Yeast diversity on grapes from Galicia, NW Spain: biogeographical patterns and the influence of the farming system

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ABSTRACT

Aim: Organic viticulture has increased in recent decades, worldwide. Farming system influences grapes’ yeast populations and, therefore, fermentation dynamics and wine quality. This work aims to study yeast diversity on grapes and musts from organic and conventional production in different areas within Galicia (NW Spain) and to evaluate the existence of yeast geographic patterns (microbial terroir) in this region.

Methods and results: During the 2015 vintage, 42 grape samples were collected from four Denominations of Origin (DOs) in Galicia. Representative cultivable yeasts from grapes and must samples were isolated and characterised at the species level in the Estación de Viticultura e Enoloxía de Galicia (EVEGA-AGACAL). Results were compared among DOs and between organic and conventional production. A total of 33 yeast species were identified. Organic production showed 27.3% higher species richness than conventional production. Accordingly, diversity indexes (H’ and E) were higher under organic cultures compared to conventional production. The highest yeast diversity was found in Rías Baixas and Ribeira Sacra DOs. The predominant yeasts were Aureobasidium spp., Metschnikowia spp., Hanseniaspora uvarum and Cryptococcus spp., although their proportion varied depending on the farming system and location. Furthermore, important differences were observed in minor species, which appeared mainly in organic cultivation. In addition, a biogeographic pattern was found: Issatchenkia terricola and Starmerella bacillaris were isolated in Rías Baixas and Ribeira Sacra DOs, whereas Lachancea thermotolerans was ligated to Monterrei and Ribeiro DOs. ANOSIM, PERMANOVA and PCA analysis confirmed this differentiation.

Conclusions: The results indicated that an organic farming system favoured yeast diversity on grapes and musts. Moreover, species distribution made it possible to establish a biogeographic pattern in the yeast population that could be associated to a particular microbial terroir and wine typicality.

Significance and impact of the study: This study reports for the first time a survey on yeast diversity on grapes and musts from organic and conventional production in different areas within Galicia. The results highlighted the role of organic culture as a tool to preserve yeast richness and the existence of biogeographic patterns of yeast communities in vineyards in NW Spain, which could contribute to wine typicality of specific areas.

KEYWORDS

organic vineyards, grapes and musts, yeast diversity, biogeographic patterns, microbial terroir, Galicia

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INTRODUCTION

Organic viticulture is increasingly popular around the world because organic practices are more respectful to the environment and health (Azabagaoglus et al., 2007; Fragoulis et al., 2009; Mariani and Vastola, 2015). Spain is the largest organic vineyard in the world with the greatest growth during the last decade (European Commission, 2016; Willer and Lernoud, 2017). The wine industry plays an important economic and social role in Galicia (NW Spain); however, certified organic production methods are applied in less than 0.5% of the total area of Galicia vineyard (78.2 ha; 36 operators, of which 16 are processors) (CRAEGA, 2017). A wide climatic and orographic heterogeneity in Galicia favours the proliferation of vine pathogens and results in irregular yields as well as problems with pest management (Fernández-González et al., 2012; Fraga et al., 2014), which leads to a difficult and expensive implementation of organic viticulture.

Yeast diversity associated with grape berries has been widely studied (for a review see Barata et al., 2012). Diversity and frequency of yeast populations on grapes are influenced by several factors including agronomical practices, climatic conditions, anthropogenic and biotic factors, maturation process and grapes sanitary conditions (Comitini and Ciani, 2008; Cordero-Bueso et al., 2011; Drumonde-Neves et al., 2016 and 2017; Garofalo et al., 2016; Grangeteau et al., 2017; Nemcová et al., 2015; Renouf et al., 2005). In particular, the influence of the farming system on yeast diversity has been reported in vineyards from several countries. The results varied among different studies and they are sometimes contradictory. Whereas some authors found a wider yeast diversity in organic vineyards (Bagheri et al., 2015; Cordero-Bueso et al., 2011; Martins et al., 2014; Setati et al., 2012 and 2015; Tello et al., 2012), others reported higher yeast abundance in conventional grapes (Grangeteau et al., 2017; Milanović et al., 2013), while Guzzon et al. (2015) found no significant differences.

The application of more natural and environmentally friendly techniques in wineries implies an increase in the use of spontaneous fermentations for organic wine elaboration. These practices are believed to contribute to creating wines with unique terroir-related characteristics. The concept of terroir involves a number of factors, including climate, soils, grape varieties, "FIGURE 1. Denominations of Origin in Galicia and climatic zones: I, coastal oceanic climate; II, indoor oceanic climate; III, mediterranean oceanic climate; IV, mountain oceanic climate. Location of sampling sites."
viticulture and oenological practices in a particular region. Furthermore, geographical differences found in vineyard yeast diversity have been associated with a microbial terroir responsible for distinct characteristics of wines from a given region (Belda et al., 2017; Bokulich et al., 2014 and 2016; Drumonde-Neves et al., 2017; Nemcová et al., 2015; Setati et al., 2015). Therefore, the knowledge of yeast distribution patterns and the factors that influence their structure and diversity is of great importance because yeasts represent a valuable resource for the production of differentiated wines (Capozzi et al., 2015; Tofalo et al., 2013). In this sense, organic vineyards and wineries have been proposed as natural reservoirs of fermentative yeasts (Cordero-Bueso et al., 2011; Tello et al., 2012). Under these conditions, yeast biodiversity could be a competitive advantage in organic musts contributing to a more complex flavour and aroma in wines due to the role of non-Saccharomyces species involved (Capozzi et al., 2015; Ciani and Comitini, 2015; Tofalo et al., 2016).

Despite the importance of wine sector in Galicia, the information available about microorganisms associated with grapes and/or wineries is scarce. Data about yeast diversity during spontaneous fermentations evidenced differences between species during fermentations from the Atlantic and the interior regions, and also between vintages (Longo et al., 1991). Apart from this, the influence of Saccharomyces and non-Saccharomyces yeast on the wine aroma profile has been reported (Lema et al., 1996). Previous studies in our laboratory have been focused on strain diversity of Saccharomyces cerevisiae in an experimental winery (Blanco et al., 2006). However, more research is required about yeast population associated with grapes from different areas of Galicia as well as the influence of the farming system on yeast diversity.

This study, carried out at Estación de Viticultura e Enoloxía de Galicia (EVEGA-AGACAL), reports for the first time a survey on yeast diversity in grapes and musts from organic versus conventional production in different areas within Galicia. It also verified the existence of biogeographic patterns of yeast populations in NW Spain vineyards.

**MATERIALS AND METHODS**

**1. Origin of grapes and sampling design**

Grapes were collected from vineyards within four Denominations of Origin (DO) from Galicia in 2015 before harvest (9 to 14 of September) (Figure 1). Representative grapevine varieties grown in Galicia were chosen: Albariño and Treixadura as white varieties and Brancellao and Mencía as red varieties. Table 1 shows the

| DO            | Grapevine variety | Farming system* | Code    | Coordinates  | Elevation (m) | Rainfall** (mm) |
|---------------|-------------------|-----------------|---------|--------------|---------------|-----------------|
| Monterrei     | Treixadura        | Org             | Mo-Trx  | 41°52'11.9"N, 7°25'49.7" W | 406           |                |
|               |                   | Con             |         | 41°52'12.8"N, 7°25'54.5" W | 402           |                |
|               | Mencia            | Org             | Mo-Men  | 41°52'11.0"N, 7°25'51.9" W | 404           |                |
|               |                   | Con             |         | 41°52'12.5"N, 7°25'56.9" W | 399           |                |
| Ribeiro       | Brancellao        | Org             | Ri-Bra  | 42°19'24.8" N, 8°6'7.8" W  | 270           |                |
|               |                   | Con             |         | 42°19'22.3" N, 8°5'51.7" W | 290           |                |
|               | Treixadura        | Org             | Ri-Trx  | 42°19'27.6" N, 8°6'2.0" W  | 278           |                |
|               |                   | Con             |         | 42°19'22.0" N, 8°5'52.4" W | 289           |                |
| Ribeira Sacra | Mencía            | Org             | RS-Men  | 42°34'11.5" N, 7°43'3.4" W | 243           |                |
|               |                   | Con             |         | 42°34'11.4" N, 7°43'3.5" W | 244           | 719             |
| Rías Baixas   | Albariño          | Org             | GB-Alb  | 42°54'7.9" N, 8°21'18.1" W | 74            |                |
|               |                   | Con             |         | 42°54'6.8" N, 8°21'16.9" W | 72            |                |
|               | Treixadura        | Org             | RB-Trx  | 42°54'8.2" N, 8°21'15.1" W | 75            |                |
|               |                   | Con             |         | 42°54'6.2" N, 8°21'17.7" W | 71            |                |

* Org, organic farming; Con, conventional farming. ** Average annual (2015) rainfall
location of vineyards, grapevine varieties sampled in each DO and their codes, plus the average annual rainfall and elevation for each vineyard. Date of grapes collection and maturation characteristics for each grapevine variety are summarised in Table S1.

For each variety, plots bordering or close to each other, but employing different farming systems, (organic and conventional) were selected. Organic producers used phytosanitary products according to legislation, which were mainly cooper, sulphur and plant extracts. In contrast, conventional producers applied these compounds and also synthetic fungicides to their vineyards. In every plot, three representative random blocks were established. Around 4 kg of grape bunches per variety were collected from at least 8 to 10 vines from each block.

2. Sample processing and yeast isolation

Grape samples were collected, transported to the laboratory in individual sterile plastic bags and processed within 2 h. A total of 42 samples were collected. To isolate yeast from the grapes, 20 healthy whole berries were aseptically separated from random bunches in the sample. Then the grapes were placed into sterile flasks with 100 mL of 2 % w/v buffered peptone water (Scharlau Microbiology, Barcelona, Spain) and incubated in an orbital shaker at 120 rpm for 2 h to dislodge yeast cells from the surface of the grape berries. In order to obtain must, the grapes were aseptically hand-destemmed and crushed. Both (grape washing solution and must) samples were diluted (10⁻¹ to 10⁻⁵) and 100 μL were homogeneously spread in duplicate on WL Nutrient Agar medium (Scharlau Microbiology) supplemented with 200 μg/mL biphenyl (Acros Organics by Thermo Fisher Scientific, Madrid, Spain) and 34 mg/L chloramphenicol (Merck KGaA, Darmstadt, Germany) to suppress moulds and bacteria, respectively. Plates were incubated at 28 °C until visible colonies appeared; those containing between 20 and 200 colonies were used for quantification of total viable cells. A representative number of yeasts from each sample were isolated in YEPD medium (1 % w/v yeast extract, 2% w/v peptone, 2 % w/v glucose, and 2 % w/v agar; all components were from Scharlau Microbiology) based on their colony aspect and frequency (10–20 for each sample). The isolates obtained were maintained at -80 °C in YEPD with 15 % (v/v) glycerol until further identification.

3. Molecular identification of yeast isolates

Yeast identification at species level was performed by PCR amplification of the 5.8S rRNA gene and the two internal (non-coding) ITS1 and ITS2 spacers using the ITS1 and ITS4 primers according to the method described by Esteve-Zarzoso et al. (1999).

PCR amplification and sequencing of D1/D2 region of 26S rDNA gene was used to confirm yeast identification. The D1 and D2 domains were amplified using NL-1 and NL-4 primers as described by Kurtzman and Robnett (1998). PCR products were purified using the PCR Extract Mini kit (5PRIME) according to the supplier’s instructions (Fisher Scientific S.L., Madrid, Spain). The same primers were used for sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems by Thermo Fisher Scientific, Madrid, Spain) and a 3130x1 Genetic Analyzer (Applied Biosystems). Sequences similarities were obtained using GenBank BLASTN search facilities (Altschul et al., 1990). The identification was considered correct when the gene sequences identity was 98% or higher.

4. Statistical analysis of biodiversity

Yeast species diversity was estimated using different classical ecology indexes. The Shannon-Wiener index (H') (Shannon, 2001) was calculated as follows:

$$H' = -\sum_{i=1}^{S} p_i \ln(p_i)$$

where $S$ is the species richness (number of species found) and $p_i = n_i/N$ is the relative abundance of species, calculated as the proportion of individuals ($n$) of species $i$ with respect to the total of individuals ($N$).

Simpson’s diversity index ($1 – D$) to evaluate yeast species dominance was calculated as follows (Simpson, 1949):

$$1 - D = 1 - \sum_{i=1}^{S} (p_i)^2 = 1 - \sum_{i=1}^{S} \left(\frac{n_i(n_i-1)}{N(N-1)}\right)$$

where $D$ is the Simpson’s index and $S$ and $p_i$ as described above.

Finally, in order to calculate the similarity or evenness of the different species abundance, a measure of Equitability (E) was calculated as follows (Pielou, 1969):

$$E = H/\ln(S) = H/H_{max}$$
Differences in yeast populations between DOs and production systems were tested by one and two-way ANOSIM (non-parametric analysis of similarity) (Clarke, 1993) and PERMANOVA (permutational multivariate analysis of variance using distance matrices) (Anderson, 2001), using the PAST 3.20 (2018) software. In addition, the similarity percentages breakdown (SIMPER) between production systems/DOs was calculated to assess the average contribution of individual species to dissimilarity (Clarke, 1993). The Bray-Curtis similarity index with 9999 permutations was used in the tests, and in some cases, Bonferroni correction was also applied. Principal component analysis (PCA) was carried out by PAST 3.20, using the species frequencies to separate samples according to DOs and culture systems.

RESULTS

1. Influence of the farming system on yeast species richness

Representative yeasts were isolated from each sample and identified using genetic techniques. These isolates belonged to 33 different yeast species within 18 genera. Of those, 30 species were identified in musts and 24 on grapes. The results showed that total yeast richness ($S$) was higher on grapes from organic origin than conventional origin for all samples collected (Figure 2A). Musts followed the same trend, although two exceptions were found (samples from Treixadura in Monterrei and Ribeiro DOs) (Figure 2B). Considering the percentage of species with respect to the total (33 species), organic production exhibited 27.3% higher species richness than conventional production:

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** Yeast diversity, expressed as species richness $S$ (number of species), found in organic and conventional samples from different DOs. A) grapes, B) musts. $g+m =$ grapes + musts.
21.2% on grapes and 18.2% in musts. Within each group these percentages increased to 29.2% and 20.0% for grapes and musts, respectively. Moreover, the results evidenced that the greatest yeast diversity was reached in musts from Rias Baixas DO under organic production; Ribeira Sacra DO also presented a high species richness (Figure 2B). In contrast, the lowest values of richness were observed in Ribeiro DO, especially with Brancellao grapes.

2. Frequency of yeast species under different farming system: geographical patterns

Frequencies of yeast species identified on grapes and in musts samples are summarised on Tables S2 and S3, respectively. Metschnikowia spp., Hanseniaspora uvarum, Cryptococcus spp., and yeast-like Aureobasidium spp. were the predominant species in all samples, although their frequency varied depending on the farming system and DO. Grape samples showed higher frequency of Aureobasidium and Cryptococcus genus compared to musts. In general, yeast-like Aureobasidium spp. reached higher frequencies in conventional grapes samples and Cryptococcus spp. in the organic samples, for instance, Cryptococcus terrestris in organic Brancellao (65.3%) (Figure 3). In contrast, an increase of fermentative yeast percentage in must samples was observed (Table S2 and S3, Figure 3).

Considering the total results for organic musts, the three predominant species H. uvarum, Metschnikowia spp. and Aureobasidium spp. showed a similar proportion (22.8%, 17.6% and

FIGURE 3. Cumulative yeast species richness (S) in organic and conventional samples from different Galician DOs (Mo, Monterrei DO; Ri, Ribeiro DO; RS, Ribeira Sacra DO; RB, Rias Baixas DO) and grapevine varieties (Trx, Treixadura; Men, Mencia; Bra, Brancellao; Alb, Albariño). A) grapes; B) musts.
However, in conventional musts their proportion was different, with a higher incidence of *Aureobasidium* spp. (45.2%) (Table S3). *Metschnikowia* spp. and *H. uvarum* were also widely distributed in musts and, to a lesser extent, on grapes, in both culture systems. As an exception, grapes from Ribeira Sacra presented a high frequency of genus *Metschnikowia*.

In addition, the results evidenced a geographical pattern of yeast species, which allowed a clear differentiation between Monterrei-Ribeiro DOs and Ribeira Sacra-Rías Baixas DOs (Figure 3). Thus, yeast-like *Aureobasidium* spp. reached very high proportions in Monterrei and Ribeiro DOs, especially on grapes (Table S2; Figure 3A). In organic musts, the presence of *Aureobasidium* spp. was lower and increased the frequency of *L. thermotolerans*, which reached 32.6% and 31.3% in Monterrei and Ribeiro organic samples, respectively (Table S3; Figure 3B). Likewise, *Metschnikowia* spp. were found at high proportions in Monterrei must samples, whereas *Candida apicola* reached 27.1% in Mencía from this DO. The presence of *Metschnikowia* spp. was also important in Ribeiro DO (26.3%), together with *Cryptococcus* spp. in organic musts (Table S3).

In contrast, yeast-like *Aureobasidium* spp. was found at lower frequencies (even it was absent in several musts) in Ribeira Sacra and Rías Baixas DOs, except in Albariño grapes (Figure 3). *H. uvarum* was the predominant yeast species isolated in musts and grapes within these DOs (Tables S2 and S3; Figure 3), at proportions higher in organic than in conventional samples, except on grapes from Treixadura. A remarkable presence of *D. hansenii* (58.7%) was found in organic Treixadura grapes from Rías Baixas DO. *Candida* spp. and *Starm. bacillaris* (formerly *C. zemplinina*) reached a high frequency in musts, especially due to its presence in Treixadura (34.1%) and Albariño (12.4%) organic samples. This later one was important in conventional Treixadura musts from Rías Baixas DO (28.2%) and Mencia from Ribeira Sacra DO (14.5%) (Table S3). *Metschnikowia* spp. and *I. terricola* also appeared at frequencies higher than 10% in musts within these DOs. Regarding Ribeira Sacra, it is worth noting that the proportion of *Metschnikowia* spp. reached 42.1% and 28.0% in conventional and organic grapes, respectively. *Pichia kluyveri* was recorded in both grapes and musts, mainly in organic grapes (17.2%).

Finally, the farming system influenced the distribution of minor species, which were more abundant under organic production. Thus, *Candida bentonensis*, *Candida oleophila*, *Candida cf. sorbostivorans*, *Pichia sporocuriosa*, *Pichia membranifaciens* and *Zygosaccharomyces bailii* were isolated always in organic samples.

**TABLE 2.** Biodiversity indexes in organic (Org) and conventional (Con) grape and must samples from different Galician DOs and grapevine varieties.

| DO-Grapevine variety | Farming system | H’ | 1 – D | E   | H’ | 1 – D | E   |
|----------------------|----------------|----|-------|-----|----|-------|-----|
|                      |                | Grape samples | Must samples |
| **Mo-Trx**           | Org            | 0.85          | 0.46          | 0.62          | 1.11 | 0.62          | 0.80          |
|                      | Con            | 0.67          | 0.36          | 0.61          | 1.07 | 0.50          | 0.60          |
| **Mo-Men**           | Org            | 1.10          | 0.48          | 0.53          | 1.36 | 0.69          | 0.76          |
|                      | Con            | 0.67          | 0.33          | 0.48          | 0.90 | 0.53          | 0.82          |
| **Ri-Trx**           | Org            | 0.84          | 0.40          | 0.52          | 1.37 | 0.74          | 0.99          |
|                      | Con            | 0.77          | 0.47          | 0.70          | 1.05 | 0.56          | 0.76          |
| **Ri-Bra**           | Org            | 0.87          | 0.51          | 0.79          | 1.50 | 0.74          | 0.83          |
|                      | Con            | 0.44          | 0.27          | 0.63          | 0.98 | 0.48          | 0.61          |
| **RS-Men**           | Org            | 1.52          | 0.73          | 0.73          | 1.52 | 0.66          | 0.63          |
|                      | Con            | 1.21          | 0.68          | 0.87          | 1.50 | 0.73          | 0.77          |
| **RB-Alb**           | Org            | 1.42          | 0.71          | 0.79          | 1.79 | 0.73          | 0.72          |
|                      | Con            | 1.06          | 0.58          | 0.77          | 1.57 | 0.78          | 0.97          |
| **RB-Trx**           | Org            | 1.45          | 0.62          | 0.63          | 1.69 | 0.73          | 0.68          |
|                      | Con            | 1.17          | 0.62          | 0.73          | 1.79 | 0.80          | 0.86          |
| **Total**            | Org            | 1.96          | 0.77          | 0.65          | 2.32 | 0.87          | 0.74          |
|                      | Con            | 1.53          | 0.64          | 0.61          | 1.93 | 0.75          | 0.67          |

Values are the average of six data.
In contrast, species such as *Torulaspora delbrueckii* was isolated only in conventional musts. The presence of *Saccharomyces cerevisiae* was restricted to Mencía grapes from Monterrei DO (Table S2).

### 3. Yeast biodiversity indexes and statistics data analysis

The proportion of different species in both organic and conventional vineyards within each DO and for the total data in musts and grapes samples allowed the calculation of biodiversity indexes: Shannon-Wiener (H’), Simpson (1-D) and Equitability (E). The global results confirmed that organic production exhibited the highest diversity Shannon-Wiener index and Equitability in musts (H’ = 2.32 and E = 0.74) and grapes (H’ = 1.96 and E = 0.65) compared to conventional vineyards (H’ = 1.93 and E = 0.67 in musts, H’ = 1.53 and E = 0.61 and on grapes) (Table 2). Similarly, the Simpson’s diversity index was higher in organic samples, i.e. the dominance was lower (D = 0.13 and D = 0.25 in organic and conventional musts, respectively; D = 0.23 and D = 0.36 in organic and conventional grapes, respectively). However, when particular DOs and/or grapevine varieties were considered, diversity indexes values were not always higher in organic samples. Although H’ was higher in all cases, except in Treixadura must from Rías Baixas, E and (1 – D) were higher in some conventional musts and grapes.

That was the case in Treixadura grapes from Ribeiro DO and in conventional musts from Rías Baixas and Ribeira Sacra DOs, which had presented the greatest yeast species richness.

Statistical analysis using ANOSIM and PERMANOVA proved the existence of differences in yeast population (species richness and frequency), especially between DOs and culture systems. Concerning species richness from the musts and grapes jointly analysed, global PERMANOVA showed significant differences among DOs (pPERMANOVA = 0.0480; F = 3.25); however, global ANOSIM results were not significant (p = 0.0652; Table 3).

**TABLE 3.** Comparison of yeast population from different DO and production systems by analysis of similarity/distance measure (ANOSIM and PERMANOVA) in musts.

|          | Mo-Org | Mo-Con | Ri-Org | Ri-Con | RS-Org | RS-Con | RB-Org | RB-Con |
|----------|--------|--------|--------|--------|--------|--------|--------|--------|
| Mo-Org   | 0.2771 | 0.2925 | 0.5782 | 0.5641 | 0.2620 | 0.8301 | 0.5981 |
| Mo-Con   | 0.0140 | 0.6165 | 0.6987 | 1      | 0.6355 | 0.8284 |        |
| Ri-Org   | 0.0084 | 0.0028 | 0.8754 | 1      | 0.7445 | 0.9996 | 0.8573 |
| Ri-Con   | 0.0028 | 0.0028 | 0.0028 | 1      | 0.7483 | 0.9463 |        |
| RS-Org   | 0.0028 | 0.0056 | 0.0028 | 0.0028 | 0.0644 |        | 0.2503 |
| RS-Con   | 0.6972 | 0.0084 | 0.0028 | 0.0028 | 0.0476 | 0.0672 | 0.3637 |
| RB-Org   | 0.0028 | 0.0028 | 0.0028 | 0.0028 | 0.0476 | 0.0672 |        |
| RB-Con   | 0.0028 | 0.0028 | 0.0028 | 0.0028 | 0.0476 | 0.0672 |        |

Statistical R- and F-values are shown above the diagonal and the level of significance (p-values) and the Bonferroni correction is shown below.
The influence of culture system on global species richness was not significant (pPERMANOVA = 0.0522; pANOSIM = 0.0681). When the farming system and DO were grouped together, both tests presented more evident significant differences (pANOSIM = 0.0143, pPERMANOVA = 0.0061; R = 0.4554, F = 5.71). In addition, the highest dissimilarity (R = 1) was observed between Rías Baixas-Ribeira Sacra DOs and Monterrei-Ribeiro DOs binomials. In contrast, no significant differences in species richness were observed when the influence of the grapevine variety factor was determined (p > 0.5942 in both statistics).

Regarding the influence of DO, culture system and grapevine variety on species frequency in musts and grapes, significant differences (p < 0.001) were obtained in global ANOSIM and PERMANOVA tests for all factors and clusters. For example, in the cluster test for the two main factors (DO and culture system) significant differences (p = 0.0001) were found in both tests. ANOSIM and PERMANOVA values obtained for musts from different DOs and culture systems pairwise are summarised in Table 3. The results evidenced similarity (low R and F statistic values) between Rías Baixas and Ribeira Sacra DOs, but they were different from the other two DOs: Ribeiro and Monterrei (high R- and F-values). The highest F-values were obtained for organic samples of Rías Baixas (F = 74.34 and 86.21) and the Ribeira Sacra (F = 58.51 and 70.65) compared to the conventional Monterrei and Ribeiro DOs respectively. Furthermore, significant differences were found between the organic and conventional production systems for all DOs. In particular, the culture system strongly influenced yeast community in Ribeiro DO and, to a lesser extent, in Ribeira Sacra DO. Tests on musts for the DO-grapevine variety cluster also showed that the differences observed were due to DOs rather than to grapevine varieties. Tests on grapes shower lower differences in yeast population (Supplementary Table S4).

Moreover, the two-way ANOSIM and the two-way PERMANOVA were applied, which both allow the simultaneous study of two factors effects. The results showed significant differences in all the factors (DO, culture system and grapevine variety) for the frequency data (Table 4). Nevertheless, tests only revealed significant differences for DO factor (and only in the PERMANOVA test) when the species richness data were considered.

In addition, SIMPER analysis confirmed that the greatest contribution to differences between musts and grapes from organic and conventional cultivation was due to *Aureobasidium pullulans*, *H. uvarum* and *Metschnikowia* spp., previously described as the dominant species (Table 5). These species were the main contributors on grapes, but with higher percentage of *Cryptococcus* spp. and a remarkable presence of *D. hansenii* compared to musts. The first five species accounted for more than 70% of the cumulative percentage on grapes from both cultivation systems. In conventional samples, only *Aureobasidium* spp. represented half of the cumulative percentage of species, so diversity was lower than in organic samples. In contrast, the influence of *Starm. bacillaris*, *L. thermostolerans*, *Cr. terrestris* and *C. apicola* were important in musts. In this case, seven species were responsible for 70% cumulative differences.

### Table 4. Two-way ANOSIM and PERMANOVA for DO, culture system and grapevine variety factors in species frequency (%) and species richness (S).

| Factors          | Two-way ANOSIM | Two-way PERMANOVA |
|------------------|----------------|-------------------|
|                  | R   | p     | F     | p     |
| **Species frequency (%)** |     |       |       |       |
| Variety          | 0.16428 | 0.0006 | 48.774 | 0.0001 |
| Farming system  | 0.51302 | 0.0001 | 86.021 | 0.0001 |
| DO               | 0.60001 | 0.0001 | 193.760 | 0.0001 |
| Farming system  | 0.37741 | 0.0001 | 169.410 | 0.0001 |
| **Species richness (S)** |     |       |       |       |
| Variety          | -0.2573 | 0.8716 | 0.2111 | 0.7069 |
| Farming system  | 1   | 0.5962 | 0.9762 | 0.1431 |
| DO               | 0.4074 | 0.0647 | 32.783 | 0.0174 |
| Farming system  | 0.7187 | 0.2301 | 48.473 | 0.0129 |
The farming system is not common. The existence of a great heterogeneity and adverse climatic and orography conditions seriously influence succession of this production system. The current study presents a comparison between yeast diversity in organic and conventional grapes/musts from four differentiated Galician areas.

The influence of farming systems (biodynamic, organic, conventional and/or integrated) on yeast species diversity have been approached in different winegrowing areas. In Galicia, our findings showed that yeast biodiversity tended to be higher in grapes and musts from organic production than in conventional production. These results agree with those reported for vineyards from Madrid, Spain, (Cordero-Bueso et al., 2011; Tello et al., 2012). In the same way, a higher yeast diversity has been described in biodynamic vineyards than in conventional and organic vineyards (Castrillo et al., 2017).

Finally, PCA of must samples using the frequency of main yeast species allowed the separation between DOs and farming systems. PC1 and PC2 explained 68% of the variance (Figure 4). PC1 separated Rías Baixas and Ribeira Sacra DOs samples (plotted on the negative side) from Ribeiro and Monterrei DOs samples (plotted on the positive side). PC2 separated organic samples (positive side) from conventional samples (negative side) in Ribeiro and Monterrei DOs, which agree with the distribution of the most relevant species as mentioned above within each DO. There was also a separation between organic and conventional production samples in the opposite areas of the PCA.

**DISCUSSION**

Organic viticulture is increasingly popular around the world; however, in Galicia its use as a farming system is not common. The existence of a great heterogeneity and adverse climatic and orography conditions seriously influence succession of this production system. The current study presents a comparison between yeast diversity in organic and conventional grapes/musts from four differentiated Galician areas.

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integrated vineyards in South Africa (Bagheri et al., 2015; Setati et al., 2012 and 2015). With regard to studies performed in France, Martins et al. (2014) confirmed that diversity index (H’) were higher in organically farmed grapes. Conversely, Grangeteau et al. (2017) observed a lower diversity under organic modality, which could be explained by possible interactions between microorganisms, but also due to the use of higher doses of fungicides based on copper in organic vineyards. The same conclusions were reported by Milanović et al. (2013), who also found less yeast species diversity on grapes from organic Italian vineyards compared to conventional ones. In contrast, Comitini and Ciani (2008) demonstrated that conventional fungicide treatments applied on grapes during ripening also caused a drastic reduction in the population and diversity of yeasts. However, in some studies, no differences were found in microbial diversity between biodynamic and traditional vineyards, which appeared to be more related to grape variety than to agronomical practices (Guzzon et al., 2015).

Yeast associated to grapes and musts has been widely reported (Barata et al., 2012; Drumonde-Neves et al., 2016; Grangeteau et al., 2017). Yeast population on grapes was dominated by the ascomycetous yeast-like fungus *Aureobasidium* spp. and by basidiomycetous yeasts (for example, *Cryptococcus* spp., *Rhodotorula* spp. and *Sporobolomyces* spp.). These species have been described as normal microbiota associated to grapes (Barata et al., 2012; Fleet, 2003; Grangeteau et al., 2017; Martins et al., 2014; Nemcová et al., 2015; Prakitchaiwattana et al., 2004; Setati et al., 2012). All are oxidative yeasts which are technologically irrelevant because they are not detected for a long period during the alcoholic fermentation; so they do not have a negative effect on wine. These species were found as predominant yeasts in most samples in our study. Furthermore, the weakly fermentative yeasts *H. uvarum* and *Metschnikowia* spp. were also identified in certain grapes samples in a high proportion (between 1.4% and 42.1%). Both species have been associated with mature or damaged grapes (Drumonde-Neves et al., 2017; Garofalo et al., 2016; Guzzon et al., 2014; Prakitchaiwattana et al., 2004; Sipiczki, 2016). *H. uvarum* was especially abundant in Ribeira Sacra and Rías Baixas DOs, whereas *Metschnikowia* spp. appeared in samples from all DO, being especially high its frequency in Mencia grapes from Ribeira Sacra DO. A widely ranged distribution frequency in *H. uvarum* and *Metschnikowia* spp. has also been reported for grapes from Azores Archipelago (Drumonde-Neves et al., 2017), Slovakia (Nemcová et al., 2015) and France (Grangeteau et al., 2017). These variations have been attributed to several factors, such as vintage, location, grape cultivar and phytosanitary treatments. In our study the differences are more related to location than grapevine variety and/or farming system.
probably due to the particular climatic conditions in each DO and their influence on grape sanitary conditions.

Other minor oxidative and/or weakly fermentative species such as *C. apicola*, *C. oleophila*, *D. hansei*, *P. kluveri* and *L. thermotolerans* appeared, although not frequently, in some grapes samples, most of them from organic production. Their presence can be explained due to nutrient availability on the berry surface, which increased with ripening grapes (Barata et al., 2012; Sipiczki, 2016). Some of these minor species as well as *Candida* spp., *Zygossaccharomycetes* spp. and *I. terricola* were also widely identified, especially in the musts from Ribeira Sacra DO and Rías Baixas DO. *Candida* spp. and *Pichia* spp. are highly heterogeneous and ubiquitous film-forming yeasts regarded as common species on grapes and musts with the ability to produce off-flavours, but good manufacturing practices can prevent their activity (Barata et al., 2012; Loureiro and Malfeito-Ferreira, 2003). The presence of *S. cerevisiae*, the major wine yeast in wineries and during fermentation, was restricted to scarce grape samples. Our results agree with the fact that *S. cerevisiae* appears with a lower frequency (less than 10–100 cfu/g of grapes), being rarely found in healthy berries (Fleet, 2003). These observations are the origin of a significant controversy about its origin in winemaking (Martini et al., 1996; Mortimer and Polsinelli, 1999).

Regarding must samples, the yeast-like *Aureobasidium* spp. was also widely identified, although less frequently than on grapes; however, the frequency of fermentative yeasts such as *H. uvarum*, *Metschnikowia* spp., *Starm. bacillus* or *L. thermotolerans* was higher than on grapes, and their distribution differed among DOs (Table S2, S3; Figure 3). In addition, other minor species were identified, mainly in organic samples. Grangeteau et al. (2017) had already described a decrease in *Aureobasidium* spp. in musts as well as the presence of some genera that had not appeared on grapes. *H. uvarum* showed high occurrence in musts and grapes from vineyards under different agronomic management (Bagheri et al., 2015; Milanović et al., 2013). These authors also detected *C. zemplinina* (*Starm. bacillus*) associated to grapes and high-sugar musts. In our study, this species is related to Ribeira Sacra and Rías Baixas DOs samples, but not all had a high-sugar concentration (Table S1), and therefore the presence of *Starm. bacillus* cannot be explained by this reason. Drumonde-Neves et al., (2017) reported *H. uvarum* and *Metschnikowia* spp. as major species, but they also found *Pichia terricola* (synonym *I. terricola*) and *Starm. bacillus* at high frequencies. In our study *I. terricola* was isolated exclusively in Rías Baixas and Ribeira Sacra DOs. However, an important incidence of *K. thermotolerans* (synonym *L. thermotolerans*) as well as *Candida stellata* (close to *Starm. bacillus*) was found in the Madrid winegrowing area (Cordero-Bueso et al., 2011; Tello et al., 2012). These authors also suggested that phytosanitary treatment affects diversity; to be more specific, organic treatments favoured the presence of fermentative yeasts. Our results support the presence of yeast species with interesting fermentative properties in organic samples.

Concerning the influence of the farming system on the frequency of certain yeast species there are diverse, even contradictory, data available. Thus, yeast-like *Aureobasidium* was associated with organic samples by Martins et al. (2014), whereas Comitini and Ciani (2008) observed the opposite: a higher incidence of *A. pullulans* in conventional samples. Our results support a higher occurrence of *Aureobasidium* in conventional samples; however, the diversity and frequency of *Cryptococcus* spp. were wider under organic production, agreeing with Grangeteau et al. (2017). *Metschnikowia* and *Hanseniaspora* have been mainly associated with the conventional protection (Grangeteau et al., 2017; Milanović et al., 2013). However, these genera were isolated mainly in control (untreated) and organic plots by Comitini and Ciani (2008). The incidence of *Metschnikowia* spp. in Galicia was lower in organic grapes, but not in musts. *H. uvarum* appeared more frequently in organic musts from Ribeira Sacra and Rías Baixas. These differences in the incidence of certain species have been attributed to the impact of copper treatments in organic farming, but also to a possible interaction between species (Grangeteau et al., 2017). In that sense, Setati et al. (2012) suggested that organic production involves an increase in richness species, including many yeasts with potential for biological control such as *H. uvarum*, *Metschnikowia* spp., *I. terricola* or *A. pullulans* (Guzzon et al., 2014; Raspor et al., 2010).
The diversity indexes (H’, D, and E) obtained in this study for specific grape varieties and DOs were similar to those reported previously in grapes and musts (Cordero-Bueso et al., 2011), whereas the global values were close to those found by Bagheri et al. (2015). Although in most cases, and at a global level, organic samples presented higher values of biodiversity indexes than their conventional counterparts, some exceptions were found. In all the exceptions, organic samples presented higher species richness, but the frequency of each species is an important factor for indexes calculation, especially for E’ and D.

Finally, statistical analysis of diversity data (species richness and species frequency) using ANOSIM and PERMANOVA showed significant differences among DOs and between culture systems; the highest values of R and F were obtained for DO factor. However, the low values of R and F in the one- and two-way tests indicated that grapevine variety does not have an important impact on yeast diversity. Therefore, these results confirmed that the factors that influenced diversity the most were the DO followed by the cultivation system and, to a lesser extent, the grapevine variety. Moreover, the results of two-way tests suggest that the species frequency found in each region had greater influence on yeast diversity than the species richness identified. In addition, PCA analysis (Figure 4) clearly separated samples of Rías Baixas and Ribeira Sacra DOs from those collected in Ribeiro and Monterrei DOs based on their species frequency. This differentiation is consistent with the values of similarity obtained by ANOSIM and PERMANOVA analysis (Table 3), and with the species patterns presented in figure 3. Differences in distribution of major yeasts and the association of certain species to specific DOs are responsible for these regional differences. Thus, our results suggest the existence of biogeographical patterns of yeast communities from different Galician DOs, although further studies including several vintages are required to confirm these findings. Recently, Bokulich et al. (2014) reported that grape microbial communities were closely related to the environmental conditions and the production area. Later studies evidenced an association between soil and grapes microorganisms and environmental conditions (microbial terroir) that contribute to the regional characteristics of wines (Belda et al., 2017, Bokulich et al., 2016; Knight et al., 2015; Mezzasalma et al., 2018). The incidence of non-\textit{Saccharomyces} fermentative yeast species, particularly in organic samples, showed that such yeasts are well adapted to these areas and could contribute to the complexity and distinctive characteristic of their wines.

**CONCLUSION**

The global results of this study confirmed that organic farming leads to an increase in the yeast diversity. The major species – \textit{Metschnikowia} spp., \textit{H. uvarum} and \textit{Cryptococcus} spp. and yeast-like \textit{Aureobasidium} spp. – were similar in almost all samples, although their proportions varied depending on the culture system and DO. The main differences in yeast biodiversity were attributed to a higher richness of minor species under organic production. In addition, certain species such as \textit{L. thermotolerans} appeared in Ribeiro and Monterrei DOs whereas \textit{Starm. bacillaris}, \textit{Candida} spp. and \textit{I. terricola} were associated to Rías Baixas and Ribeira Sacra DOs. These findings allowed the establishment of a geographical yeast species pattern (microbial terroir), which could influence wine typicality at regional level.

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