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# Genetic and phenotypic diversity of autochthonous cider yeasts in a cellar from Asturias

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ABSTRACT

This paper analyses yeast diversity and dynamics during the production of Asturian cider. Yeasts were isolated from apple juice and at different stages of fermentation in a cellar in Villaviciosa during two Asturian cider-apple harvests. The species identified by ITS-RFLP corresponded to *Hanseniaspora valbyensis*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Pichia guilliermondii*, *Candida parapsilosis*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus/Saccharomyces pastorianus/Saccharomyces kudriavzevii/Saccharomyces mikatae*. The species *C. parapsilosis* is reported here for the first time in cider. The analysis of *Saccharomyces* mtDNA patterns showed great diversity, sequential substitution and the presence of a small number of yeast patterns (up to 8), present in both harvests. Killer (patterns nos. 22′ and 47), sensitive (patterns nos. 12, 15, 33 and 61) and neutral phenotypes were found among the *S. cerevisiae* isolates. The detection of β-glucosidase activity, with arbutin as the sole carbon source, allowed two *S. cerevisiae* strains (patterns nos. 3′ and 19′) to be differentiated by means of this enzymatic activity. Yeast strains producing the killer toxin or with β-glucosidase activity are reported for the first time in autochthonous cider yeasts.



## 1. Introduction

Asturias, a region in northern Spain, is the country's main producer of natural cider (Suárez and Picinelli, 2001). Cider production depends on the cider-apple yield, which is characterized by a biennial crop. Consequently, the production of cider apple in Asturias varies between 40,000–50,000 tons in odd years and 7000–20,000 tons in even years. Natural cider is produced by the spontaneous fermentation of apple juice. This step involves both alcoholic and malolactic fermentation carried out by the sequential action of different yeasts and bacteria originating from the fruit and the cider-making equipment (Beech, 1972; Salih et al., 1988; Michel et al., 1988; Cabranes et al., 1990; Dueñas et al., 1994). Yeasts are primarily responsible for alcoholic fermentation and hence for the taste and flavour characteristic of products (Beech and Davenport, 1970; Mafart, 1986; Le Queré and Drilleau, 1993; Cabranes et al., 1997; Suárez et al., 2005). Therefore, spontaneous fermentations are of particular interest in order to ascertain the yeast species associated with the fermentation processes.

Preliminary studies on population dynamics in cider have shown that the composition of yeast flora can vary according to climatic conditions, apple varieties, geographic location and the cider-making technology employed (Cabranes et al., 1990; del Campo et al., 2003; Suárez et al., 2007a). Autochthonous non-*Saccharomyces* yeasts with a low fermentation capacity usually develop during the first stages of the process (Michel et al., 1988; Morrissey et al., 2004; Coton et al., 2006; Suárez et al., 2007a). Strains with a greater tolerance to ethanol (*Saccharomyces* spp.) subsequently complete the fermentation. On the other hand, studies carried out to examine the dynamics and variability of wild *Saccharomyces* spp. (Suárez et al., 2007b) have determined that the number of strains observed was higher than those reported for *Saccharomyces* populations in some wine-growing regions (Frezier and Dubourdieu, 1992; Querol et al., 1994; Gutiérrez et al., 1999; Torija et al., 2001; Schuller et al., 2005).

In the present study, PCR amplification and restriction pattern analysis of the ITS region (Esteve-Zarzoso et al., 1999; Fernández-Espinar et al., 2000) were used to identify yeast associated with the natural fermentation of Asturian cider. Differentiation of *Saccharomyces* strains was achieved by mitochondrial DNA (mtDNA) restriction enzyme analysis (Querol et al., 1992a; López et al., 2001). Subsequently, the different isolated yeast strains were physiologically

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characterized by killer and β-glucosidase activity and $H_2S$ production.

## 2. Materials and methods

### 2.1. Cider fermentations

The present study was carried in a cellar in Villaviciosa (Asturias) where commercial dry yeast had not been used in the 2001 and 2003 harvests. The cider-making processes were carried out under the same conditions in both years and one vat was analysed per year. Apple juice was obtained from a mixture of cider apples using an automatic hydraulic press. Fermentations were carried out in 20,000 l stainless-steel tanks, at cellar temperature (12–15 °C), without the addition of $SO_2$. Alcoholic fermentations rates were monitored by density measuring.

### 2.2. Yeast sampling and isolation

Samples were taken aseptically at different stages of the cider-making process: A (density: 1051–1048 g $l^{-1}$), B (density: 1047–1044 g $l^{-1}$), C (density: 1032–1030 g $l^{-1}$), D (density: 1020–1019 g $l^{-1}$), E (density: 1005–1003 g $l^{-1}$) and F (density: ≤1000 g $l^{-1}$). At each stage, aliquots of several dilutions were spread onto Malt Extract Agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar) supplemented with 25 mg $l^{-1}$ penicillin G potassium salt (Calbiochem, Nottingham, UK) and 100 mg $l^{-1}$ streptomycin sulphate (Acofarma, Madrid, Spain) to inhibit bacterial growth. Plates were incubated for colony development at 28 °C for two days. Fifty colonies from each of the samples were randomly selected. The isolated yeasts were preserved on Yeast Extract Peptone Dextrose (YEPD) agar slants and stored at 4 °C.

### 2.3. Molecular identification of yeast

Yeast colonies were identified by amplification and restriction of the rRNA gene region (Esteve-Zarzoso et al., 1999) using the primers ITS1 and ITS4. Amplification, restriction and electrophoresis conditions as well as the comparison of the DNA fragments against reference strains and the database were carried out following Esteve-Zarzoso et al. (1999) and Fernández-Espinar et al. (2000). Certified yeast strains of different species obtained from the Spanish Type Culture Collection (CECT) were used as reference strains in each amplification and restriction reaction. The strains were: *Hanseniaspora uvarum* (CECT 1444), *Hanseniaspora valbyensis* (CECT 10122), *Metschnikowia pulcherrima* (CECT 10408), *Candida parapsilosis* (CECT 1449), *Saccharomyces cerevisiae* (CECT 1883) and *Saccharomyces bayanus* (CECT 1969).

### 2.4. Mitochondrial DNA (mtDNA) restriction analysis of Saccharomyces strains

DNA extraction and determination of mitochondrial DNA restriction patterns of *Saccharomyces* strains were performed (López et al., 2001) using the restriction endonuclease *Hinf* I (Roche, Mannheim, Germany). The different patterns obtained in the 2001 harvest were indicated by Arabic numerals followed by a prime symbol (′), while the patterns in the 2003 harvest were indicated solely by Arabic numerals.

### 2.5. Physiological trait

#### 2.5.1. Killer phenotype

The strains tested for their ability to produce killer toxin were pre-cultured for 24 h in liquid "GPY" medium (4% glucose, 0.5% peptone, 0.5% yeast extract). The killer (ref: CECT 1893, *S. cerevisiae*) or sensitive (ref: CECT 1407, *S. cerevisiae*) reference strains were inoculated separately onto plates containing "MBM" medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar, 0.015% methylene blue, 1 M phosphate–citrate buffer pH = 4.5). The killer reference strain was added to pre-cooled MBM medium, while the sensitive reference strain was seeded as a lawn onto plates. The strains to be tested were loaded (5 μL) onto the two seeded agar plates and incubated at 23 °C for 3 days. Killer activity is recognized when a strain grows on both plates and a clear growth inhibition halo is also exhibited around the colony in sensitive lawn cultures. The strains that were unable to grow on the plate seeded with the killer reference strain were considered sensitive, whereas the strains that grew on both plates were considered neutral.

#### 2.5.2. β-Glucosidase activity

Screening of enzymatic activity of strains was carried out using arbutin as the substrate and carbon source in an agar slant tube. The medium was composed of: 0.1% yeast extract, 0.5% arbutin, 0.14% iron chloride and 2% agar (pH = 5.0). 48-h-old cultures on "GPY" medium were inoculated on the agar surface. The agar tubes were incubated at 28 °C for 15 days. Strains with β-glucosidase activity hydrolysed the arbutin and a dark brown colour developed in the agar slant tube. Non-inoculated tubes and pure enzyme (EC.3.2.1.21, Sigma–Aldrich, Germany) were used as negative and positive control, respectively.

#### 2.5.3. Hydrogen sulphide production

The ability of yeast strains to produce $H_2S$ was tested using the bismuth-containing indicator medium "BiGGY" agar (Becton Dickinson). Plates were incubated at 28 °C for 24 h and production of $H_2S$ was revealed by the darkening of colonies. The following scale was established: white colour, 0 (no production); light brown colour, 1 (low production); dark brown colour, 2 (medium production); black colour, 3 (high production).

## 3. Results

### 3.1. Molecular characterization of yeast strains

The ITS1 and ITS4 primers were used to identify a total of 600 isolated yeast colonies (300 in each harvest). The approximate length of the amplified products and the restriction fragments observed after digestion for all the strains studied are summarized in Table 1.

The yeast species identified and the isolation frequencies obtained during the spontaneous fermentations are shown in Table 2. A wide variety of yeast species, including *Saccharomyces* and non-*Saccharomyces*, were found throughout the cider-making process. Only non-*Saccharomyces* yeasts were isolated in the apple juice. The species *H. valbyensis* was predominant in both years (84% in 2001 and 66% in 2003), while the secondary species were *M. pulcherrima* (16%) in the 2001 harvest and *H. uvarum* (30%) in that of 2003. In the former year, a high abundance of non-*Saccharomyces* yeasts was detected at the beginning of alcoholic fermentation (density: 1047–1044 g $l^{-1}$), the isolates belonging to the species *C. parapsilosis* (84%), *H. valbyensis* (14%) and *Pichia guilliermondii* (2%). In 2003, however, the non-*Saccharomyces* yeasts constituted a minority, while *Saccharomyces* yeasts represented 92% of the isolates. In both years, *Saccharomyces* spp. became predominant from this stage onwards

**Table 1**
Size of the PCR and the restriction fragments of the species identified in this study.

| AP (bp) | Size of the restriction fragments (bp) | | | | | Identification species | Number of isolates | |
|---|---|---|---|---|---|---|---|---|
| | *CfoI* | *Hae III* | *Hinf I* | *DdeI* | *HpaII* | | 2001 | 2003 |
| 400 | 205, 100, 95 | 280, 100 | 200, 190 | | | *M. pulcherrima* | 8 | 3 |
| 625 | 300, 265, 60 | 400, 115, 90 | 320, 300 | | | *P. guilliermondii* | 1 | 0 |
| 750 | 630, 120 | 750 | 250, 220, 170, 105 | | | *H. valbyensis* | 55 | 37 |
| 750 | 320, 310, 105 | 750 | 350, 200, 180 | 300, 180, 95, 90, 85 | | *H. uvarum* | 0 | 19 |
| 550 | 300, 240 | 400, 115 | 290, 260 | | | *C. parapsilosis* | 42 | 0 |
| 850 | 385, 365 | 320, 230, 180, 150 | 365, 155 | | 725, 125 | *S. cerevisiae* | 131 | 197 |
| 850 | 385, 365 | 500, 220, 145 | 365, 155 | | | *S. bayanus/S. pastorianus/ S. kudriavzevii/S. mikatae* | 63 | 44 |

AP: 5.8S-ITS amplified product size.
*M: Metschnikowia*; *P:Pichia*; *H: Hanseniaspora*; *S: Saccharomyces*; *C: Candida.*

until the end of the fermentation process. The *Saccharomyces* species isolated were *S. cerevisiae*, and at a lower frequency *S. bayanus/Saccharomyce pastorianus/Saccharomyces kudriavzevii/Saccharomyce mikatae*. At the end of fermentation in 2001, the isolation percentages of the two species were similar (56% *S. cerevisiae*, 44% *S. bayanus/Saccharomyces pastorianus/S. kudriavzevii/S. mikatae*), while, in 2003, the proportions were very different (90% *S. cerevisiae*, 10% *S. bayanus/S. pastorianus/S. kudriavzevii/S. mikatae*). The kinetics of the yeast species in this study revealed that the *Hanseniaspora* genus coexisted with the *Saccharomyces* yeast during the beginning of fermentation and the rapid fermentation stage (samplings: B–D).

A total of 435 *Saccharomyces* yeasts were isolated during the two harvests. Discrimination between these autochthonous *Saccharomyces* species at the strain level was performed using molecular mtDNA-RFLP analysis. Table 3 shows the references of mtDNA patterns obtained in both years at each stage of the cider fermentations.

The mtDNA restriction patterns (194 in 2001 and 241 in 2003) revealed 48 and 94 different profiles, respectively. The variability, measured as the percentage of different patterns found among the colonies analysed, ranged between 29 and 42% for *S. cerevisiae* and 16–27% for *S. bayanus/S. pastorianus/S. kudriavzevii/S. mikatae* in the 2001 and 2003 harvests, respectively. A progressive decrease in the ratio of the number of patterns detected to the number of isolates analysed was observed in each species as the fermentation process developed, indicating a decrease of intraspecific diversity.

In 2001, 50% (24 patterns) were isolated in one sampling. Among the remaining patterns, 10 were isolated during one stage of fermentation, eight of these at a low frequency (<7%) and two (patterns 3′ and 54′) with frequencies of 16 and 20%, respectively. The rest of the patterns were isolated in more than one sampling; their isolation percentages in the several samplings are shown in Table 3. Analysis of the different patterns revealed a substitution of strains throughout the fermentation. Pattern no. 8′ was present at significant frequencies (10%) in samplings C and D. This pattern, together with that denoted as no. 21′, constituted the majority of isolates in sampling D. From that moment onwards, none of these patterns were found. In sampling E, 78% of the isolates were represented by 3 patterns (nos. 4′, 42′, and 52′). At the end of alcoholic fermentation, the 50 colonies analysed belonged to 4 different patterns, three of which (patterns nos. 4′, 50′ and 52′) were isolated previously. These colonies represented 80% of the isolates, while the remaining 20% was represented by pattern no. 54′, which was only isolated in this sampling.

In the 2003 harvest, more than half of the patterns (59) were represented by one colony, seven being isolated in one stage of the fermentation process, with a frequency of less than 9%. Twenty-three out of the 28 patterns isolated in more than one sampling were identified as *S. cerevisiae* and five as *S. bayanus/S. pastorianus/ S. kudriavzevii/S. mikatae* (Table 3). In this harvest, four patterns (nos. 4, 14, 30 and 34) were present in all the samples, with isolation frequencies lower than 13%. Throughout the fermentation (samplings B, C, D, E), most of the strains were isolated with frequencies lower than 7%; only the isolation percentages of patterns no. 39 (sampling C) and nos. 4 and 24 (sampling E) were noteworthy. At the end of the alcoholic fermentation stage (sampling F), 19 restriction patterns were isolated, seven of which were present in this sampling only, while 50% of the isolates was represented by four patterns (nos. 4, 14, 29 and 40).

Comparing the results of mtDNA restriction analysis, it was observed that 8 restriction patterns were identical in both years (these patterns are represented in the tables by bold numbers).

### 3.2. Physiological characterization of strains

The 134 different *Saccharomyces* strains were tested for their ability to produce hydrogen sulphide, killer toxin and β-glucosidase

**Table 2**
Distribution of yeast species (%) during spontaneous fermentation of apple juice at different sampling stages.

| Yeast species | 2001 | | | | | | 2003 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sampling | | | | | | Sampling | | | | | |
| | A | B | C | D | E | F | A | B | C | D | E | F |
| *Metschnikowia pulcherrima* | 16 | | | | | | 4 | 2 | | | | |
| *Pichia guilliermondii* | | 2 | | | | | | | | | | |
| *Hanseniaspora valbyensis* | 84 | 14 | | 12 | | | 66 | 2 | 6 | | | |
| *Hanseniaspora uvarum* | | | | | | | 30 | 4 | 2 | 2 | | |
| *Candida parapsilopsis* | | 84 | | | | | | | | | | |
| *S. cerevisiae* | | | 62 | 72 | 72 | 56 | | 78 | 78 | 84 | 64 | 90 |
| *S. bayanus/S. pastorianus/S. kudriavzevii/S. mikatae* | | | 38 | 16 | 28 | 44 | | 14 | 14 | 14 | 36 | 10 |

Samplings: A (density: 1051–1048 g $l^{-1}$), B (density: 1047–1044 g $l^{-1}$), C (density: 1032–1030 g $l^{-1}$), D (density: 1020–1019 g $l^{-1}$), E (density: 1005–1003 g $l^{-1}$) and F (density: ≤1000 g $l^{-1}$).

**Table 3**
References and representative frequencies (%) of the mtDNA patterns.

| | Sampling | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Patterns isolated one time | | | | | | |
| 2001 | I | – | 14′, **15**′, 16′ | – | – | – |
| | II | – | 6′, **17**′, 19′, 22′, 23′ 24′, 25′, 26′, 27′, 28′ | 29′, 33′, 34′, 38′, 40′, 42′ | 35′, 46′, 49′ 51′, 53′ | – |
| 2003 | I | 25, 32 | **53**, 54 | 75 | 85, 87 | – |
| | II | 2, 5, 6, 7, 8, 9, 15, 18, 20, 21, 22, 23, 26, 33, **35** | 37, 41, 43, 44, 47, 49, 50 | 55, 56, 57, 59, 60, 61, 62, 63, 64, 65, 67, 69, 70, 71, 72, 73, 74 | 76, 77, 78, 80, 81, 82, 83 | 89, 90, 91 **92**, 93, 94 |
| Patterns isolated in one sampling | | | | | | |
| 2001 | I | – | 2′, 9′, **13**′, 18′ | – | – | – |
| | II | – | 3′ | **30**′, 31′, 36′, **39**′ | – | 54′ |
| 2003 | I | – | – | – | – | – |
| | II | 12, 19, 31 | 51 | 68 | **84** | **88** |
| Isolation percentage of patterns isolated in several samplings | | | | | | |
| 2001 | | | | | | |
| 4′ | I | 0 | 2 | 2 | 28 | 42 |
| 7′ | I | 0 | 2 | 4 | 0 | 0 |
| 8′ | I | 0 | 10 | 10 | 0 | 0 |
| 1′ | II | 0 | 8 | 6 | 0 | 0 |
| 5′ | II | 0 | 2 | 4 | 0 | 0 |
| 10′ | II | 0 | 4 | 2 | 0 | 0 |
| 11′ | II | 0 | 4 | 2 | 2 | 0 |
| **12**′ | II | 0 | 4 | 8 | 2 | 0 |
| 20′ | II | 0 | 2 | 6 | 0 | 0 |
| **21**′ | II | 0 | 2 | 10 | 0 | 0 |
| **32**′ | II | 0 | 0 | 2 | 6 | 0 |
| 42′ | II | 0 | 0 | 2 | 20 | 0 |
| 50′ | II | 0 | 0 | 0 | 2 | 22 |
| 52′ | II | 0 | 0 | 0 | 30 | 16 |
| 2003 | | | | | | |
| 4 | I | 4 | 6 | 4 | 12 | 10 |
| 17 | I | 2 | 0 | 2 | 0 | 0 |
| 24 | I | 4 | 2 | 2 | 18 | 0 |
| **36** | I | 2 | 2 | 0 | 0 | 0 |
| 66 | I | 0 | 0 | 4 | 2 | 0 |
| 1 | II | 6 | 0 | 4 | 0 | 0 |
| 3 | II | 2 | 2 | 0 | 0 | 0 |
| 10 | II | 2 | 0 | 2 | 0 | 0 |
| 11 | II | 2 | 0 | 0 | 2 | 0 |
| 13 | II | 2 | 0 | 4 | 6 | 6 |
| 14 | II | 6 | 2 | 4 | 6 | 10 |
| 16 | II | 2 | 4 | 0 | 0 | 0 |
| 27 | II | 2 | 6 | 2 | 0 | 0 |
| 28 | II | 2 | 2 | 0 | 0 | 0 |
| 29 | II | 4 | 0 | 6 | 0 | 12 |
| 30 | II | 2 | 6 | 6 | 4 | 4 |
| 34 | II | 2 | 4 | 4 | 4 | 6 |
| **38** | II | 0 | 2 | 2 | 4 | 0 |
| 39 | II | 0 | 12 | 0 | 2 | 0 |
| 40 | II | 0 | 6 | 0 | 4 | 18 |
| **42** | II | 0 | 4 | 0 | 2 | 0 |
| 45 | II | 0 | 4 | 6 | 2 | 0 |
| 46 | II | 0 | 2 | 2 | 0 | 0 |
| 48 | II | 0 | 2 | 0 | 4 | 2 |
| 52 | II | 0 | 2 | 2 | 0 | 2 |
| 58 | II | 0 | 0 | 2 | 2 | 6 |
| 79 | II | 0 | 0 | 0 | 2 | 2 |
| 86 | II | 0 | 0 | 0 | 2 | 2 |

I: *S. bayanus/S. pastorianus/S. kudriavzevii/S. mikatae*; II: *S. cerevisiae*; Bold numbers: patterns common to both harvests (**17**′ and **92**), (**39**′ and **84**), (**32**′ and **88**), (**15**′ and **53**), (**12**′ and **42**), (**30**′ and **35**), (**13**′ and **36**) and (**21**′ and **38**). Samplings: B (density: 1047–1044 g $l^{-1}$), C (density: 1032–1030 g $l^{-1}$), D (density: 1020–1019 g $l^{-1}$), E (density: 1005–1003 g $l^{-1}$) and F (density: $\leq$1000 g $l^{-1}$).

activity. Table 4 summarizes the physiological properties of the different analysed strains.

The production of $H_2S$ was assessed by pigmentation of the different strains in "BiGGY" agar. Six *S. cerevisiae* strains developed black colonies and were considered as high $H_2S$ producers, forty-three strains (10 *S. bayanus/S. pastorianus/S. kudriavzevii/S. mikatae* and 33 *S. cerevisiae*) were non-$H_2S$ producers and the rest were classified as low and medium producers. Killer toxin was only detected in two *S. cerevisiae* strains, denoted as no. 22′ and no. 47, which were classified as medium and low hydrogen sulphide producers, respectively. The majority of the yeasts (95%) had a neutral phenotype and only four *S. cerevisiae* strains had a sensitive phenotype. Arbutin was chosen as substrate for screening β-glucosidase activity, with weak enzymatic activity only being detected in two *S. cerevisiae* strains (patterns no. 3′ and 19′) isolated during the 2001 harvest.

**Table 4**
Physiological characteristics of the different *Saccharomyces* strains.

| Strain reference | β-Glucosidase activity | Killer phenotype | $H_2S$ production |
|---|---|---|---|
| 3′ | + | N | 0 |
| 19′ | + | N | 2 |
| 22′ | – | K | 2 |
| 47 | – | K | 1 |
| 12 | – | S | 1 |
| 15 | – | S | 3 |
| 33, 61 | – | S | 0 |
| 1′, 2′, 4′, 5′, 6′, 7′, 8′, 9′, **13′**, 14′, **15′**, 26′, 54′, 1, 2, 4, 9, 13, 14, 16, 19, 20, 29, **36**, 37, 39, 43, 44, 49, 50, **53**, 54, 62, 63, 70, 76, 78, 81, 83, 94 | – | N | 0 |
| 11′, 16′, 18′, 23′, 24′, 31′, 33′, 35′, 36′, **39′**, 49′, 51′, 3, 5, 7, 10, 11, 17, 21, 22, 23, 24, 25, 26, 27, 28, 30, 31, 32, 34, 40, 41, 45, 46, 48, 51, 52, 55, 56, 57, 58, 59, 60, 64, 65, 66, 67, 68, 71, 72, 73, 74, 77, 79, 80, 82, **84**, 85, 86, 87, 91, 93 | – | N | 1 |
| **12′**, **17′**, 20′, **21′**, 25′, 27′, 29′, **30′**, **32′**, 34′, 37′, 38′, 40′, 42′, 46′, 50′, 52′, 53′, 8, 18, **35**, **38**, **42,** 75, **88**, 90, **92** | – | N | 2 |
| 10′, 28′, 6, 69, 89 | – | N | 3 |

+: β-glucoside activity; –: no β-glucoside activity; N: neutral phenotype; K: killer phenotype; S: sensitive phenotype; 0: no $H_2S$ production; 1: low $H_2S$ production; 2: middle $H_2S$ production; 3: high $H_2S$ production; Bold numbers: patterns common to both harvests (**17′** and **92**), (**39′** and **84**), (**32′** and **88**), (**15′** and **53**), (**12′** and **42**), (**30′** and **35**), (**13′** and **36**) and (**21′** and **38**).

## 4. Discussion

Yeasts associated with natural cider fermentation were analysed in one cellar from a molecular and physiological perspective. The observed cider microflora showed a high diversity in terms of both yeast species and *Saccharomyces* patterns.

Non-*Saccharomyces* species were isolated mainly in the apple juice and in the early stages of the cider-making process. The absence of *Saccharomyces* yeasts in the must has previously been explained by the use of a fast pressing system (Suárez et al., 2007a). Within the non-*Saccharomyces* yeasts, the isolation of *C. parapsilosis* strains in one sampling at a high percentage is worth noting. This finding is an original feature, as this species had never been isolated in cider fermentations until now. The presence of this species type did not affect the sensorial characteristics of the final cider, according to a trained expert panel (data not shown). *C. parapsilosis* has previously been described in other fermented products such as white wine, in the appellation of origin "Valle de Güimar", Tenerife (González et al., 2007), orange juice (Arias et al., 2002), carrot juice (Senses-Ergul et al., 2006) and cocoa beans (Jespersen et al., 2005). The rest of non-*Saccharomyces* species isolated in this study had already been found in ciders or apple must in England, France, Ireland and Spain (Beech and Davenport, 1970; Michel et al., 1988; Morrissey et al., 2004; Coton et al., 2006; Suárez et al., 2007a). On the other hand, the presence of *Hanseniaspora* yeasts in advanced stages of fermentation are in agreement with previous studies on ciders (Cabranes et al., 1990; Suárez et al., 2007a) and can be explained by the minor sugar content of the apple must with regard to the grape juice and the non-addition of $SO_2$.

Irrespective of the harvest, both *S. cerevisiae* and *S. bayanus/S. pastorianus/S. kudriavzevii/S. mikatae* were detected in spontaneous cider fermentation. The coexistence of these isolated *Saccharomyces* species have been previously reported in cider (Coton et al., 2006; Suárez et al., 2007a).

The degree of *Saccharomyces* strain variability, which indicates the number of strains participating in the fermentation process, was high. This fact had been associated with the uncommon addition of commercial starter yeasts (Querol et al., 1992b; Torija et al., 2001; Beltran et al., 2002) and/or with the low frequency of the killer phenotype in autochthonous yeasts (Cabranes, 1994). In fact, the majority of the strains (95%) had a neutral phenotype and only two strains were found to have the killer phenotype in this study. The variability detected within *Saccharomyces* spp. was higher in the 2003 harvest than in that of 2001. In this respect, it has been reported that factors such as the specific year studied (Gutiérrez et al., 1999) may influence the yeast flora or the number and the percentage of different *S. cerevisiae* strains in spontaneous alcoholic fermentations. Our ability to isolate new patterns shows that the yeast population changes from year to year. However the isolation of 8 patterns common to both harvests indicates the presence of resident patterns in the cellar (Vezinhet et al., 1992; Sabate et al., 1998), whose development has been associated in other studies with the different conditions of each harvest (Epifanio et al., 1999; Torija et al., 2003). Regarding the sequential substitution of patterns detected throughout fermentation, these results have been previously reported by several researchers (Querol et al., 1994; Gutiérrez et al., 1999; Santamaría et al., 2005; Suárez et al., 2007b).

Screening for the production of extracellular hydrolytic enzymes permitted the detection of two *S. cerevisiae* strains with 1,4-β-glucosidase activity. Research on β-glucosidase in yeast has revealed that the majority of *Saccharomyces* isolates do not show activity in a natural substrate like arbutin and that such activity is more frequent in non-*Saccharomyces* yeast strains (Rosi et al., 1994; Rodríguez et al., 2004; Fia et al., 2005). However, Mateo and DiStefano (1997) demonstrated the hydrolysis of grape glycosides by crude extracts of *Saccharomyces* strains. This activity was suggested to be related to exo-β-glucanase activity (EXG1) in *S. cerevisiae* (Gil et al., 2005). A study to determine glucoside hydrolase activity in *Saccharomyces* and *Brettanomyces* brewing yeasts showed pronounced β-glucosidase activity only in *Brettanomyces* yeasts (Daenen et al., 2008a). Our study corroborated the infrequent presence of β-glucosidase in *S. cerevisiae*, as only two strains presented this activity. The significance of this activity lies in its capacity to release flavour compounds from natural glucosides, thus increasing the concentration of volatile compounds and thereby improving the organoleptic quality of beverages (Gunata et al., 1988; Arévalo Villena et al., 2005; Daenen et al., 2008b). On the other hand, the formation of hydrogen sulphide by yeasts is related to off odours in fermented beverages and has been shown to be strain-dependent (Guidici et al., 1993).

The two strains with β-glucosidase activity and medium and low $H_2S$ production as well as the two strains producing the killer toxin showed interesting technological characteristics. This study is the first step to preserve cider yeast biodiversity and requires more research to determine the technological characteristics of these strains that have been included in the SERIDA collection of autochthonous yeasts.

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