which results in different wines derived from the different compositions and origins of the soils and the climate conditions.

The study of the ecology and biodiversity of the yeast population during alcoholic fermentation is an interesting and important step in the research into, and understanding of, a winegrowing area and should be a step prior to the selection and subsequent employment of the yeasts isolated from that area as starters. The aim of this work was to study the ecology of spontaneous fermentations in 11 wineries from all over the Rioja Designation of Origin during 3–4 consecutive years in order to establish the existence of typical strains belonging to wineries, sub-zones, and regional ecosystems. The wineries under study were distributed throughout the three sub-zones of the Rioja designation of origin.

2. Study on the ecology of S. *cerevisiae* in the Rioja qualified designation of origin

2.1 Material and methods

2.1.1 Sample collection

Samples were taken from 11 wineries (A–K) located in three different sub-zones of the Rioja designation of origin (Rioja Alta, Rioja Oriental, and Rioja Alavesa)

over a period of 3 or 4 consecutive years (**Figure 1**). None of the wineries studied had ever used a commercial starter yeast. Wineries A and B were new; wineries C, E, and K were about 20 years old, while the others were over 50 years old.

Alcoholic fermentation (AF) was carried out by the destemming and crushing method in stainless steel (wineries A, B, C, D, and F) or wooden vats (winery H). In the other wineries, AF was carried out following the traditional carbonic maceration method (whole grape) in open concrete vats (wineries E, G, I, J, and K). The wines underwent spontaneous AF with the indigenous microbiota in all cases.

In each winery, one fermentation tank was monitored in each year studied. The sampling was carried out 24 hours after vatting, in tumultuous AF (density 1025 g/L) and final AF.

2.1.2 Microbial analyses: strain typing of S. cerevisiae

Samples collected in sterile bottles were taken to the laboratory and processed as follows: serial decimal dilutions were performed, and the samples were seeded onto plates containing a chloramphenicol glucose agar medium (CGA). The plates were incubated at 28°C for 48 h. Plates containing between 30 and 300 colonies were examined, and 10 colonies were randomly isolated from each CGA plate. The colonies were analyzed in order to identify *Saccharomyces* and non-*Saccharomyces* yeast and the clonal distribution of the *S. cerevisiae* by mitochondrial DNA (mtDNA) restriction analysis. Yeast cells were grown overnight in a culture of 5 ml YPD. DNA extraction and mtDNA restriction were determined by the method described by Querol and Barrio [12]. The DNA was digested with the restriction endonucleases Alu I, RSa I, and Hinf I, in accordance with the supplier's instructions (Boehringer Mannheim). The restriction fragments were separated by electrophoresis in agarose 1% gels and visualized on a UV transilluminator after ethidium bromide staining.

The different clones isolated in each fermentation were named with the letter of the cellar (A–K), followed by a Roman numeral and year of harvest (1–4).

The clonal variability of each fermentation was determined as a percentage of different *S. cerevisiae* genotypes compared to the total colonies *S. cerevisiae* identified.

The index of diversity (I.D.) [13] was calculated with the different *S. cerevisiae* strains identified in the tumultuous and final stages of fermentation, according to the following equation:

$$\text{I.D.} = 1 - \frac{\sum 3\, n_j\,(n_j - 1)}{N\,(N - 1)} \tag{1}$$

where N is the total number of *S. cerevisiae* strains and n_j is the number of *S. cerevisiae* strains with the same electrophoretic profile.

2.2 Results and discussion

2.2.1 Ecology of the yeast population during alcoholic fermentation in Rioja Oriental sub-zone

In this sub-zone, four wineries were sampled (A–D). In all of them, the vinification took place after destemming and crushing the grapes.

As expected, and concurring with previous studies on the ecology of alcoholic fermentation (FA) [14, 15], non-*Saccharomyces* yeasts were detected mainly in the first stages of the fermentation (data not shown). The rest of the yeasts isolated in this sub-zone (316 colonies) were identified as belonging to the *S. cerevisiae* species.

Figure 1.
Location of the wineries in the three different sub-zones of the Rioja designation of origin.

The clonal variability in this species was different depending on the winery and the year studied within each winery (**Table 1**). Thus, while in cellars A and B the total clonal variability was 13.8 and 21.8%, respectively, in the other two (C and D), it was around 35%. Because of the lower variability in the first two wineries, the index of diversity was also smaller.

A percentage of the isolated microbiota in each harvest had already been identified in previous years (**Table 1**). This percentage was higher in wineries A and B, while in the others, this proportion was lower. However, the evolution in the population of the strains during fermentation was similar in all the wineries, since in all of them the fermentation in general was carried out by one or two majority yeasts (**Table 2**) associated with a variable number of minority strains (**Table 3**). This majority strain(s) represented between 50 and 82% of the total isolated in each vinification (**Table 2**). The only exception was found in vintage 2 of cellar C, in which there were three major strains with a percentage of 14.3% each. Previous research on the *S. cerevisiae* population in spontaneous fermentations also showed the existence of a great diversity of strains. Among them, one or two represented more than 50% of the total [16, 17].

Nonetheless, only in wineries A and B, there was one common majority strain in the vinifications studied in the 3–4 years (it is highlighted in the same color in **Table 2**). The fact that the same strains were predominant in successive campaigns in the same winery has already been described in other works [4, 18], for which reason they considered that these yeasts were representative of the winery or the processing area. In contrast, in other studies, common yeasts were not found from one campaign to another or were only found in small proportions [19, 20].

The results obtained could suggest that in wineries A and B, there are yeasts characteristic of the winery that lead the fermentation, in proportions that vary depending on the characteristics of the vintage. However, in the other two wineries, the fermentations were carried out by a succession of strains, among which one or two were majority, but different from one vintage to another.

The main difference between the four wineries studied in the Rioja Oriental sub-zone was their age. While wineries A and B were new (this study began in its third and first harvest, respectively), cellar C had been producing wine for 15 years and D for 50. This means that the latter two had more complex ecosystems, which have been formed over successive harvests, as evidenced by the greater number of clones that participated in the fermentations and the higher diversity index (**Table 1**).

Winery	Years	Isolates of *S. cerevisiae* analyzed	Number of different clones	Clonal variability (%)	Index of diversity	Genotypes detected in previous years	
						Number	%
A	1	29	6	20.7	0.65	–	–
	2	28	7	25.0	0.77	3	71.4
	3	22	4	18.2	0.33	2	86.3
	4	30	6	20.0	0.46	3	83.2
	Total	**109**	**15**	**13.8**	**0.64**		
B	1	–	–	–	–	–	–
	2	20	5	25.0	0.76	–	–
	3	30	9	30.0	0.84	2	36.6
	4	28	8	28.6	0.77	3	63.8
	Total	**78**	**17**	**21.8**	**0.83**		
C	1	–	–	–	–	–	–
	2	21	11	53.4	0.94	–	–
	3	22	8	36.4	0.77	2	13.6
	4	24	11	45.8	0.87	3	45.8
	Total	**67**	**25**	**37.3**	**0.92**		
D	1	–	–	–	–	–	–
	2	20	10	50.0	0.86	–	–
	3	22	5	22.7	0,58	1	9.1
	4	20	8	40.0	0.82	2	10.0
	Total	**62**	**20**	**32.3**	**0.91**		

Table 1.
Clonal diversity of S. cerevisiae yeasts in Rioja Oriental wineries in 4 consecutive years (1–4).

In a later study, carried out by our research group in cellar B [21], 72 colonies of the species *S. cerevisiae* were isolated in three fermentation tanks analyzed. The results showed 41 different clones, which provided a clonal variability of 56.9% and a biodiversity index of 0.98. These data would indicate that the fermentations were carried out by the succession of different clones and that there were no dominant ones. On the other hand, the comparison of the restriction profiles of this year's clones with those identified in the years specified in **Tables 1** and **2** showed that there was no correspondence with any of them, and therefore the majority strain (**Table 2**) that was isolated in the first three vintages as representative of the winery did not appear years later. Therefore, the ecology of the fermentations of this last year in cellar B was similar to those found in cellars C and D in the period shown previously. The situation in winery A could be similar, but it could not be proven since in the following years, commercial yeasts were inoculated, and the study could not be carried out. Taking into account the results shown, the presence of a dominant strain in the elaborations of wineries A and B could be due to the fact that they are new wineries and not to the existence of representative yeasts.

On the other hand, the comparison of the different restriction profiles obtained in the four wineries during the 3–4 years of study showed that only two yeasts were common among the different wineries (**Table 3**). Therefore, the results obtained in this study did not show the existence of typical strain/s of this sub-zone. In the wine

Years	1		2		3		4	
Winery	**Strain**	**%**	**Strain**	**%**	**Strain**	**%**	**Strain**	**%**
A	A-I_1	55.2	A-I_2	42.9	A-IV_3	81.8	A-I_4	73.3
	A-II_1	24.1	A-II_2	21.4				
	Total	**79.3**		**64.3**		**81.8**		**73.3**
B	–	–	B-I_2	35.0	B-I_3	33.3	B-II_4	21.4
	–	–	B-IV_2	35.0	B-$VIII_3$	20.0	B-IV_4	42.3
	Total	–		**70.0**		53.3		**63.7**
C	–	–	C-IV_2	14.3	C-I_3	40.9	C-I_4	33.3
	–	–	C-V_2	14.3	C-IV_3	27.3	C-IX_4	16.7
			C-X_2	14.3				
	Total	–		**42.9**		**68.2**		**50.0**
D	–	–	D-V_2	35.0	D-II_3	63.6	D-I_4	35.0
	–	–	D-VI_2	15.0			D-III_4	25.0
	Total	–		**50.0**		**63.6**		**60.0**

Clones with box in gray within the same winery indicate that they are the same strain, according to their mtDNA restriction profiles

Table 2.
Major S. cerevisiae strains in the fermentations of Rioja Oriental in 4 consecutive years (1–4).

Winery A				Winery B			Winery C			Winery D		
1	**2**	**3**	**4**	**2**	**3**	**4**	**2**	**3**	**4**	**2**	**3**	**4**
A-I_1	A-I_2	A-I_3	A-I_4	**B-I_2**	B-I_3	B-I_4	C-I_2	C-I_3	C-I_4	D-I_2	D-I_3	D-I_4
A-II_1	**A-II_2**	A-II_3	**A-II_4**	B-II_2	B-II_3	B-II_4	C-II_2	C-II_3	**C-II_4**	D-II_2	D-II_3	D-II_4
A-III_1	A-III_2	A-III_3	A-III_4	B-III_2	B-III_3	B-III_4	C-III_2	C-III_3	C-III_4	D-III_2	D-III_3	D-III_4
A-IV_1	A-IV_2	A-IV_3	A-IV_4	B-IV_2	B-IV_3	B-IV_4	C-IV_2	C-IV_3	C-IV_4	D-IV_2	D-IV_3	D-IV_4
A-V_1	A-V_2		***A-V_4***	B-V_2	B-V_3	B-V_4	C-V_2	C-V_3	C-V_4	D-V_2	D-V_3	D-V_4
A-VI_1	A-VI_2		A-VI_4		B-VI_3	**B-VI_4**	C-VI_2	C-VI_3	C-VI_4	D-VI_2		D-VI_4
	A-VII_2				B-VII_3	B-VII_4	C-VII_2	C-VII_3	C-VII_4	D-VII_2		D-VII_4
					B-$VIII_3$	B-$VIII_4$	C-$VIII_2$	C-$VIII_3$	C-$VIII_4$	D-$VIII_2$		D-$VIII_4$
					B-IX_3		C-IX_2		C-IX_4	D-IX_2		
							C-X_2		C-X_4	D-X_2		
							C-XI_2		C-XI_4			

Clones in bold and with box in gray in different columns show the same clones in different cellars

Table 3.
S. cerevisiae clones identified in each elaboration of Rioja Oriental in 4 consecutive years (1-4).

area of Charentes in Cognac, a strain widely distributed throughout the area had been described, which was considered representative of the wine region. But unlike our data, this strain was the dominant one in all the samples where it appeared [18]. Likewise, in other ecological studies, widely disseminated strains were isolated in an area, and it was thought that they may be typical strains of that area [22].

2.2.2 Ecology of the yeast population during alcoholic fermentation in Rioja Alta sub-zone

In this sub-zone, five wineries were sampled. Vinification was by both the destemming and crushing (F and H) and the carbonic maceration methods (E, G, and I).

As happened in the Rioja Oriental, alcoholic fermentation in the Rioja Alta sub-zone was carried out by yeasts of the *S. cerevisiae* species, since the non-*Saccharomyces* group was identified mainly in the early stages of the process. However, in the third harvest of cellars E and F, three colonies belonging to non-*Saccharomyces* genera were isolated in tumultuous fermentation (data not shown). The detection of these yeasts in advanced stages of winemaking had also been reported in other works [23, 24]. In total, 450 yeasts belonging to the *S. cerevisiae* species were studied.

Clonal variability was high in this sub-zone in all the wineries (**Table 4**). From the 450 isolated colonies of *S. cerevisiae*, 177 different clones were identified, resulting in high clonal variability in Rioja Alta, which ranged between 35% in winery H and 39% in winery F, with the exception of cellar I, in which 50% was reached. It is noteworthy that clonal variability depended on the year analyzed, being generally lower the first year and higher the third. These results could be related to the climatological characteristics of the harvest.

The index of diversity was high and similar for all the wineries with a value of 0.95–0.96. These results were due to the high number of different strains that participated in each fermentation, which in the case of wineries F and I was favored by the low presence of common clones in different campaigns within the same winery (**Table 4**). In wineries E, G, and H, although there were more strains that appeared in different campaigns, they did so in a low percentage, and, therefore, the index remained high.

The population evolution in this sub-zone was similar to Rioja Oriental, since the alcoholic fermentation was carried out by different clones during the fermentation process. In most of the vinifications, 1–3 majority clones were detected, representing at least 43% of the population (**Table 5**). However, in this sub-zone certain exceptions were found. Thus, in the first year analyzed in winery F, one clone represented 65% of the total and so was therefore dominant and responsible for the fermentation. This could be due to the special characteristics of this campaign (frosts at the end of April, high rainfall and strong winds in spring, hail storms and low temperatures in summer) that negatively influenced the grape ripening and meant that few yeasts were able to adapt and develop to carry out the fermentation. These conditions were more adverse in winery F, due to its geographic situation. It is the winery located at higher altitude and closer to the mountains.

On the other hand, in wineries G and I, there were vinification processes in which no major clones were isolated, which could be related to the high number of different clones that participated in the fermentations. What these wineries have in common is that they follow the carbonic maceration method and have been making wine for more than 100 years. Santamaría et al. [15] showed that the number of different *S. cerevisiae* strains and the frequency of their appearance varied according to age.

When comparing the clones found in the five wineries (**Table 6**), only pattern I, coming from the last year studied in the four wineries, was common in all of them. When comparing the restriction profiles of the four strains, all of them showed the same electrophoretic pattern (**Figure 2**).

The presence of the same strain in four wineries could be due to the fact that the villages where wineries G, H, and I are located are geographically very close to each other (**Figure 1**). In cellar F, a little further away, a part of the grapes came from the same area as the other three. In this fourth year of study, strong winds were reported by grape growers in September and October. These winds could be responsible for transporting yeasts from one area to another, which would explain the appearance

Winery	Years	Isolates of *S. cerevisiae* analyzed	Number of different clones	Clonal variability (%)	Index of diversity	Genotypes detected in previous years	
						Number	%
E	1	30	10	33.3	0.80	–	–
	2	30	14	46.7	0.93	2	6.6
	3	12	8	66.7	0.91	3	41.6
	4	20	8	40.0	0.87	1	5.0
	Total	**92**	**34**	**37.0**	**0.96**		
F	1	20	5	25.0	0.57	–	
	2	23	10	43.5	0.91	0	0
	3	19	9	47.4	0.85	0	0
	4	20	9	45.0	0.79	1	5.0
	Total	**82**	**32**	**39.0**	**0.95**		
G	1	30	13	43.3	0.93	–	–
	2	23	11	47.8	0.92	0	0
	3	21	12	57.1	0.92	5	57.3
	4	30	12	40.0	0.85	3	40.0
	Total	**104**	**40**	**38.5**	**0.95**		
H	1	20	9	45.0	0.88	–	–
	2	30	13	43.3	0.92	3	36.6
	3	20	11	55.0	0.88	4	50.0
	4	30	13	43.3	0.80	4	23.2
	Total	**100**	**35**	**35.0**	**0.95**		
I	1	–	–	–	–	–	–
	2	22	9	40.9	0.82	–	–
	3	30	20	66.7	0.97	3	13.3
	4	20	11	55.0	0.76	1	5.0
	Total	**72**	**36**	**50.0**	**0.96**		

Table 4.
Clonal diversity of S. cerevisiae yeasts in Rioja Alta wineries in 4 consecutive years (1–4).

of the same strain in the four wineries. This yeast could come from the grapes, since in none of the three previous vintages had this strain been isolated in any of the cellars. This strain dominated the beginning of the fermentations and was later replaced by other indigenous strains, since in no case was it isolated at the end of the fermentation processes. These results would concur with those of Schütz and Gafner [19], who consider that the population of yeasts can be considered dependent on the harvest and the vineyard, and also with Le Jeune et al. [25], who indicated that the populations involved in spontaneous alcoholic fermentation result from a balance between the *S. cerevisiae* strains present in the grape and in the cellar.

The data obtained would show the existence of extensive microbiota in each winery, which, together with the microflora that accompanies the grapes, will develop according to the characteristics of the harvest. The fermentations would be the result of the sequence of different yeasts, and despite having found a widely spread yeast in the sub-zone 1 year, it did not appear in any other winery in the

Years	**1**		**2**		**3**		**4**	
Winery	**Strain**	**%**	**Strain**	**%**	**Strain**	**%**	**Strain**	**%**
E	$E\text{-}I_1$	40.0	$E\text{-}I_2$	13.3	$E\text{-}I_3$	25.0	$E\text{-}I_4$	20.0
	$E\text{-}II_1$	20.0	$E\text{-}IV_2$	16.7	$E\text{-}III_3$	25.0	$E\text{-}IV_4$	25.0
			$E\text{-}XI_2$	13.3			$E\text{-}V_4$	20.0
	Total	**60.0**		**43.3**		**50.0**		**65.0**
F	$F\text{-}III_1$	65.0	$F\text{-}II_2$	13.0	$F\text{-}I_3$	25.0	$F\text{-}I_4$	45.0
			$F\text{-}III_2$	21.8	$F\text{-}V_3$	30.0		
			$F\text{-}VIII_2$	17.4				
	Total	**65.0**		**52.2**		**55.0**		**45.0**
G	$G\text{-}I_1$	20.0	$G\text{-}V_2$	20.0	$G\text{-}I_3$	14.3	$G\text{-}I_4$	33.3
			$G\text{-}VII_2$	15.0	$G\text{-}IV_3$	28.6	$G\text{-}III_4$	20.0
			$G\text{-}VII_2$	15.0			$G\text{-}VI_4$	17.4
	Total	**20.0**		**50.0**		**42.9**		**70.7**
H	$H\text{-}II_1$	25	$H\text{-}III_2$	20.0	$H\text{-}I_3$	30.0	$H\text{-}I_4$	43.3
	$H\text{-}III_1$	20	$H\text{-}X_2$	13.3	$H\text{-}II_3$	20.0	$H\text{-}XI_4$	13.3
	Total	**55**		**33.3**		**50.0**		**56.6**
I	–	–	$I\text{-}I_2$	18.2	$I\text{-}XV_3$	15.0	$I\text{-}I_4$	50.0
			$I\text{-}IV_2$	36.4				
	Total	–		**54.6**		**15.0**		**50.0**

Table 5.
Major S. cerevisiae strains in the fermentations of Rioja Alta in 4 consecutive years (1–4).

other three campaigns studied. So, we do not consider that in Rioja Alta, there are representative strains from either the wineries or the sub-zone.

2.2.3 Ecology of the yeast population during alcoholic fermentation in the Rioja Alavesa sub-zone

In this sub-zone, two wineries (J and K) were sampled, where wines were produced by the carbonic maceration method. As in the other two sub-zones, the non-*Saccharomyces* yeasts participated at the beginning of the alcoholic fermentation, and later they were replaced by *S. cerevisiae* strains, which were the ones that directed and carried out the process until the end. In total, 149 colonies of these species were isolated.

The level of clonal variability was very high in both cellars, especially in winery K, where it reached 60% (**Table 7**). It is noteworthy that the highest percentage of clonal variability in both wineries was obtained in the third year, as in the vinifications in the Rioja Alta, which confirms the importance of the characteristics of the harvest in the microbiota that drives the fermentations.

As in the rest of the sub-zones, the high number of different clones identified in each fermentation and the few common yeasts between campaigns (**Table 7**) provided high indexes of diversity, which reached values of 0.95 and 0.98 for cellars J and K, respectively. These indices were of the same order as those found in Rioja Alta and higher than those of Rioja Oriental. Likewise, the number of yeasts that participated in more than one campaign was small in the two wineries, with the

Winery E				Winery F				Winery G				Winery H				Winery I		
1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	2	3	4
$E\text{-}I_1$	$E\text{-}I_2$	$E\text{-}I_3$	$E\text{-}I_4$	$F\text{-}I_1$	$F\text{-}I_2$	$F\text{-}I_3$	F-I4	$G\text{-}I_1$	$G\text{-}I_2$	$G\text{-}I_3$	$G\text{-}I_4$	$H\text{-}I_1$	$H\text{-}I_2$	$H\text{-}I_3$	$H\text{-}I_4$	$I\text{-}I_2$	$I\text{-}I_3$	$I\text{-}I_4$
$E\text{-}II_1$	$E\text{-}II_2$	$E\text{-}II_3$	$E\text{-}II_4$	$F\text{-}II_1$	$F\text{-}II_2$	$F\text{-}II_3$	$F\text{-}II_4$	$G\text{-}II_1$	$G\text{-}II_2$	$G\text{-}II_3$	$G\text{-}II_4$	$H\text{-}II_1$	$H\text{-}II_2$	$H\text{-}II_3$	$H\text{-}II_4$	$I\text{-}II_2$	$I\text{-}II_3$	$II\text{-}II_4$
$E\text{-}III_1$	$E\text{-}III_2$	$E\text{-}III_3$	$E\text{-}III_4$	$F\text{-}III_1$	$F\text{-}III_2$	$F\text{-}III_3$	$F\text{-}III_4$	$G\text{-}III_1$	$G\text{-}III_2$	$G\text{-}III_3$	$G\text{-}III_4$	$H\text{-}III_1$	$H\text{-}III_2$	$H\text{-}III_3$	$H\text{-}III_4$	$I\text{-}III_2$	$I\text{-}III_3$	$II\text{-}III_4$
$E\text{-}IV_1$	$E\text{-}IV_2$	$E\text{-}IV_3$	$E\text{-}IV_4$	$F\text{-}IV_1$	$F\text{-}IV_2$	$F\text{-}IV_3$	$F\text{-}IV_4$	$G\text{-}IV_1$	$G\text{-}IV_2$	$G\text{-}IV_3$	$G\text{-}IV_4$	$H\text{-}IV_1$	$H\text{-}IV_2$	$H\text{-}IV_3$	$H\text{-}IV_4$	$I\text{-}IV_2$	$I\text{-}IV_3$	$II\text{-}IV_4$
$E\text{-}V_1$	$E\text{-}V_2$	$E\text{-}V_3$	$E\text{-}V_4$	$F\text{-}V_1$	$F\text{-}V_2$	$F\text{-}V_3$	$F\text{-}V_4$	$G\text{-}V_1$	$G\text{-}V_2$	$G\text{-}V_3$	$G\text{-}V_4$	$H\text{-}V_1$	$H\text{-}V_2$	$H\text{-}V_3$	$H\text{-}V_4$	$I\text{-}V_2$	$I\text{-}V_3$	$II\text{-}V_4$
$E\text{-}VI_1$	$E\text{-}VI_2$	$E\text{-}VI_3$	$E\text{-}VI_4$		$F\text{-}VI_2$	$F\text{-}VI_3$	$F\text{-}VI_4$	$G\text{-}VI_1$	$G\text{-}VI_2$	$G\text{-}VI_3$	$G\text{-}VI_4$	$H\text{-}VI_1$	$H\text{-}VI_2$	$H\text{-}VI_3$	$H\text{-}VI_4$	$I\text{-}VI_2$	$I\text{-}VI_3$	$II\text{-}VI_4$
$E\text{-}VII_1$	$E\text{-}VII_2$	$E\text{-}VII_3$	$E\text{-}VII_4$		$F\text{-}VII_{98}$	$F\text{-}VII_3$	$F\text{-}VII_4$	$G\text{-}VII_1$	$G\text{-}VII_2$	$G\text{-}VII_3$	$G\text{-}VII_4$	$H\text{-}VII_1$	$H\text{-}VII_2$	$H\text{-}VII_3$	$H\text{-}VII_4$	$I\text{-}VII_2$	$I\text{-}VII_3$	$II\text{-}VII_4$
$E\text{-}VIII_1$	$E\text{-}VIII_2$	$E\text{-}VIII_3$	$E\text{-}VIII_4$		$F\text{-}VIII_2$	$F\text{-}VIII_3$	$F\text{-}VIII_4$	$G\text{-}VIII_1$	$G\text{-}VIII_2$	$G\text{-}VIII_3$	$G\text{-}VIII_4$	$H\text{-}VIII_1$	$H\text{-}VIII_2$	$H\text{-}VIII_3$	$H\text{-}VIII_4$	$I\text{-}VIII_2$	$I\text{-}VIII_3$	$II\text{-}VIII_4$
$E\text{-}IX_1$	$E\text{-}IX_2$				$F\text{-}IX_2$	$F\text{-}IX_3$	$F\text{-}IX_4$	$G\text{-}IX_1$	$G\text{-}IX_2$	$G\text{-}IX_3$	$G\text{-}IX_4$	$H\text{-}IX_1$	$H\text{-}IX_2$	$H\text{-}IX_3$	$H\text{-}IX_4$	$I\text{-}IX_2$	$I\text{-}IX_3$	$II\text{-}IX_4$
$E\text{-}X_1$	$E\text{-}X_2$				$F\text{-}X_2$			$G\text{-}X_1$	$G\text{-}X_2$	$G\text{-}X_3$	$G\text{-}X_4$		$H\text{-}X_2$	$H\text{-}X_3$	$H\text{-}X_4$		$I\text{-}X_3$	$II\text{-}X_4$
	$E\text{-}XI_2$							$G\text{-}XI_1$	$G\text{-}XI_2$	$G\text{-}XI_3$	$G\text{-}XI_4$		$H\text{-}XI_2$	$H\text{-}XI_3$	$H\text{-}XI_4$		$I\text{-}XI_3$	$II\text{-}XI_4$
	$E\text{-}XII_2$							$G\text{-}XII_1$		$G\text{-}XII_3$	$G\text{-}XII_4$		$H\text{-}XII_2$		$H\text{-}XII_4$		$I\text{-}XII_3$	
	$E\text{-}XIII_2$							$G\text{-}XIII_1$					$H\text{-}XIII_2$		$H\text{-}XIII_4$		$I\text{-}XIII_3$	
	$E\text{-}XIV_2$																$I\text{-}XIV_3$	
																	$I\text{-}XV_3$	
																	$I\text{-}XVI_3$	
																	$I\text{-}XVII_3$	
																	$I\text{-}XVIII_3$	
																	$I\text{-}XIX_3$	
																	$I\text{-}XX_3$	

Clones with box in gray in different columns show the same clone in different cellars

Table 6.
S. cerevisiae clones identified in each elaboration of Rioja Alta in 4 consecutive years (1–4).

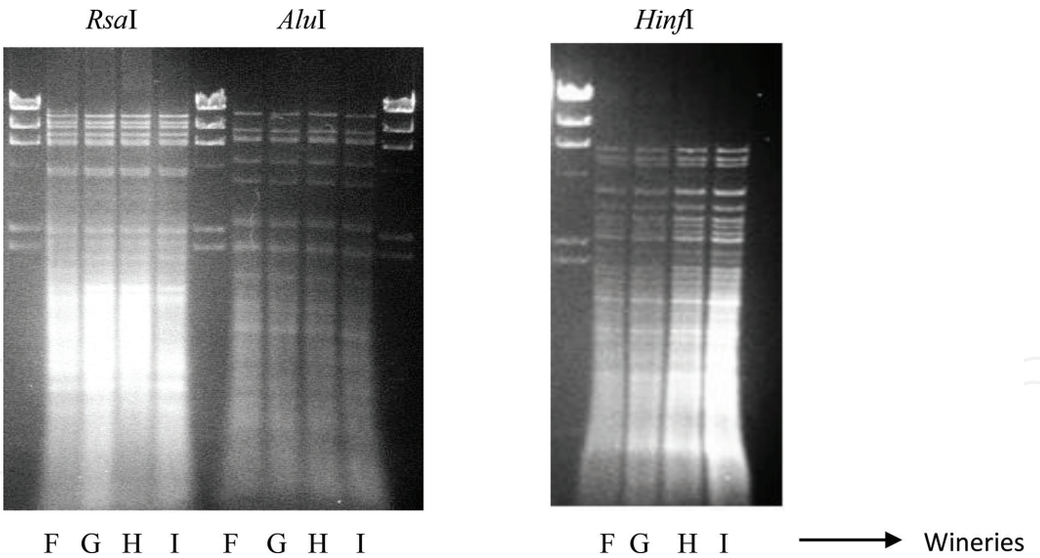

Figure 2.
Restriction patterns obtained with different restriction endonucleases, from the patterns I isolated in four Rioja Alta wineries in year 4.

Winery	Years	Isolates of *S. cerevisiae* analyzed	Number of different clones	Clonal variability (%)	Index of diversity	Genotypes detected in previous years	
						Number	%
J	1	29	15	51.7	0.93	–	–
	2	30	13	43.3	0.92	3	29.9
	3	20	14	70.0	0.94	5	50.0
	Total	**79**	**34**	**43.0**	**0.95**		
K	1	20	12	60.0	0.95	–	–
	2	30	14	46.7	0.92	0	0
	3	20	19	95.0	0.99	3	15.0
	Total	**70**	**42**	**60.0**	**0.98**		

Table 7.
Clonal diversity of S. cerevisiae yeasts in Rioja Alavesa wineries in 3 consecutive years (1–3).

exception of the third year of study in winery J. Therefore, the microbiota responsible for the fermentation was different in each harvest.

Regarding the population dynamics of the fermentation, it was observed that in the two wineries, the fermentations were carried out by different *S. cerevisiae* strains that followed each other during the different fermentative phases. In each vinification process, several major clones were detected, which represented at least 30% of the yeasts that carried out the fermentation (**Table 8**). The exception was the third year, in which most of the strains that were found in a fermentative phase were replaced by others in the following stage, particularly in winery K. This fact was also found in some of the vinifications in wineries G and I in Rioja Alta. All four wineries are over a hundred years old and conduct vinifications using the carbonic maceration method.

The comparison of the different strains found in the two cellars during the 3 years of study (**Table 9**) showed the existence of a single common clone, corresponding to the fermentations of the second year. Therefore, the data obtained would show the existence of extensive microbiota in each winery. The fermentations would be the result of the sequence of different populations of yeasts, as there are no representative strains of the sub-zone.

Years	1		2		3	
Winery	**Strain**	**%**	**Strain**	**%**	**Strain**	**%**
J	J-IV_1	20.7	J-III_2	13.3	J-III_3	25
	J-I_1	13.8	J-IV_2	13.3		
			J-V_2	13.3		
			J-VI_2	16.6		
	Total	**34.5**		**56.5**		**25**
K	K-VI_1	15.5	K-III_2	16.7	K-II_3	6.7
	K-XI_1	15.0	K-IV_2	16.7		
			K-VI_2	13.3		
	Total	**30.5**		**46.7**		**6.7**

Table 8.
Major S. cerevisiae strains in the fermentations of Rioja Alavesa in 3 consecutive years (1–3).

Winery J			Winery K		
1	**2**	**3**	**1**	**2**	**3**
J-I_1	**J-I_2**	J-I_3	K-I_1	K-I_2	K-I_3
J-II_1	J-II_2	J-II_3	K-II_1	**K-II_2**	K-III_3
J-III_1	J-III_2	J-VI_3	K-III_1	K-III_2	K-IV_3
J-IV_1	J-IV_2	J-VII_3	K-IV_1	K-IV_2	K-V_3
J-V_1	J-VI_2	J-IX_3	K-V_1	K-V_2	K-VI_3
J-VI_1	J-VII_2	J-X_3	K-VI_1	K-VI_2	K-$VIII_3$
J-VII_1	J-$VIII_2$	J-XII_3	K-VII_1	K-VII_2	K-IX_3
J-$VIII_1$	J-IX_2	J-$XIII_3$	K-$VIII_1$	K-$VIII_2$	K-X_3
J-IX_1	J-X_2	J-XIV_3	K-IX_1	K-IX_2	K-XI_3
J-X_1	J-$XIII_2$		K-X_1	K-X_2	K-XII_3
J-XI_1			K-XI_1	K-XI_2	K-$XIII_3$
J-XII_1			K-XII_1	K-XII_2	K-XV_3
J-$XIII_1$				K-$XIII_2$	K-XVI_3
J-XIV_1				K-XIV_2	K-$XVII_3$
J-XV_1					K-$XVIII_3$
					K-XIX_3

Clones In bold in different columns show the same clone in different cellars

Table 9.
S. cerevisiae clones identified in each elaboration of Rioja Alavesa in 3 consecutive years (1–3).

3. Conclusions

As in other ecological studies of wine fermentations, spontaneous alcoholic fermentations in the Rioja qualified designation of origin are mainly conducted by yeasts of the *S. cerevisiae* species, and the non-*Saccharomyces* species have only been detected in the early stages. These fermentations have been carried out by different

S. cerevisiae strains that have appeared throughout the different stages of the process. Out of the 915 colonies of *S. cerevisiae* analyzed, 330 different clones have been identified, which means a very high clonal diversity.

Different agronomic and technological factors can influence in the diversity of the yeasts present in each vinification, such as the age of the winery, the winemaking system employed, and the climate conditions that prevailed during the ripening period of the grapes. The vinifications carried out in newly constructed wineries presented a lower clonal diversity than those which took place in older wineries. The clonal diversity was higher in vinifications conducted by carbonic maceration than in those carried out after crushing and destemming grapes. Unfavorable climatology during the vegetative period decreased the number of strains that participated in the fermentation.

There were very few common strains that participated in the fermentations carried out in successive years within the same winery, and hardly any common strains were detected in different wineries of the same sub-zone during the 4 years studied. All this allows us to affirm that there are no representative "typical" strains of the wineries, nor of the sub-zones and, therefore, of the Rioja designation of origin.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

[1] Varela C, Siebert T, Cozzolino D, Rose L, McLean H, Henschke PA. Discovering a chemical basis for differentiating wines made by fermentation with wild indigenous and inoculated yeasts: Role of yeasts volatile compounds. Australian Journal of Grape and Wine Research. 2009;**15**:238-248. DOI: 10/1111/j.1755-0238.2009.00054.x

[2] Santamaría P, López R, López E, Garijo P, Gutiérrez AR. Permanence of yeast inocula in the winery ecosystem and presence in spontaneous fermentations. European Food Research and Technology. 2008;**227**(5):1563-1567. DOI: 10.1007/s00217-008-0855-5

[3] Gutiérrez AR, Santamaría P, Epifanio S, Garijo P, López R. Ecology of spontaneous fermentation in one winery during five consecutive years. Letters in Applied Microbiology. 1999;**29**:411-415. DOI: 10.1046/j.1472-765X.1999.00657.x

[4] Vezinhet F, Hallet JN, Valade M, Poulard A. Ecological survey of wine strains by molecular methods of identification. American Journal of Enology and Viticulture. 1992;**43**:83-86

[5] Blanco P, Ramilo A, Cerdeira M, Orriols I. Genetic diversity of wine *Saccharomyces cerevisiae* strains in an experimental winery from Galicia (NW Spain). Antonie Van Leeuwenhoek. 2006;**89**:351-357. DOI: 10.1007/s10482-005-9038-6

[6] Torija M, Rozès N, Poblet M, Guillamón JM, Mas A. Yeast population dynamics in spontaneous fermentations: Comparison between two different wine-producing areas over a period of three years. Antonie Van Leeuwenhoek. 2001;**79**:345-352. DOI: 10.1023/A:1012027718701

[7] Knight S, Klaere S, Fedrizzi B, Goddard MR. Regional microbial signatures positively correlate with differential wine phenotypes: Evidence for a microbial aspect to terroir. Scientific Reports. 2015;**5**:14233. DOI: 10.1038/srep14233

[8] Capece A, Granchi L, Guerrini S, Mangani S, Romaniello R, Vincenzini M, et al. Diversity of *Saccharomyces cerevisiae* strains isolated from two italian wine-producing regions. Frontiers in Microbiology. 2016;**7**:1-11. DOI: 10/3389/fmicb.2016.01018

[9] Tristezza M, Fantastico L, Vetrano C, Bleve G, Corallo D, Grieco F, et al. Molecular and technological characterization of *Saccharomyces cerevisiae* strains isolated from natural fermentation of Susumaniello grape must in Apulia, southern Italy. International Journal of Microbiology. 2014:897428. DOI: 10.1155/2014/897428

[10] Schvarczová E, Stefaniková J, Jankura E, Kolek E. Selection of autochthonous *Saccharomyces cerevisiae* strains for production of typical Pinot gris wines. Journal of Food and Nutrition Research. 2017;**56**(4):389-397

[11] Orlic S, Vojvoda T, Babic KH, Arroyo-López FN, Jeromel A, Kocina B, et al. Diversity and oenological characterization of indigenous *Saccharomyces cerevisiae* associated with Zilavka grapes. World Journal of Microbiology and Biotechnology. 2010;**26**:1483-1489. DOI: 10.1007/s11274-010-0323-9

[12] Querol A, Barrio E. A rapid and simple method for the preparation of yeast mitocondrial DNA. Nucleic Acids Research. 1990;**18**:1657

[13] López I. Detección y control por técnicas de la Biología Molecular de bacterias lácticas autóctonas responsables de la fermentación maloláctica en vinos de la D.O.Ca. Rioja [thesis]. University of La Rioja; 2004

[14] Clemente-Jiménez JM, Mingorance-Cazorla L, Martínez-Rodríguez S, Las Heras-Vázquez FJ, Rodríguez-Vico F. Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. Food Microbiology. 2004;**21**:149-155. DOI: 10.1016S0740-0020(03)00063-7

[15] Santamaría P, Garijo P, López R, Tenorio C, Gutiérrez AR. Analysis of yeast population during spontaneous alcoholic fermentation: Effect of the age of the cellar and the practice of inoculation. International Journal of Food Microbiology. 2005;**103**:49-56. DOI: 10.1016/j.ijfoodmicro.2004.11.024

[16] Jemec KP, Cadez N, Zagorc T, Vuvic V. Yeast population dynamics in five spontaneous fermentations of Malvasia musts. Food Microbiology. 2001;**18**(3):247-259. DOI: 10.1016/fmic.2001.0396

[17] Sangorrín M, Zajonskovsky I, van Broock M, Caballero A. The use of killer biotyping in an ecological survey of yeast in an old Patagonian winery. World Journal of Microbiology and Biotechnology. 2002;**18**(2):115-120. DOI: 10.1023/A:101441722 2890

[18] Versavaud A, Courcoux P, Roulland C, Dulau L, Hallet JN. Genetic diversity and geographical distribution of wild *Saccharomyces cerevisiae* strains from the wine-producing area of Charentes, France. Applied Environmental Microbiology. 1995;**61**(10):3521-3529

[19] Schütz M, Gafner J. Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. Letters in Applied Microbiology. 1994;**19**(4):253-257. DOI: 10.1111/j.1472-765X.1994.tb00957.x

[20] Granchi L, Ganucci D, Viti C, Giovannetti L, Vincenzini M. *Saccharomyces cerevisiae* biodiversity in spontaneous commercial fermentations of grape musts with adequate and inadequate assimilable-nitrogen content. Letters in Applied Microbiology. 2003;**36**:54-58. DOI: 10.1046/j.1472-765X.2003.01263.x

[21] Garijo P. Estudio del aire como vía de diseminación de microorganismos enológicos [thesis]. University of La Rioja; 2013

[22] Van der Westhuizen TJ, Augustyn OPH, Pretorius IS. Geographical distribution of indigenous *Saccharomyces cerevisiae* strains isolated from vineyard in the Coastal Regions of the Western Cape in South Africa. South African Journal of Enology and Viticulture. 2000;**21**(1):3-9. DOI: 10.21548/21-1-2179

[23] Fleet GH. Yeast interactions and wine flavour. International Journal of Food Microbiology. 2003;**86**(1-2):11-22. DOI: 10.1016/S0168-1605(03)00245-9

[24] Ocón E, Garijo P, López I, Santamaría P, Gutiérrez AR, Tenorio C, et al. Quantitative and qualitative analysis of non-*Saccharomyces* yeasts in spontaneous alcoholic fermentations. European Food Research and Technology. 2010;**230**(6):885-891. DOI: 10.1007/s00217-010-1233-7

[25] Le Jeune C, Claude E, Demuyter C, Lollier M. Evolution of the population of *Saccharomyces cerevisiae* from grape to wine in a spontaneous fermentation. Food Microbiology. 2006;**23**:709-716. DOI: 10.1016/j.fm.2006.02.007