OENO One
VINE AND WINE OPEN ACCESS JOURNAL
By IVES International Viticulture & Enology Society

DOI: https://doi.org/10.20870/oeno-one.2022.56.4.4898

OPEN ACCESS

ORIGINAL RESEARCH ARTICLE

Influence of vintage, geographical location and agricultural management on yeast populations in Galician grape musts (NW Spain)

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Associate editor:
Isabelle Masneuf-Pomarede

Received:
2 November 2021

Accepted:
5 October 2022

Published:
2 November 2022

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ABSTRACT

The influence of vintage, geographical location and farming system on yeast populations was evaluated in grape musts from Galicia (NW Spain) in three consecutive years. Grape samples were taken in organic and conventional vineyards from four Denominations of Origin (DOs) during the 2013, 2014 and 2015 vintages. Cultivable yeast populations were characterised at the quantitative and qualitative level. The general results show the existence of significant differences for yeast counts in musts among vintages and DOs, but not between farming systems. Given the geographical location of the grapes, the influence of vintage was significant in all DOs; however, the farming system only had a significant impact on viable yeasts in Rías Baixas, although the number of yeasts tended to be higher in organic samples than in conventional ones in most cases. Species richness was location dependent, with Rías Baixas and Ribeira Sacra showing the highest values. In addition, the type of yeasts varied between Denominations of Origin. From a total of 39 different yeast species identified, *Metschnikowia* spp., *Hanseniaspora uvarum* and *Aureobasidium* spp. were the prevailing species. These major yeasts were found to be widely distributed. However, species such as *Issatchenkia terricola*, *Starmerella bacillaris* and different species of *Candida*, *Pichia* and *Zygosaccharomyces* genera were mainly found in Rías Baixas and Ribeira Sacra DOs. In contrast, *Lachancea thermotolerans* was isolated in Monterrei and Ribeiro DOs only. Accordingly, the ANOSIM and PERMANOVA analyses evidenced significant differences in species richness among different DOs and, to a lesser extent, among vintages, but not between farming systems.

KEYWORDS: yeast population, grape must, vintage, geographical location, farming system, Galicia

INTRODUCTION

Grapes are a primary source of the yeasts involved in must fermentation and influence wine quality. The yeast populations associated with grapes and the factors involved in their diversity have been widely examined (for a review see Barata *et al*., 2012). In general terms, the amount of yeasts increases throughout the maturation process of grapes and also depends on their sanitary conditions (Fleet, 2003; Martins *et al*., 2014; Prakitchaiwattana *et al*., 2004; Renouf *et al*., 2005). Typically, the surface of healthy grape berries is dominated by basidiomycetous yeasts, such as *Cryptococcus* spp., *Rhodotorula* spp. and the yeast-like fungus *Aureobasidium pullulans*. With ripening, the nutrient availability on the grape surface increases, as does the presence of oxidative or weakly fermentative ascomycetous populations, such as *Hanseniaspora* spp., *Metschnikowia* spp., *Pichia* spp. or *Candida* spp. (Barata *et al*., 2012). On damaged grapes the available high sugar concentration favours the growth of higher fermentative yeasts like *Pichia* spp., *Lachancea* spp., *Torulaspora* spp., *Zygosaccharomyces* spp. and *Saccharomyces cerevisiae*. Moreover, the diversity of yeast species in grape musts can also be influenced by many other factors, including geographical location of the vineyard, agronomic management, grapevine cultivar, climatic conditions and vintage (Fleet *et al*., 2002).

The relationship between the regional characteristics of a wine and a specific microbial community has been long thought to exist and only recently has it been possible to prove. Gayevskiy and Goddard (2012) reported the existence of geographical patterns for cultivable yeast in grapes from different areas/regions and they suggested that microbes contribute to terroir. Taking advantage of next-generation sequencing approaches, Bokulich *et al*. (2014) were able to demonstrate that the fungal and bacterial communities associated with grapes were correlated with regional, varietal and climatic factors. Their findings were confirmed by other authors and the biogeographical patterns of yeast diversity found in the vineyard have been associated with a *microbial terroir* responsible for the much appreciated regional character of wines (Belda *et al*., 2017; Bokulich *et al*., 2016; Drumonde-Neves *et al*., 2017; Knight *et al*., 2015).

There is a close relationship between climatic conditions and the yeast populations on grapes and in musts. Parameters such as rainfall and temperature, UV exposure, sunlight and wind all influence the sanitary status of grapes and therefore the proliferation of yeasts. For example, rainy vintages have been found to be associated with higher yeast counts on grapes (Čadež *et al*., 2010; Combina *et al*., 2005; Longo *et al*., 1991). In addition, differences in yeast diversity among vintages can be attributed to climatic conditions, which affect berry integrity (Bagheri *et al*., 2015; Combina *et al*., 2005; De La Torre *et al*., 1999; Grangeteau et *al.,* 2017b; Nemcová et *al.,* 2015). In this context, Vigentini et *al.* (2015) found that vintage, rather than t*erroir,* had a more significant impact on yeast diversity in grapes.

Finally, the societal demand for environmentally friendly agricultural practices has led to an increase in the area of organic vineyards in recent decades, with Spain having the largest surface area devoted to this type of farm management in the world (Willer and Lernoud, 2020). This increase is due to the benefits in terms of not only environment and health, but also the biodiversity and socioeconomic added value that are generated (Mariani and Vastola, 2015; Moscovici *et al*., 2020; Provost and Pedneault, 2016; Schäufele and Hamm, 2018). Indeed, the type of farming system has an influence on the grape yeast population. Several studies have shown that organic and/or biodynamic vineyards have a higher yeast diversity than conventional vineyards (Agarbati *et al*., 2019a; Bagheri *et al*., 2015; Cordero-Bueso *et al*., 2011; Martins *et al*., 2014; Morrison-Whittle *et al*., 2017; Setati *et al*., 2012). Conversely, other authors have reported the same or a greater yeast abundance in conventional grapes (Grangeteau *et al*., 2017b; Guzzon *et al*., 2016; Milanović *et al*., 2013). The lower yeast biodiversity in organic vineyards may be due to the sensitivity of yeasts to copper and sulfur, the only fungicides permitted in organic farming (Grangeteau *et al*., 2017a). However, many other factors may have contributed to these contradictory results (Setati *et al*., 2015).

In the region of Galicia in Northwest Spain, the wine industry plays an important economic and social role, with a vineyard area of approximately 24.134 ha. This region is characterised by a high heterogeneity in orography, soil and climatic conditions, which sometimes hinder vineyard management (Blanco-Ward *et al*., 2007; Fernández-González *et al*., 2012; Fraga *et al*., 2014). This could be the reason why certified organic production is restricted to less than 0.5 % of Galicia's total vineyard surface area (82.6 ha; 26 operators, of which 16 are processors) (CRAEGA, 2020). Regarding the role of microorganisms, there is little information available on the yeast populations associated with grapes and/or wineries in different areas of Galicia and on how different factors affect them. In spontaneous fermentations, Longo *et al*. (1991) found differences between species from the Atlantic region and the interior regions, as well as between vintages. A recent study has suggested the existence of a biogeographical pattern in the distribution of yeast populations from grapes from Galicia (Castrillo *et al*., 2019). The results also indicate that the organic farming system increased yeast diversity in the grapes and musts. In addition, a survey in organic wineries from Galicia showed that this practice favoured *S. cerevisiae* strain diversity (Castrillo *et al*., 2020).

In this context, this study reports the influence of vintage, geographical location and farming system on yeast population (at quantitative and qualitative level) in grape musts from different areas within Galicia. The study was carried out at Estación de Viticultura e Enoloxía de Galicia (EVEGA) over a period of three years.

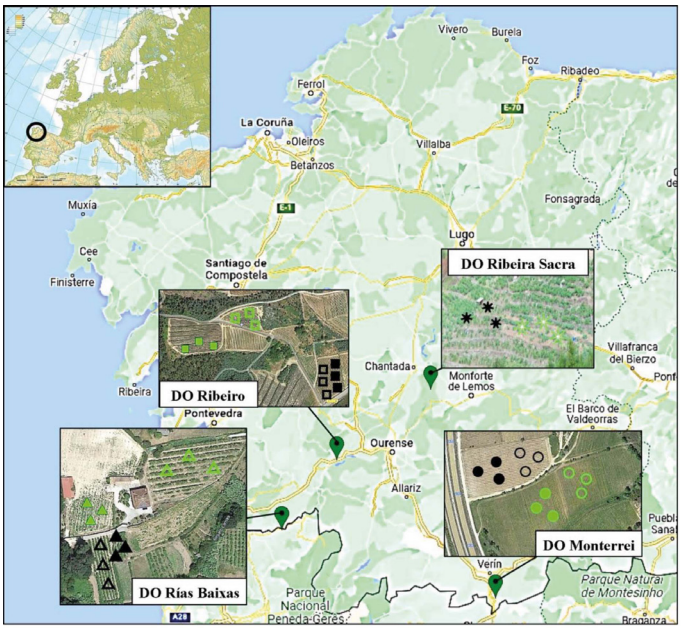


FIGURE 1. Geographical location of the sampling sites within four Denominations of Origin (DO) in Galicia (NW Spain). Black symbols = conventional samples; green symbols = organic samples; *= Mencía in Ribeira Sacra; ○ = Treixadura and ● = Mencía in DO Monterrei; □ = Treixadura and ■ = Brancellao in DO Ribeiro; Δ = Treixadura and ▲= Albariño in DO Rías Baixas.

MATERIALS AND METHODS

1. Grape origin and climatic conditions

The grapes used in this study were picked from different vineyards within four Denominations of Origin (DO) in Galicia in three consecutive years (2013, 2014 and 2015) (Figure 1). Grapevine cultivars traditionally grown in each DO were chosen: Albariño and Treixadura in DO Rías Baixas; Mencía in DO Ribeira Sacra; Brancellao and Treixadura in DO Ribeiro; and Mencía and Treixadura in DO Monterrei. For each DO and cultivar, the selected plots bordered or were next to each other, but their farming systems were different (organic or conventional). The characteristics of the vineyards (DO, cultivar, farming system, location and elevation) have been described previously (Castrillo *et al.*, 2019). In addition, climatic data were obtained from MeteoGalicia (2015). For each DO, data were calculated from recordings made by two meteorological stations closest to the plots in each agricultural year. Table 1 summarises the monthly temperature and annual precipitation in 2013, 2014 and 2015.

TABLE 1. Climatic data calculated from recordings made by the two meteorological stations closest to each plot (MeteoGalicia) for each agricultural year: from September to September.

Denomination of Origin (Meteorological stations)	Year	Monthly temperature (ºC)			Rainfall (L/m^2)		
		min	mean	max	min	mean	max
Monterrei (Verín-Vilela)	2013	5.6	12.7	22.3	0	166	787
	2014	3.6	12.8	20.2	7	171	928
	2015	3.4	13.0	21.9	0	150	595
Ribeiro (Prado-Evega)	2013	7.5	14.3	23.4	0	244	1211
	2014	6.0	14.4	21.2	17	291	1351
	2015	5.8	14.5	22.9	2	193	752
Ribeira Sacra (Míllara-San Fiz)	2013	6.8	13.5	23.9	2	215	1075
	2014	6.2	13.7	20.5	13	263	1197
	2015	5.7	13.8	21.8	7	197	719
Rías Baixas (Meder-Cequeliños)	2013	8.2	14.8	23.2	0	350	1489
	2014	7.2-	14.9	20.8	23	361	1649
	2015	7.0	15.1	21.5	6	284	1071

Temperature and rainfall are indicated as minimum (min), mean and maximum (max) monthly values each year.

2. Sampling and yeast isolation

The plots were divided into three representative random blocks excluding the external rows and vine plants to avoid an edge effect (Figure 1). In each block, 4 kg of grape bunches were collected from at least 8 to 10 vines. Thus, a total of 42 grape samples were collected each year: 3 samples per 7 organic plots and 3 samples per 7 conventional plots (39 samples in 2014, because Mencía conventional grapes from DO Monterrei were not available). All samples were transported to the laboratory in individual sterile plastic bags and processed within 2 h to limit mould growth. The grapes were aseptically destemmed and crushed by hand inside the sterile bags to obtain must. These must samples were adequately diluted and 100 µL were homogeneously spread in duplicate on a WL Nutrient Agar medium (Scharlau Microbiology) supplemented with 200 µg/mL biphenyl to suppress the growth of moulds. The plates were incubated at 28 °C until visible colonies appeared, and those containing between 20 and 200 colonies were used for the quantification of total viable cells (expressed as number of colonies forming units (CFU) per mL). In addition, a representative number of yeasts from each sample was isolated based on their colony morphology (10-20 per sample). The yeasts were isolated in a YEPD medium (1 % w/v yeast extract, 2 % w/v peptone, 2 % w/v glucose, and 2 % w/v agar) and the pure culture was maintained at -80 °C in YEPD with 15 % (v/v) glycerol until further identification by molecular techniques. Species richness (S) was defined as the number of different yeast species found in a given sample based on their colony morphology on WL Nutrient Agar plates.

3. Yeast identification

Yeasts were identified at species level following the methodology described by Esteve-Zarzoso *et al*. (1999). Thus, the 5.8S rRNA gene and the two internal (non-coding) ITS1 and ITS2 spacers were amplified by PCR using the ITS1 and ITS4 primers. The PCR products were digested with the restriction endonucleases FastDigest *Hinf* I, *Bsu* RI and *Hha* I (Thermo Fisher Scientific, Madrid, Spain) and the fragments obtained were separated in a 3 % agarose gel in 1X TBE buffer. DNA pattern bands were visualised under UV light and documented using a Molecular Imager® Gel DocTM XR+ imaging system (BIO-RAD, Madrid, Spain).

In addition, the identification of at least 2-3 yeast pure cultures per rRNA28S ITS1-ITS4 restriction profile was confirmed by PCR amplification and sequencing of D1/D2 region of 26S rDNA gene. The D1 and D2 domains were amplified using NL-1 and NL-4 primers as described by Kurtzman and Robnett (1998). The PCR products were purified with the PCR Extract Mini kit (5PRIME) according to the supplier's instructions, and the sequences were obtained as indicated in Castrillo *et al*. (2019). Sequence similarities were obtained by means of GenBank BLASTN search facilities (Altschul *et al*., 1990). The identification was considered correct when the gene sequences' identity was 98 % or higher, in which case, the isolates showing the same profile were assumed to be the identified species. The unique or very scarce isolates were all sequenced.

4. Statistical analysis

Differences in yeast population at the quantitative level between samples from organic and conventional production were tested by variance analysis (ANOVA) using SPSS (v.18.0) software. In addition, a two-way ANOVA analysis was performed on all factors using the PAST (v.4.02; 2020) software.

To obtain an overview of efficiency of the sampling carried out, a species richness study (theoretical count of the different species) inferred from a rarefaction curve was performed with EstimateS 9.1.0 software (Colwell and Elsensohn, 2014). In addition, eight non-parametric estimators of species richness were calculated with 1000 randomisations: ACE (Abundance Coverage-based Estimator), ICE (Incidence Coverage-based Estimator), Chao 1, Chao 2, Jack 1, Bootstrap and Michaelis Menten (MM: MMRuns and MMMeans). Sampling efficiency was calculated as number of yeast species observed divided by mean of the estimators. The general yeast diversity of the vineyards was calculated using Shannon (H′) and Simpson (1-D) indices, where D is the dominance and 1/D is the Simpson inverse diversity (Castrillo *et al.*, 2019). However, diversity indices were not calculated for the grape variety factor, as the samples were not a homogeneous group.

Differences in yeast species richness due to DOs, production system, varieties and vintage were tested by one-way and two-way Bray Curtis-ANOSIM (non-parametric analysis of similarity), using the software PAST 4.02. The distances are converted to ranks. Pairwise ANOSIMs between all pairs of groups are provided as a post-hoc test. Significant comparisons are considered at $p < 0.05$. PERMANOVA analyses were also performed; however, data are only shown in Table S2, because they were very similar to those obtained with ANOSIM. The Bonferroni correction (which is known to be too strict as it multiplies p-values with the number of comparisons) was calculated when it was necessary to compare or confirm differentiation between factors that had high significant differences (since it produces large p-values). The results of these tests show the probability of observing significant differences in yeast populations by chance using permutations based on any distance measure (Anderson and Walsh, 2013; Clarke, 1993). Distances are converted to a rank Bray-Curtis similarity matrix to create null distributions. In addition, Principal Component Analyses (PCA) were carried out by PAST 4.02 using the main species richness to separate samples from different DOs, culture systems and vintages. The main species were defined as having a frequency higher than 5 %; that is, the percentage of samples in which a given species appeared taking into account 100 % of the total number of samples. This value was considered an appropriate threshold for separating minority and majority species based on previous SIMPER-based studies (Castrillo *et al*., 2019). Finally, a Pearson's test was performed to assess the correlation between the main climatic factors and species richness.

TABLE 2. Yeast population (Log CFU/mL) in must samples from organic (Org) and conventional (Con) grapes in 2013, 2014 and 2015.

Denomination of Origin	Cultivar	2013		2014		2015		Factors significance		
		Org	Con	Org	Con	Org	Con	Year	FS	Year x FS
Monterrei	Treixadura[b]	6.76 ± 2.14	4.64 ± 0.92	7.77 ± 0.29	6.69 ± 0.07	4.01 ± 0.16	3.82 ± 0.24	***	ns	ns
	Mencía[ac]	6.42 ± 0.28	7.22 ± 0.05	7.72 ± 0.14		6.03 ± 0.32	5.16 ± 0.09			
Ribeiro	Treixadura	5.60 ± 0.80	4.85 ± 0.25	6.14 ± 0.04	6.50 ± 0.49	4.55 ± 0.96	4.85 ± 0.08	***	ns	*
	Brancellao	5.13 ± 0.20	4.55 ± 0.50	5.72 ± 0.12	5.89 ± 0.26	4.73 ± 0.35	4.92 ± 0.23			
Ribeira Sacra	Mencía[c]	6.11 ± 1.14	7.52 ± 0.11	6.69 ± 0.16	6.52 ± 0.31	6.33 ± 0.12	5.15 ± 0.41	**	ns	**
Rías Baixas	Treixadura	6.33 ± 1.15	6.81 ± 0.46	7.34 ± 0.41	6.95 ± 0.50	6.55 ± 0.22	5.99 ± 0.70	*	*	ns
	Albariño[bc]	4.89 ± 0.42	4.83 ± 0.22	6.71 ± 0.17	5.49 ± 0.11	6.12 ± 0.25	4.12 ± 0.36			

Data are the mean of three replicates ± standard deviation. The superscript a, b, c in the cultivar indicates statistically significant differences according to Tukey´s test ($p < 0.05$) between organic and conventional cultivation in 2013, 2014 and 2015 for this cultivar respectively. ns, *, ** and *** indicate no significant or statistically significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. FS = farming system.

RESULTS

1. Influence of vintage, farming system and location on viable yeast populations

The effect of vintage and farming system on yeast populations in musts from different DO and cultivars at the quantitative level is summarised in Table 2. The values observed ranged between 7.77 and 3.82 log CFU/mL. The highest counts were achieved in 2014, which also registered the highest rates of rainfall (Table 1). Accordingly, the lowest values were obtained in 2015 for both number of yeasts and precipitation.

When the geographical location of grapes was considered, the influence of vintage was significant in all Denominations of Origin; however, the farming system only had a significant impact on viable yeasts in Rías Baixas ($p < 0.05$) (Table 2). Organic samples tended to show a higher number of viable yeasts than those of conventional origin, especially in 2013 and 2015. DO Ribeiro was an exception, showing an opposite tendency: higher counts in conventional must in 2013 and 2015 and in organic musts in 2014. Considering specific cultivars, the amount of yeasts from Albariño must in 2014 and 2015 was higher in the organic samples. The musts from Treixadura followed the same trend, although in this case the differences were not significant. Accordingly, organic must from Treixadura in Monterrei also showed a higher number of viable yeasts than the conventional ones (significant in 2014). Conversely, in Ribeiro, the number of viable yeasts was higher in the conventional samples in 2014 and 2015, although these differences were not statistically significant. Similar behaviour was observed in 2013 for Mencía musts from Ribeira Sacra and Treixadura musts from Rías Baixas. The results also showed that the interaction between farming system and vintage was significant in Ribeiro and Ribeira Sacra (Table 2).

Finally, taking all the results together, the two-way ANOVA analysis confirmed the existence of significant differences in the number of yeasts in musts among vintages and DOs, but not between farming systems (Figure 2). Moreover, we detected interactions between vintages and farming systems and between vintages and DOs, but not between farming system and DOs. Significant differences were observed among all vintages ($p < 0.01$). Regarding DOs, the results showed significant differences among Ribeiro and Rías Baixas or Ribeira Sacra; however, the number of yeasts in must did not differ between Ribeiro and Monterrei or between Rías Baixas and Ribeira Sacra.

2. Quantitative evaluation of yeast species richness

The yeasts isolated from each sample were identified using genetic techniques. These isolates belonged to 39 different yeast species within 18 genera (Table S1). The statistical analysis of the sampling showed an efficiency of 77 %, which indicated that the sampling was acceptable. The rarefaction curve is shown in Figure 3A.

The results showed that total yeast richness (S) was higher in musts of organic origin than those of conventional origin (Figure 3B). Thus, 32 species (82 % respect to the total number of species-39) were found in organic samples compared to 28 species (72 %) identified in conventional musts. However, the ANOVA test did not show any significant differences for the farming system parameter ($p = 0.2330$) or for grapevine varieties in terms of species richness ($p = 0.1913$). Conversely, ANOVA showed significant differences in the year factor ($p = 0.0055$ in the overall value, highlighting the p value = 0.0038 between the pairwise 2013-2015 according to Tukey's pairwise test. Similarly, for geographical location the differences were significant between all DOs ($p = 0.0002$) and between RS-Mo, RS-Ri and RB-Ri pairwise at $p < 0.05$ in the Tukey's test. Considering each DO separately, only Rías Baixas showed a higher number of species in organic samples than in the conventional ones. The diversity was similar for both type of cultures in DO Monterrei and DO Ribeira Sacra, but lower under organic production in DO Ribeiro. Figure 3B also showed that Rías Baixas was the DO with the highest species richness, followed by Ribeira Sacra, whereas the lowest number of species was found in Monterrei and Ribeiro.

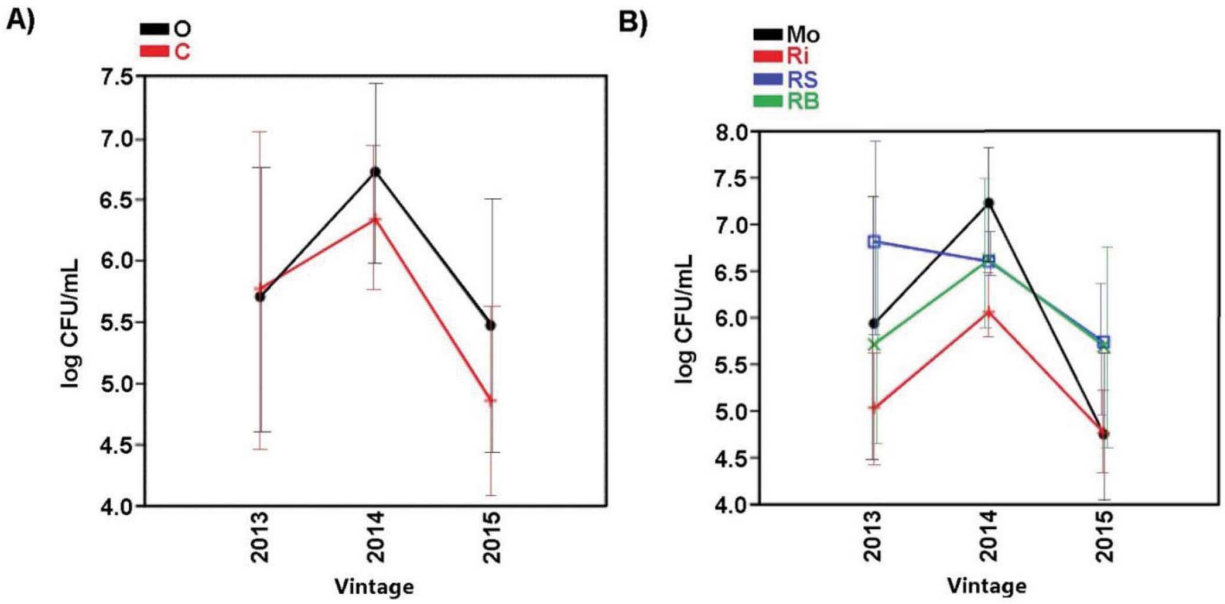


FIGURE 2. Influence of different factors on viable yeast population (log CFU/mL). A) Interaction between farming system and vintages, and B) Interaction between Denomination of Origin and vintage: O = Organic; C = Conventional; Mo = Monterrei, Ri = Ribeiro, RS = Ribeira Sacra and RB = Rías Baixas.

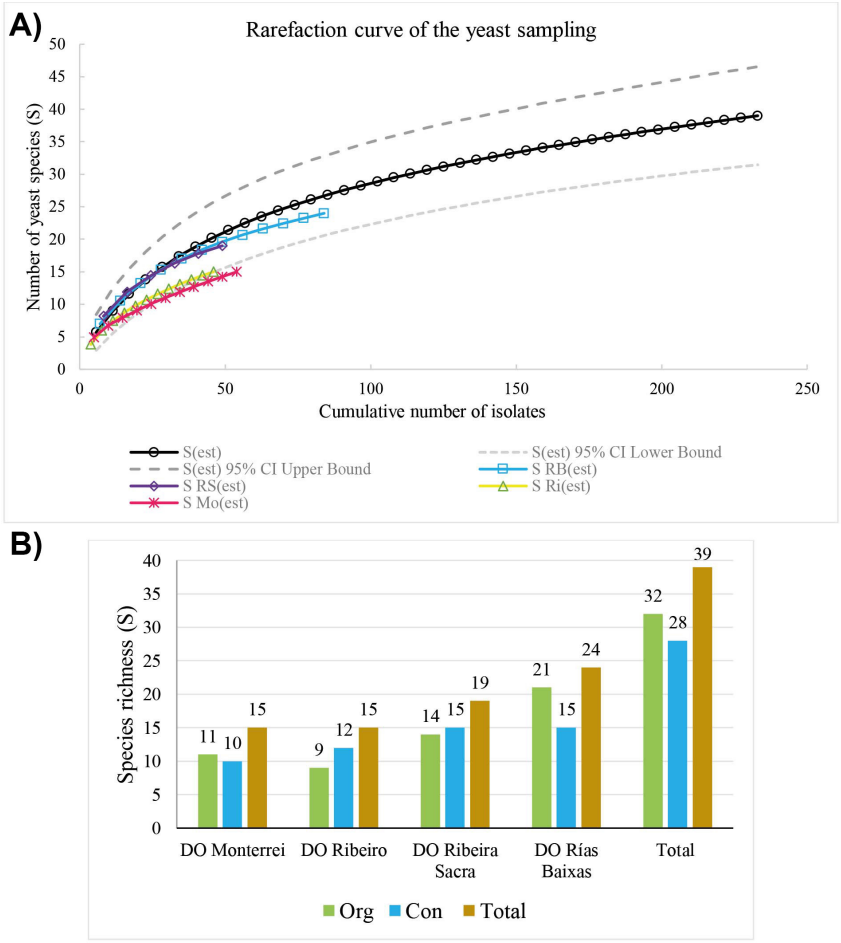


FIGURE 3. A) Rarefaction curve (number of yeast species observed (S) versus cumulative number of yeast isolates for each Denomination of Origin (DO) sampled: Mo (DO Monterrei); Ri (DO Ribeiro); RS (DO Ribeira Sacra) and RB (DO Rías Baixas). B) Species richness, expressed as number of different yeast species (cumulative count) found in organic (Org) and conventional (Con) must samples from different Denominations of Origin (DO) and in total in Galicia.

TABLE 3. Biodiversity indices for species richness (S) for each year, DO and farming system.

Factor		Shannon (H′)	Simpson	
			(1-D)	(1/D)
Year	General/Total	3.02	0.923	13.03
	2013	2.18	0.847	6.54
	2014	2.78	0.916	11.84
	2015	3.08	0.941	16.84
Denomination of origin	DO Monterrei	2.18	0.847	7.36
	DO Ribeiro	2.33	0.872	7.84
	DO Ribeira Sacra	2.74	0.925	13.27
	DO Rías Baixas	2.83	0.924	13.16
Farming System	Total organic	2.98	0.925	13.28
	Total conventional	2.85	0.915	11.83

Climatic conditions also have an impact on species richness. In this study, rainfall and temperature were area- and vintage-dependent (Table 1). A positive moderate correlation was found between general species richness and temperature (r = 0.424). However, no correlation was found between rainfall and species richness (Pearson = 0.19)

Moreover, the yeast diversity of the vineyards was evaluated using Shannon's and Simpson's indices (Table 3). The values obtained for organic and conventional samples, as well as for each DO, confirmed the tendencies observed in Figure 3B.

When the grapevine cultivars and vintage were considered separately, all tendencies were observed (Figure 4). Within DO Monterrei, Treixadura organic musts had higher species richness than the conventional ones in 2013 and 2014, but not in 2015 (Figure 4A). In contrast, musts from Mencía variety showed a higher yeast population in organic cultivation in 2015.

In DO Ribeiro the opposite trend was observed, especially for Treixadura musts (Figure 4B). Richness was similar between both culture systems (2014) or higher in conventional samples (2013 and 2015). In contrast, musts from Brancellao showed higher richness under organic production in 2014 and 2015, but not in 2013 (Figure 4B).

Mencía musts from DO Ribeira Sacra showed higher richness under organic production, except in 2013, when no differences were found (Figure 4C). Nevertheless, the total richness was higher in the conventional samples. Finally, the musts from DO Rías Baixas showed a higher number of yeasts species under the organic culture system, except Treixadura in 2013 (Figure 4D). That year, lower richness

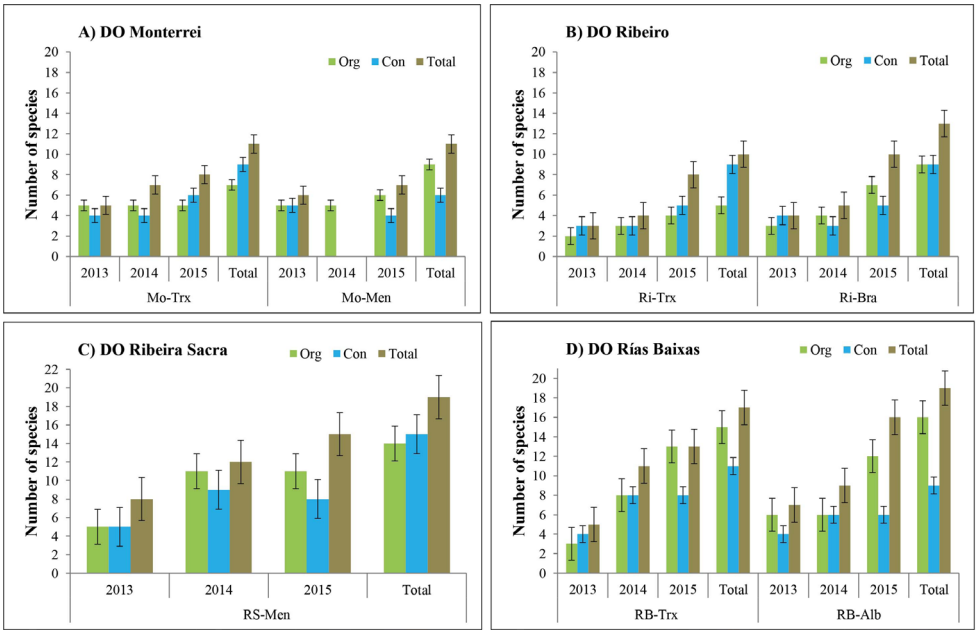


FIGURE 4. Number of yeast species in organic (Org) and conventional (Con) musts from different cultivars and DOs: A) Monterrei, B) Ribeiro, C) Ribeira Sacra and D) Rías Baixas in 2013, 2014 and 2015.

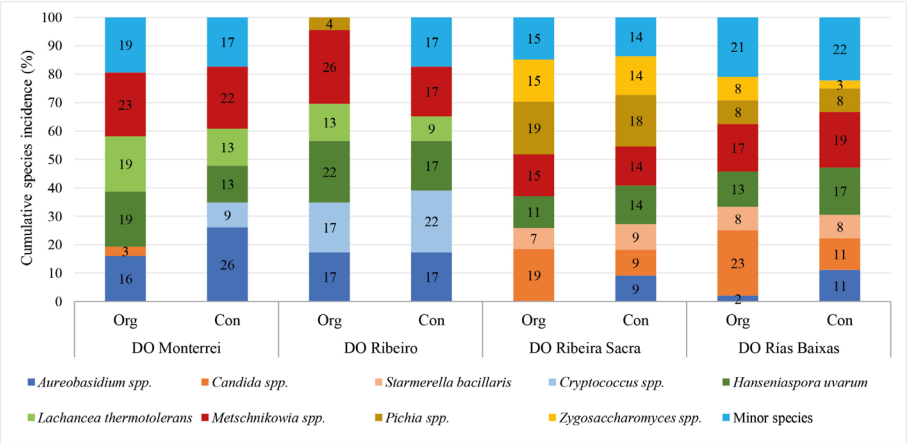


FIGURE 5. Incidence of a given yeast species in must samples from different Denominations of Origin and farming systems. Org = organic, Con = conventional. Minor species include the sum of those strains that appeared in less than 10 samples.

was observed in the organic musts, as in Treixadura from the Ribeiro DO. No richness differences were observed in 2014 in the Rías Baixas samples.

Moreover, the results showed that the highest yeast diversity was reached in Rías Baixas DO in the Albariño and Treixadura varieties under organic production (16 and 15 species respectively). The Mencía samples from Ribeira Sacra DO also showed a high species richness, with similar numbers in both culture systems (14 and 15 species in organic and conventional samples respectively). However, in the conventional samples of Rías Baixas DO, only 9 and 11 species were found in Albariño and Treixadura respectively. The latter values were similar to those obtained in the Monterrei and Ribeiro DO in terms of total richness (organic and conventional samples). Finally, the lowest values of diversity were observed in the Ribeiro DO with Brancellao (9 species) and Treixadura (5 species) under organic cultivation, while in conventional cultivation both varieties had 9 species (Figure 4).

3. Influence of the different factors on yeast species distribution

Table S1 summarises the yeast species identified in musts, including the samples in which they were present. *Aureobasidium* spp., *Metschnikowia* spp., *Hanseniaspora uvarum* were widely distributed in all DOs and vintages regardless of farming system. Moreover, the results indicated that the presence of other species was linked to the geographical location (DO) of samples (Figure 5). Thus, *Cryptococcus* spp. was found mainly in the Ribeiro DO in 2015 and to a lesser extent in DO Monterrei. *Issatchenkia terricola, Zygoascus hellenicus/meyerae, Starmerella bacillaris* and different species of *Candida, Pichia* and *Zygosaccharomyces* genera were isolated in Ribeira Sacra and Rías Baixas (Table S1, Figure 5, Figure S1). In contrast, *Lachancea thermotolerans* was found in Monterrei and Ribeiro DOs, but not in Ribeira Sacra or Rías Baixas. Within these DOs, the above-mentioned species were found in almost all samples, whereas others were scarcely isolated (Table S1, Figure S1). For instance, in Rías Baixas and Albariño *Candida apicola* and *Candida cf. sorbosivorans* were found in 2015 only, *Candida pyralidae* in 2013 and *Naganishia diffluens* in 2014*; Pichia kudriavzevii* was found in Treixadura in 2014. Species such as *Pichia membranifaciens*, *Pichia sporocuriosa, Rhodotorula nothofagi* and *Torulaspora delbrueckii* were only isolated in Mencía from Ribeira Sacra, whereas *Rhodotorula mucilaginosa* was present in the sample of Mencía from Monterrei. Despite their minor occurrence, all these species contributed to increasing the yeast diversity in those DOs.

The farming system also influenced the distribution of yeast species. Thus, *Candida* spp., *Zygosaccharomyc*es spp. and *L. thermotolerans* appeared mainly in organic samples (Figure 5). In contrast, more conventional samples than organic ones contained *Aureobasidium* spp. Regarding minor species, the results showed that *Candida bentonensis*, *C. pyralidae*, *C. cf. sorbosivorans*, *Cryptococcus victoriae, N. diffluens, P. kudriavzevii, P. sporocuriosa, R. mucilaginosa, Zygosaccharomyces bailii, Z. hellenicus/meyerae* were always isolated in the organic must samples (Table S1, Figure S1). However, species such as *Cryptococcus carnescens* and *Cr. stepossus, Cystofilobasidium macerans* (the sexual-anamorphic stage of *Cryptococcus macerans*), *R. nothofagi, T. delbrueckii* and *Saccharomyces cerevisiae* were identified in conventional must samples only.

Regarding vintage, the results showed the highest yeast diversity in 2015. A total of 30 different species were identified during this vintage, compared to 15 and 23 species found in 2013 and 2014 respectively. Thus, the Shannon indices were: H′ = 3.08 in 2015, H′ = 2.78 in 2014 and H′ = 2.18 in 2013 (Table 3). Figure 6 shows that a higher percentage of samples contained *Aureobasidium, Candida*, and *Rhodotorula* spp.

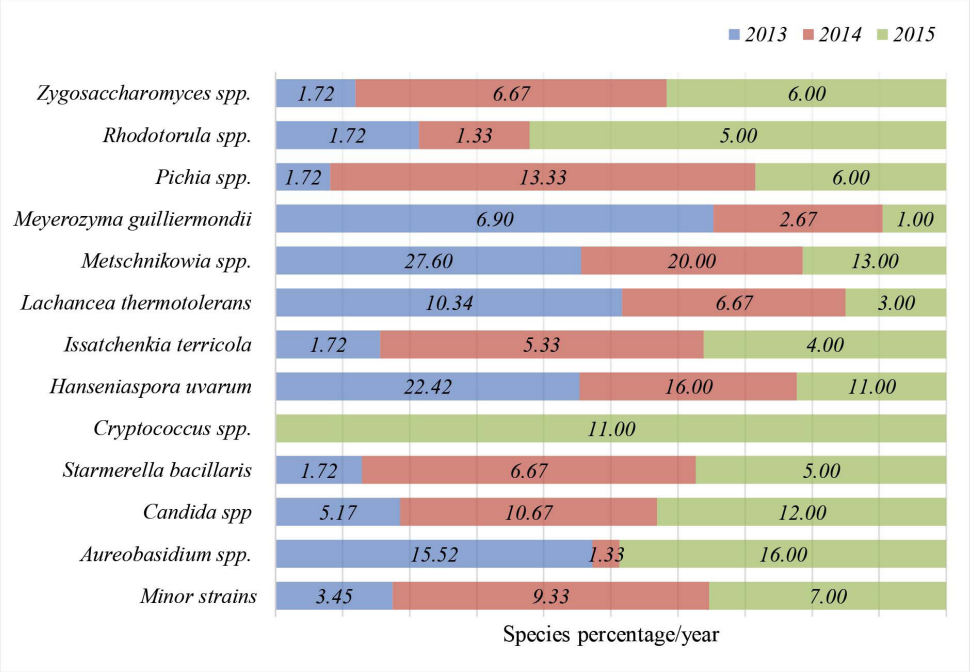


FIGURE 6. Influence of the vintage on the incidence of yeast species in must samples.

in 2015 compared to the other vintages. In addition, all *Cryptococcus* spp. found were isolated in DO Ribeiro that year. In contrast, in 2014, the incidence of fermentative species was remarkable with higher percentages of samples containing *Pichia* spp., *I. terricola*, *Starm. bacillaris* and minor strains such as *S. cerevisiae* (Figure 6, Table S1). Must samples from 2013 contained the highest percentages of the main species, including *Metschnikowia* spp., *H. uvarum* and *L. thermotolerans* (Figure 6). Most of them also contained *Meyerozyma guilliermondii* and *Aureobasidium* spp.

The Principal Component Analysis (PCA) of musts using yeast species richness was carried out to separate the samples according to their origin (DOs) and vintage. PC1 and PC2 explained 70.6 % of the variance (Figure 7). Must samples from Ribeira Sacra and Rías Baixas were mainly plotted on the positive side of PC1, which is characterised by the presence of *Starm. bacillaris* and different species of *Candida, Pichia* and *Zygosaccharomyces* genera. In contrast, all samples from Ribeiro and Monterrei DOs were located on the negative side of PC1, which is associated with a higher presence of *Aureobasidium* spp., *Cryptococcus* spp., and *L. thermotolerans*. Regarding vintages, samples from 2015 were mainly plotted in the positive part of PC2, whereas 2013 and 2014 samples were in the negative part with overlapping

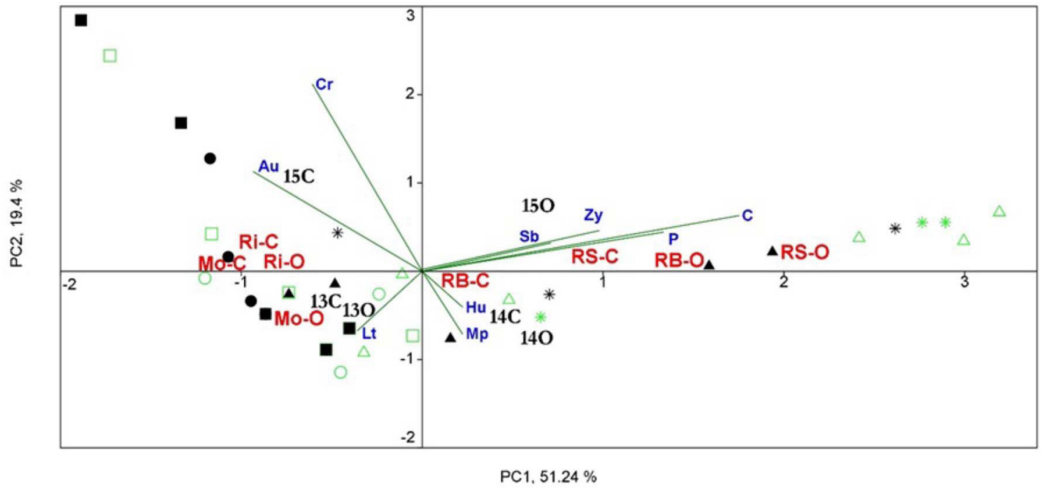


FIGURE 7. Principal component analysis (PCA) of organic (O) and conventional (C) musts based on main yeast species richness (> 5 %) in different Denominations of Origin (DO) and years. Black symbols = conventional samples; green symbols = organic samples; * = Ribeira Sacra DO; ○ = Monterrei DO; □ = Ribeiro DO; Δ = Rías Baixas DO; 13, 14 and 15 = 2013, 2014 and 2015 respectively. Species name abbreviations in blue: Au = *Aureobasidium* spp.; C = *Candida* spp.; Cr = *Cryptococcus* spp.; Hu = *H. uvarum*; Lt = *L. thermotolerans*; Mp = *Metschnikowia* spp.; P = *Pichia* spp.; Sb = *Starm. bacillaris*; Zy = *Zygosaccharomyces* spp.

areas (Figure S2). The species richness did not enable the discrimination between organic and conventional samples that had a disperse distribution in the plot.

4. Statistical evaluation of the impact of different factors on species richness

The analysis of the DO-Farming System-Variety data from all three years by one-way ANOSIM showed significant differences among them at a general level ($p = 0.0033$, $R = 0.1471$), but not between them pairwise.

The statistical analysis using one-way ANOSIM proved the existence of significant differences in species richness among vintages at a general level ($p = 0.0014$, $R = 0.1724$). Similarly, differences were also found between years (binomial), when all years were compared pairwise even with the Bonferroni correction: 2013-2014 ($p_{Bonferrroni} = 0.0273$, $R = 0.1715$), 2014-2015 ($p_{Bonferrroni-} = 0.0396$, $R = 0.1754$) and 2013-2015 ($p_{Bonferrroni} = 0.0036$, $R = 0.1877$). The largest differences were found in descending order in the 2013-2015, 2013-2014 and 2014-2015 pairwise. However, the R values were not very high ($R < 0.2$), indicating that vintage is not a very strong factor in terms of its influence on species richness.

In addition, the data analysis revealed that the geographical location of samples (Denomination of Origin) had a stronger effect on species richness than vintage ($p = 0.0001$, $R = 0.3364$), which confirms the biogeographical character of yeasts. Thus, significant differences were found between all DOs except for the pairwise Rías Baixas - Ribeira Sacra (Table 4). The pairwise Ribeira Sacra-Monterrei, Ribeira Sacra-Ribeiro and Rías Baixas-Monterrei had the lowest similarity (highest R value > 0.4).

Regarding the farming systems, the analysis of the overall data did not show any significant differences for species richness between organic and conventional musts ($p = 0.1624$; $R = 0.0734$). Nor were differences observed in species richness between cultivars ($p = 0.4370$; $R = 0.0037$). However, when farming system and DO factors were grouped, the results evidence differences in a general comparison ($p = 0.0001$) and in many of the pairwise comparisons (Table S2). This approach revealed significant differences between Monterrei or Ribeiro when compared to Ribeira Sacra or Rías Baixas for must samples under both culture systems. No differences were found between Monterrei and Ribeiro or between Ribeira Sacra and Rías Baixas. Therefore, we suggest that the differences in yeast species are due to the geographical location, since they follow the pattern of this dominant factor and because no differences were found between org-con within the same DO. Similarly, when the factors farming system and vintage were grouped, the ANOSIM results evidenced differences at a general level ($p = 0.0041$). In the pairwise comparison, these differences were observed when 2015 was compared to 2013 and 2014 and between conventional samples from 2013 and 2014 (data not shown).

Furthermore, the effect of two factors simultaneously on species richness was studied by two-way ANOSIM. The results evidenced significant differences among DO and vintages, but not between the farming systems in any of the cases. The influence of the geographical location of the samples was stronger than the vintage and cultivation system. The analysis also confirmed an interaction between vintage and DO, but not between these factors and the farming system.

DISCUSSION

Yeast diversity in grapes and musts is influenced by many factors, including geography, climatic conditions, grapevine variety, agronomic practices and other human activities (Barata *et al*., 2012; Drumonde-Neves *et al*., 2016; Grangeteau *et al*., 2017b). This work evaluated the yeast population in grape musts from different DOs in Galicia in three consecutive years and how it was affected by vineyard location, vintage and farming system. We used culture-dependent methods; therefore, it is important to bear in mind that some yeast diversity, such as rare or slow-growing species, as well as non-culturable yeasts, might have been lost. Although this type of approach does not provide a full picture of yeast communities, it allows yeast diversity among different vineyards, vintages and culture systems to be compared (Drumonde-Neves *et al*. 2017).

At a quantitative level, the values of viable yeast were within a wider range (10^3 and 10^7 cells/mL) than the typical counts for fresh grape juice (10^4-10^6) (Bagheri *et al*., 2015; Combina *et al*., 2005; Milanović *et al*., 2013; Zott *et al*., 2008). The data analysis indicates that vintage and DO had a stronger influence than farming system on yeast counts. The high

TABLE 4. Analysis of similarity (One-way ANOSIM) on species richness between Denominations of Origin: Monterrei, Ribeiro, Ribeira Sacra and Rías Baixas.

R\ p-value	Monterrei	Ribeiro	Ribeira Sacra	Rías Baixas
Monterrei		*0.0394*	*0.0003*	*0.0001*
Ribeiro	0.0959		*0.0042*	*0.0003*
Ribeira Sacra	0.8307	0.4321		0.2000
Rías Baixas	0.4967	0.2973	0.0661	

Analyses were performed with 9999 permutations. ANOVA: mean rank (within: 306.6 and between: 444.5); the level of significance (p-values) is shown above the diagonal (italics indicates significant differences ($p < 0.05$ between groups) and Statistical R- is shown below the diagonal.

number of yeasts in samples from 2014 was probably related to the poor sanitary conditions of grapes in that vintage due to the adverse climatic conditions at harvest time. Indeed, the climatic data showed that the highest precipitation values were recorded in 2014 (Table 1). It is known that in damaged grapes there is an increase in nutrient availability on the grape surface; therefore, the proliferation of microorganisms is favoured (Combina *et al*., 2005). Accordingly, several authors have associated rainy vintages with higher yeast counts on grapes (Čadež *et al*., 2010; Combina *et al*., 2005; Longo *et al*., 1991), but the opposite has also been observed (Comitini and Ciani, 2006; Rementeria *et al*., 2003); an increase in antifungal treatments during rainy campaigns could explain this reduction in yeast population.

The farming system also influences the yeast population in grape musts. We observed that its impact varied depending on the location of the vineyard, vintage and grape cultivar. In general, most organic musts contained a higher number of viable yeasts than their conventional counterparts, in agreement with data reported previously (Bagheri *et al*., 2015; Comitini and Ciani, 2008; Martins *et al*., 2014); however, no significant differences were found in 75 % of the comparisons, as observed by other authors (Escribano-Viana *et al*., 2018; Setati *et al*., 2012). In contrast, other studies have reported lower values for yeast populations in biodynamic production depending on the grape variety, and in organic vineyards compared to traditional ones (Guzzon *et al.*, 2016; Milanović *et al.*, 2013). These differences have been attributed to the sensitivity of grape yeasts to copper and sulfur, which are the only permitted fungicides in organic viticulture (Grangeteau *et al*., 2017a; Milanović *et al*., 2013).

The characterisation of yeast population in musts at a qualitative level, including both number and type of yeast species, produced some interesting results. Total species richness (S) was higher in musts of organic origin than in those of conventional origin mainly due to Rías Baixas samples, which also showed this trend and was the DO with the highest species richness (Figure 3B). However, Monterrei and Ribeira Sacra contained a similar number of species between samples from different agronomic systems; in Ribeiro, conventional must samples achieved higher species richness than organic ones. Moreover, the general yeast diversity of the vineyards was high (H′ = 3.02; 1-D = 0.923; 1/D = 13.03) compared to other similar studies (Bagheri *et al.*, 2015; João Drumonde-Neves *et al.*, 2016; Vigentini *et al.*, 2015).

Accordingly, the results reported by different authors in studies on the influence of farming system on yeast diversity are also contradictory. We found higher diversity in the organic samples (H′ = 2.98) than in the samples from conventional production (H′ = 2.85). Several studies found that organic and/or biodynamic practices favoured yeast diversity compared to conventional management (Agarbati *et al*., 2019a; Bagheri *et al*., 2015; Cordero-Bueso *et al*., 2011; Martins *et al*., 2014; Morrison-Whittle *et al*., 2017; Setati *et al*., 2012; Tello *et al*., 2011). In contrast, some authors have found greater yeast abundance in conventional grapes (Grangeteau *et al*., 2017b; Milanović *et al*., 2013), while Guzzon *et al*. (2016) found that the differences in microbial diversity appeared to be more related to grape variety than to agricultural practice. Thus, the influence of other factors, such as geographical location, vintage, climatic conditions and grape cultivar, could explain these findings and our contradictory ones. Setati *et al*. (2015) suggested that sampling method, identification techniques and intra-vine variation could also contribute to such differences.

Regarding the type of yeast species, the results showed *Metschnikowia* spp. and *H. uvarum* to be widely distributed in samples from all DOs and vintages, regardless of farming system. These species have been described as being normal microbiota associated with mature grapes (Drumonde-Neves *et al*., 2017; Garofalo *et al*., 2016; Nemcová *et al*., 2015; Prakitchaiwattana *et al*., 2004). In contrast, Milanović *et al*. (2013) reported the presence of *H. uvarum* in both culture systems, while *Metschnikowia pulcherrima* was isolated mainly in conventional samples. *Aureobasidium* spp., also appeared in all DOs, although it was identified in a higher number of samples in Monterrei and Ribeiro than in Ribeira Sacra and Rías Baixas, and particularly associated with conventional samples as reported by other authors (Agarbati *et al*., 2019a; Agarbati *et al*., 2019b; Comitini and Ciani, 2008). In contrast, Martins *et al*. (2014) associated *Aureobasidium* spp. with organic samples. Some species showed a distribution pattern that corresponded with the geographical origins of the samples, confirming our previous observation regarding a particular vintage, for which type of species in the samples, in addition to its abundance, were considered (Castrillo *et al*., 2019). Here, the pattern of a yeast species associated with a given DO was observed during all vintages under study. Thus, *I. terricola, Z. hellenicus/meyerae, Starm. bacillaris* and different species of *Candida, Pichia* and *Zygosaccharomyces* genera were isolated in Ribeira Sacra and Rías Baixas and found to contribute to increasing yeast diversity in those DOs. In contrast, *L. thermotolerans* was found in Monterrei and Ribeiro DOs, especially in the organic samples. Pinto *et al*. (2015) also found *Lachancea* to be differentially associated with grape growing regions from Portugal. Other studies have reported the presence of *K. thermotolerans* (synonym *L. thermotolerans*) as the most abundant species in vineyards from Madrid, Spain (Cordero-Bueso *et al*., 2011; Tello *et al*., 2011), regardless of the farming system. Agarbati *et al*. (2019a) and Agarbati *et al*. (2019b) isolated *L. thermotolerans* only in must from conventional treatments; however, they found species such as *Starm. bacillaris* and *Z. meyerae* in the organic or non-treated musts, as we also observed in musts from Galicia. In particular, *Starm. bacillaris* have been associated with high-sugar grape musts or botrytised grapes (Bagheri *et al*., 2015; Milanović *et al*., 2013; Sipiczki, 2003; Tofalo *et al*., 2009). In our study, the incidence of this species was higher in 2014 (Figure 5), being favoured by the poor sanitary conditions of grapes, which were damaged or had a higher incidence of botrytis

due the adverse climatic conditions close to harvest time. Drumonde-Neves *et al*. (2017) reported *Starm. bacillaris* and *Pichia terricola* (syn. *I. terricola*) at high frequencies in vineyards in the Azores Archipelago. The presence of minor species, such as *T. delbrueckii*, was influenced by the farming system; this yeast was only isolated in Ribeira Sacra and always from conventional sampled as reported by several authors (Agarbati *et al*., 2019a; Bagheri *et al*., 2015; Cordero-Bueso *et al*., 2011). As already mentioned regarding viable counts, the differentiation in the incidence of certain species could be due to the impact of copper/sulfur treatments in organic farming, as well as to a possible interaction between species (Grangeteau *et al*., 2017b).

Regarding vintages, the highest number of species was observed in 2015 for all DOs when the lowest counts of viable yeast were obtained. These results indicate that the number of viable yeasts in must was not correlated with species richness. In addition, the species richness in the samples also varied among vintages: while 2013 was characterised by less species diversity, a higher number of samples contained the dominant species *Metschnikowia* spp., *H. uvarum* and *L. thermotolerans*. In 2014, more samples contained fermentative species (*Pichia* spp., *I. terricola*, *Starm. bacillaris* and minor strains, such as *S. cerevisiae*) associated with the poor sanitary conditions of the grapes in this campaign; they also contained higher amounts of cells due to nutrient availability which favoured the growth of this type of yeasts (Barata *et al*., 2012). In contrast, in 2015 a higher percentage of samples contained *Aureobasidium* spp., *Candida* spp., and *Rhodotorula* spp. Moreover, all the *Cryptococcus* spp. that were found were isolated in DO Ribeiro from the 2015 vintage. Differences in yeast diversity among consecutive years have been previously reported (Bagheri *et al*., 2015; Combina *et al*., 2005). and Vigentini *et al*. (2015) demonstrated that vintage has a more significant impact on yeast community than terroir. Such variations among vintages have been attributed to changes in climatic conditions (Combina *et al*., 2005; Drumonde-Neves *et al*., 2017). The vineyards sampled in the present study are located in different areas of Galicia; therefore their characteristics (including climatic conditions, Table1), are quite heterogeneous depending on the area and vintage. During our study, there was a significant increase in rainfall in 2014, especially on the dates close to the harvest. Accordingly, the amount of viable yeast in musts was higher in that year compared to the previous one and to 2015. Moreover, higher yeast diversity was observed in areas with more rainfall, such as Rías Baixas (Vélez *et al.,* 2021). Previous studies within this area have also evidenced a significant increase in the number of species during the vintage with more rainfall (Longo *et al*., 1991). However, while we found no correlation between rainfall and species richness (for instance, the year with the highest precipitation (2014) did not show the highest overall S), species richness was positively correlated with the temperature, which was related to the rainfall factor ($r = 0.81$). The propagation of correlations between factors suggests that other factors are involved. For instance, Vélez *et al*. (2021) found the existence of a positive linear correlation or stable trend over the years (Pearson's $r = 0.39$, $p = 0.01$) between vegetative development (NDVI) and species richness for each DO, both influenced by higher mean rainfall and temperature. Other authors claim that the reports on species diversity are not conclusive in relation to rainfall in large scale studies (Barata *et al*., 2012): increased rainfall has been found to involve more fungicide treatments which affect yeast population (Grangeteau *et al*., 2017b; Milanović *et al*., 2013); meanwhile, years with higher rainfall close to harvest have been observed to favour nutrient availability on the grape surface and, therefore, the proliferation of microorganisms (Combina *et al*., 2005).

The statistical analyses of the data confirmed that both the geographical location of the samples and the vintage, and their interaction, significantly impacted the richness and distribution of yeast species in musts. However, the influence of the farming system was not significant. Likewise, no differences have been found in terms of yeast species richness in grapes between cultivation systems in previous studies; nonetheless, when the abundance of a given yeast species was considered, the differences were significant for both DO and farming system, especially within DO Ribeiro (Castrillo *et al*., 2019; Vélez *et al*., 2021). Morrison-Whittle *et al*. (2017) also found differences for both type and abundance of fungi in samples from different habitats in the vineyard, but not between types of system management for juice.

Finally, it is worth mentioning the concept of *terroir* which involves climate, soils, grape varieties, viticulture and oenological practices in each region. All these factors have been correlated with the fungal and bacterial community of grapes and have confirmed the existence of a *microbial terroir* (Bokulich *et al*., 2014; Grangeteau *et al*., 2017b). Furthermore, the microbial diversity found in the vineyard is considered to be responsible for the regional character of wines (Belda *et al*., 2017; Bokulich *et al*., 2016; Drumonde-Neves *et al*., 2017; Knight *et al*., 2015). This study evidenced the influence of geographical location and vintage on yeast communities in musts in Galicia. Regarding the biogeographical factor, similar and higher diversity was observed in the RB-RS DO pairwise ($H' = 2.83$ and 2.74 respectively) than in the Ri-Mo DO pairwise ($H' = 2.33$ and 2.18 respectively) in agreement with previous studies (Castrillo *et al*., 2019). The existence of a geographical pattern of yeast distribution may contribute to the distinctive characteristic of wines from a given DO. Although to a lesser extent, the farming system also affected the presence of specific yeast species and their occurrence. These findings reinforce our previous results that indicate that the biogeographical factor has a greater influence than the culture system on yeast species richness (Castrillo *et al*., 2019). Cordero-Bueso *et al*. (2011) reported that organic treatments favoured the presence of fermentative yeasts. Accordingly, in the present study, the use of culture-dependent techniques allowed species with oenological potential to be isolated, such as *Metschnikowia* spp., *Starm. bacillaris*, *L. thermotolerans,*

T. delbrueckii and *Pichia* spp. Further evaluation and application of these species would reinforce the regional characteristics of wines.

CONCLUSIONS

Our results indicate that the geographical location of a vineyard has a significant influence on yeast populations in musts at a quantitative and qualitative level. The effect of vintage on yeast species richness is also significant but to a lesser extent than location, whereas no significant differences were observed regarding agricultural management and grapevine variety.

ACKNOWLEDGEMENTS

This study was funded by the RTA2012-00021-C03-01 project of INIA (Instituto Nacional de Tecnología Agraria y Alimentaria) co-financed with FEDER funds (European Regional Development Fund). David Castrillo wishes to thank INIA and the European Social Fund for his FPI predoctoral contract. We would like to thank the wine producers Esther Teixeiro Lemos, Roberto Regal, Cooperativa Cume do Avia, Víctor Diéguez, Begoña Troncoso, María del Carmen Álvarez, José Luis Mateo and José dos Santos Diz for allowing us to collect samples from their vineyards. We also thank Ana Lamas, Mónica Cid and Mar Sánchez for their technical assistance in the vineyard and laboratory.

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