International Journal of Food Microbiology 249 (2017) 9–17

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Physiological characterization of *Saccharomyces uvarum* and *Saccharomyces eubayanus* from Patagonia and their potential for cidermaking

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ARTICLE INFO

Article history:
Received 28 October 2016
Received in revised form 9 February 2017
Accepted 28 February 2017
Available online 01 March 2017

Keywords:
Cryotolerant yeast
Cider
Aroma
Diversity

ABSTRACT

A diversity of yeast strains belonging to the cryotolerant fermentative species *S. uvarum* and *S. eubayanus* have been recovered from natural habitats and traditional fermentations in North Patagonia. The aim of this work was to evaluate the most relevant physiological features in a set of Patagonian strains belonging to *S. uvarum* and *S. eubayanus*, in order to analyze their potentiality to be used as starter cultures for cidermaking elaborated at low temperature.

We evidenced that *S. uvarum* strains isolated from natural habitats (*Araucaria araucana* bark) showed similar physiological features to *S. eubayanus* strains obtained from the same habitat, and different from *S. uvarum* strains from fermentative environments (apple *chichas*). We also confirm the capacity of *S. uvarum* to produce high glycerol levels, low acetic acid and elevated production of the higher alcohol 2-phenylethanol and 2-phenylethyl acetate and demonstrated similar properties in *S. eubayanus*. Finally, we evidenced for the first time the antagonistic activity of *S. eubayanus* and selected three strains (two *S. uvarum* and one *S. eubayanus*) bearing the best combination of features to be used as a starter culture in cidermaking.

1. Introduction

Apple (*Malus domestica* Borkh) is one of the most important fruit crops in temperate regions worldwide. Apple-based beverages such as ciders have been consumed for centuries by the peoples of Eurasia, even before the advent of the cultivated apple (Cornille et al., 2014). Cider is an alcoholic beverage typically produced in many European countries such as Germany, England, Scotland, France, Spain, Ireland and Slovenia. After colonization it was introduced in temperate regions of America and the earliest reports about cider elaboration in Argentina are associated with the Paraná River delta after 1900 a.C. Nowadays, the cider production is mainly concentrated in North Patagonia (86% of the national production) and Cuyo, regions that concentrate the production of pears and apples (Barbagelata, 2010).

As most fermented beverages, the alcoholic fermentation responsible for cider elaboration can be carried out spontaneously, by making use of the naturally occurring indigenous yeast species (Morrissey et al., 2004), or conducted by a selected yeast starter culture.

The spontaneous or natural biological conversion of apple must into cider is known to involve a complex mixture of many different yeast species that can vary according to climatic conditions, apple varieties and applied technology (Cabranes et al., 1990, del Campo et al., 2003; Suárez-Valles et al., 2007). However, those species belonging to *Saccharomyces* genus – and particularly *Saccharomyces cerevisiae* – dominate the alcoholic fermentation from the middle stages until the finalization of the process (Morrissey et al., 2004; Pando Bedriñana et al., 2010; Suárez-Valles et al., 2007). Due to its excellent fermentative behavior, selected strains of this species have been the traditionally chosen microorganisms to perform starter cultures for ciders and wines (Pretorius, 2000; Suárez-Valles et al., 2005). The use of these pure cultures of yeasts, generally in the form of active dry yeast, provide a useful tool

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.02.018
0168-1605/

for standardizing the product allowing the obtention of beverages with similar features year after year (Fleet and Heard, 1993).

Although *S. cerevisiae* has been isolated from most natural cider fermentations, another species of the genus known as *Saccharomyces uvarum* (in old publications is also referred as *Saccharomyces bayanus* var. *uvarum*) has been obtained from ciders elaborated in specific regions, particularly from those conducted at low temperatures (Naumov et al., 2001; Suárez-Valles et al., 2007). This species has been also associated with certain wines as Tokaj (Hungary, Slovakia), Amarone (Italy) and Txakoli (Spain), as well as from French wines as Sauternes and the whites in Burgundy, Champagne, Val de Loire and Alsace (Demuyter et al., 2004; Naumov et al., 2000; Naumov et al., 2002; Rementeria et al., 2003; Sipiczki et al., 2001; Torriani et al., 1999).

S. uvarum has a particular fermentation profile characterized by, besides its preferences for low temperature fermentation, a lower acetic acid and ethanol but more glycerol and succinic acid concentrations than *S. cerevisiae*, while synthesizing malic acid without posterior degradation (Bertolini et al., 1996; Giudici et al., 1995; Kishimoto et al., 1993). Moreover, *S. uvarum* produces high concentrations of desirable volatile fermentative compounds such as phenylethanol and its acetate (Masneuf-Pomarède et al., 2010) as well as volatile thiols (Masneuf et al., 2002).

An appreciable number of strains belonging to *S. uvarum* species have been isolated from fermented beverages in Europe and only a few strains were obtained from natural environments in both Europe and North America. In South America the situation is different; a huge natural population of *S. uvarum* has been found in Patagonian forests together with its sibling species *Saccharomyces eubayanus* (Almeida et al., 2014; Libkind et al., 2011; Rodríguez et al., 2014), and only a few were obtained from traditional apple chicha fermentations (Rodríguez et al., 2017).

S. eubayanus has never been found in association with fermented beverages and, besides Patagonian strains, this species was only recovered from the Tibetan Plateau in West China (Bing et al., 2014), North America (Peris et al., 2014; Peris et al., 2016) and New Zealand (Gayevskiy and Goddard, 2016). However, this species has been proposed to constitute one of the parental species of the natural hybrids *S. pastorianus* and *S. bayanus* (Libkind et al., 2011), associated with the elaboration of diverse alcoholic fermented beverages including beer, cider and wine (Pérez-Través et al., 2014). Due to its recent discovering, no works about the potential use of *S. eubayanus* in the elaboration of fermented beverages have been published until now. However, due to its close phylogenetic relationship with *S. uvarum*, *S. bayanus* and *S. pastorianus*, this species could become a biotechnologically relevant microorganism for the fermented beverages industry.

In our laboratory, we possess a diversity of both *S. uvarum* and *S. eubayanus* strains recovered from natural habitats as well as from traditional fermentations that could exhibit interesting features for the development of starter cultures. The presence of *S. uvarum* in ciders and apple *chichas* make us to think about the potential of this two species in cidermaking. The aim of this work was to evaluate the most relevant physiological features in a set of Patagonian strains belonging to *S. uvarum* and *S. eubayanus*, in order to evaluate their potentiality to be used as starter cultures for cidermaking.

2. Materials and methods

2.1. Yeast strains

Nineteen *S. uvarum* and thirteen *S. eubayanus* strains previously isolated, identified and molecularly characterized in our laboratory were used in this study. The strains were obtained from both *A. araucana* trees and traditionally produced apple *chicha* (Table 1). An indigenous *S. cerevisiae* strain obtained from North Patagonian natural Malbec wine fermentations was used with comparative purpose. All strains are deposited in the North Patagonian Culture Collection (NPCC), Neuquén, Argentina.

2.2. Laboratory scale fermentations

Laboratory scale fermentations were carried out in 50 mL flasks containing 35 mL of sterilized (120 °C, 15 min) Royal Gala apple juice from harvest 2012 (°Brix: 11.9, density: 1.056 g L^{-1}, total reducing sugars: 125.7 g L^{-1}; total acidity: 4.73 g L^{-1}; total SO_2: 112 mg L^{-1}; free SO_2: 70 mg L^{-1}; pH: 3.56). Fermentations were inoculated individually with $2 \cdot 10^6$ CFU mL^{-1} of the respective yeast strain and incubated at 25 °C without shaking. The fermentation evolution was daily followed by weight loss until constant weight during two consecutive measures. Experiments were carried out in triplicate.

Scaled up fermentations (1 L flasks containing 800 mL must) were carried out with a strain of each species and origin in two different apple juices: Granny Smith (°Brix: 12.5, density: 1.058 g L^{-1}, total reducing sugars: 132.3 g L^{-1}; total acidity: 5.58 g L^{-1}; total SO_2: 90 mg L^{-1}; free SO_2: 58 mg L^{-1}; pH: 3.54) and the same Royal Gala previously described. The fermentations were conducted at 25 °C and in this case, fermentation evolution was monitored by daily measuring °Brix. Fermentations were carried out in duplicate.

2.3. Enological parameters

Most general oenological parameters were determined according to the methods proposed by Ribereau-Gayon et al. (2003). Ethanol concentration was determined by steam distillation and expressed as Gay Lussac degrees (°GL or % v/v). Volatile acidity was determined by steam distillation followed by titration with NaOH 0.1 N and it was expressed as acetic acid (g L^{-1}). Total acidity was determined by direct titration using NaOH 0.1 N at 20 °C and expressed as tartaric acid (g L^{-1}). Free SO_2 was determined by direct titration with iodine and total SO_2 was determined by sample treatment with KOH (1 N) followed by titration with iodine. Glucose, fructose and glycerol concentrations were determined enzymatically using Boehringer Mannheim commercial kits.

Table 1
List of yeast strains used in this study.

Species	Strain (NPCC number)	Origin[a]	Sources
S. eubayanus	1282–1287, 1291–1292, 1294, 1296–1297, 1301–1302	*Araucaria araucana*	Rodríguez et al. (2014)
S. uvarum	1288–1290, 1293, 1298	*Araucaria araucana*	Rodríguez et al. (2014)
	1309, 1311, 1314–1317, 1320–1324, 1328–1330	Apple *chicha*	Rodríguez et al. (2017)
S. cerevisiae	1178 (MMf9)	Wine	Lopes et al. (2002)

NPCC: North Patagonian Culture Collection, Neuquén, Argentina.

[a] *Araucaria araucana*: yeast strains were obtained from bark or seed samples collected from three different sampling areas in Northwestern Patagonia, Argentina: Caviahue (*S. eubayanus* NPCC 1282–1287), Tromen (*S. eubayanus* NPCC 1291, 1292, 1294, 1296, 1297, 1301, 1302; *S. uvarum* NPCC 1288, 1290, 1298) and Huechulafquen (*S. uvarum* NPCC 1289, 1293) during 2011 summer season. Apple *chicha*: yeast strains were isolated from musts obtained from feral apples collected in three different areas of Chile: Tirúa (*S. uvarum* NPCC 1315, 1316, 1320–1322), Villarrica (*S. uvarum* NPCC 1309, 1311, 1323, 1324) and Pucón (*S. uvarum* NPCC 1314, 1317) during 2011 summer season. Wine: *S. cerevisiae* NPCC 1178 was isolated from Malbec grape must in Cipolletti, Río Negro, Argentina, during 1999 autumn season.

2.4. Higher alcohols, esters, acetaldehyde and terpenes determinations

Aliquots of the fermented product were analyzed by headspace solid-phase-microextraction sampling (SPME) using 50/30 μm DVB/CAR/PDMS fibers (Sigma-Aldrich) and GC according to Rojas et al. (2001). Aliquots of 1.5 mL of the samples were placed in 15 mL vials and 0.3 g of NaCl and 15 μL of 0.1% (v/v) 2-octanol in ethanol were added as internal standard. The vials were closed with screwed caps and 3 mm thick teflon septa. Fibers were injected through the vial septum and exposed to the headspace for 30 min and then desorbed during 10 min in an HP 7890 series II gas chromatograph equipped with an HP Innowax column (Hewlett-Packard) (length, 60 m; inside diameter, 0.32 mm; film thickness, 0.50 μm). The injection block and detector (FID) temperatures were kept constant at 220 and 250 °C, respectively. The oven temperature was programmed as follows: 40 (7 min) to 180 °C at 5 °C min^{-1}, and 200 to 260 °C at 20 °C min^{-1} and kept 15 min at 260 °C. Total running time: 75 min.

The following standards were purchased from Sigma Aldrich: isobutilic alcohol, isoamylic alcohol, 1-hexanol, bencylic alcohol, 2-phenyl ethanol, ethyl acetate, isobutyl acetate, ethyl lactate, isoamyl acetate, hexyl acetate, diethyl succinate, bencyl acetate, ethyl caprylate, ethyl 3-hydroxibutanoate, 2-penylethyl acetate, 4-terpineol, limonene, linalool, nerol and geraniol. All standards were of >99% purity. Values calculated for each different compound were the average of two independent assays.

2.5. Organic acids determinations

Organic acids were determined by HPLC using a Liquid Chromatograph with UV–visible detector (Shimadzu), according to Official Methods of Analysis AOAC International. The equipment also contained Bomb LC 20AT, Column oven CTO 6A, Controller CBM 20A, autoinjector SIL 10 A, Detector Diode Array SPD-M10A and a computer software data acquisition LC Solution. The samples were filtered through a 0.45 μm nylon filters and directly injected (10 μL) onto the chromatographic column. Standard solutions (malic acid, lactic acid, acetic acid and shikimic acid) were prepared by dilution of the individual compounds in ultrapure water.

2.6. Enzyme screening methods

Qualitative assays of enzymatic activity detection were performed on solid media using fresh yeast cultures (24–48 h cultures in GPY).

2.6.1. Protease activity

Skim milk agar plates containing basic medium agar (w/v: 0.67% YNB, 0.2% glucose and 2% agar) were supplemented with 1% w/v skim milk powder, inoculated with fresh yeast cultures and incubated at 26 °C for 3 days. The appearance of clear halos around the yeast streak was indicative of proteolytic activity (González et al., 2004).

2.6.2. Pectinase activity

Pectinase activity was evaluated using basic medium supplemented with 1% w/v apple pectin (pH 4.5). Plates were incubated at 26 °C for 5 days. Enzyme activity was evidenced by the formation of a clear zone around the colonies against a purple-brown background on pectin plate after Lugol's solution addition (Fernandes-Salomão et al., 1996).

2.6.3. Glycosidase activities

Tubes (1.5 mL) containing 500 μL of basic medium agar added with 1 mM

p-nitrophenyl-β-D-glucopyranoside (pNPG) and *p*-nitrophenyl-β-D-xyloside (pNPX) to evaluate β-glucosidase (βGl) and β-xylosidase (βXy) activities, respectively (Rodríguez et al., 2004). Cultures were incubated at 26 °C for 5 days. Yeast strains showing enzymatic activities developed a yellow coloration in the culture medium after addition of 250 mM Na_2CO_3 buffer (100 μL). Agar esculin medium (w/v: 0.67% YNB, 0.5% esculin, 0.1% glucose, 0.02% ferric ammonium citrate, 2% agar; pH 5) was also tested to confirm βGl activity. In this case, strains showing βGl activity produced a dark brown halo around the colony (Hernández et al., 2003).

2.7. Antagonist activity

Antagonistic activity was evaluated using the seeded-agar-plate technique on YEPD-MB medium (w/v: 1% yeast extract, 2% glucose, 2% peptone, 2% agar, 0.0003% methylene blue) buffered at pH 4.6 with 0.5 M phosphate–citrate (Sangorrín et al., 2002). The collection strains *S. cerevisiae* P351 (PROIMI yeast collection) and *Candida glabrata* NCYC 388 were used as sensitive yeasts.

2.8. Statistical analysis

2.8.1. Kinetic parameters in microfermentations (50 mL)

Kinetic parameters were calculated from each fermentation individually using the amount of CO_2 lost daily by the system and the reparametized Gompertz equation proposed by Zwietering et al. (1990):

$$y = A * \exp(-\exp(((\mu max * e)/A) * (\lambda - t) + 1))$$

where y = ln(Nt / N0), N0 is the initial weight of the system (g) and Nt weight at time t; A = ln(N∞/N0) is the maximum CO_2 production with N∞ as the asymptotic maximum, μmax is the maximum fermentation rate (h^{-1}), and λ the period of time needed to start the vigorous fermentation. Growth data from each treatment and yeast were fitted by a non-linear regression, minimizing the sum of squares of the difference between experimental data and the fitted model (observed − predicted)^2. This analysis was carried out using the non-linear module of the Statistica 7.0 software package and its Quasi-Newton option.

2.8.2. Kinetic parameters in 1 L fermentations

°Brix decrease during 1 L fermentations was fitted to the Exponential decay function previously used by Arroyo-López et al. (2008): $Y = D + S * e - K * t$. Where Y is the final °Brix value, t is the time (h), D is a specific value when $t \rightarrow \infty$, S is the estimated value of change, and K is the kinetic constant (h^{-1}).

ANOVA and Tukey honest significant difference tests (HSD) with $\alpha = 0.05$ were performed by mean comparison of kinetic and physiological analysis. The data normality and variance homogeneity in the residuals were verified by Lilliefors and Bartlet tests respectively. Principal component analysis (PCA) and clusters analysis (Unweighted Pair Group Method with Arithmetic Mean, UPGMA) were performed on kinetic and physicochemical variables using the NTSYS program (Numerical Taxonomic System version 2.11) (Rohlf, 2000). RStudio.Version (3.3.2) with pvclust statistical package was used for bootstrapping (R Core Team, 2016).

3. Results

3.1. Physiological characterization

3.1.1. Enological features in laboratory scale fermentations

All 32 *Saccharomyces* strains belonging to different cryotolerant yeast species (*S. uvarum* and *S. eubayanus*) and origin (*A. araucana* and *chicha*) were tested for their main enological properties in microfermentation trials (50 mL flasks) using Royal Gala apple juice. Kinetic and physicochemical parameters obtained from these fermentations are shown in Supplementary material Table 1. As a general rule, all strains completed the alcoholic fermentations, evidencing residual amounts of total reducing sugars (glucose + fructose) lesser than 0.6 g L^{-1}. Main differences among strains were observed in the kinetic

parameters A (maximum CO_2 production) and λ (time required to start the vigorous fermentation). While *S. cerevisiae* showed the highest λ value, both *S. uvarum* and *S. eubayanus* from natural habitats evidenced, in general, the highest A values. This last value is directly related to the estimated amount of ethanol produced (Supplementary material Table 1). Volatile acidity (acetic acid) levels were also a difference between ciders obtained with *S. cerevisiae* and the two criotolerant species, being the first species the highest producer.

Due to the huge amount of data obtained in this stage, we decided to use multivariate analysis (Principal Component Analysis, PCA) for data analysis. Fig. 1A shows the graph obtained from the first two principal components that described 62,2% of the total data variability. This analysis allowed us to evidence that musts fermented with yeasts isolated from natural habitats (*A. araucana*), including both *S. uvarum* and *S. eubayanus*, appeared separated from those obtained from man-made environments (in our study apple chicha for *S. uvarum* and wine for *S. cerevisiae*). By means of UPGMA analysis, we confirmed the presence of different clusters (Fig. 1B). Cluster I, composed by ciders fermented with *S. eubayanus* and *S. uvarum* strains from *A. araucana*, was mainly characterized by fermentations with the greatest λ values (Fig. 1A and C). Cluster II, containing all *S. uvarum* strains from apple chicha (except for the strain NPCC 1317 included in cluster III), was characterized by fermented products with the highest concentration of glycerol and total acidity levels. Finally, cluster III was composed only by the cider elaborated with *S. uvarum* strain NPCC 1317 from *chicha* and evidenced high total acidity; and cluster IV contained the strain *S. cerevisiae* NPCC 1178, characterized by the highest fermentation rate (μ) (Fig. 1A and C).

3.1.2. Enzymatic and antagonist activities

Other physiological traits relevant from the technological point of view, are the presence of some enzymatic activities including protease, pectinase, β-glucosidase (βGl) and β-xylosidase (βXy) as well as the antagonist activity of the yeasts.

Protease activity was not detected among *S. eubayanus* strains, although this activity was evidenced in all *S. uvarum* strains from *A. araucana* and 12 out of 14 *S. uvarum* strains from chicha. Contrarily, pectinase activity was found in almost all strains from both *S. eubayanus* and *S. uvarum* species (Table 2).

Independently from the species and origin, all strains exhibited βGl using the synthetic substrate *p*-nitrophenyl-glucopyranoside (*p*NPG); this activity was not detected in the same amount of strains when using esculin as a substrate. Finally, with the exception of two *S. uvarum* strains from chicha (NPCC 1315 and 1322), all strains were able to hydrolyze the synthetic substrate pNPX evidencing βXy (Table 2).

Regarding the antagonist activity of the evaluated yeast strains, only a few of them (six *S. eubayanus* and five *S. uvarum* strains) were able to inhibit the growth of the *S. cerevisiae* reference sensitive strain. However, only two strains *S. eubayanus* NPCC 1297 and 1302, were also active against *Candida glabrata* sensitive strain.

From all previous results, we selected one strain from each species and origin, with the best combination of physiological features, in order to carry out a new set of fermentations directed to evaluate both their ability to conduct successfully fermentations using different apple musts and their ability to produce desirable volatile aroma compounds. The strains *S. eubayanus* NPCC 1292 and *S. uvarum* NPCC 1290 from *A. araucana* located in cluster I (Fig. 1), were mainly selected because their combination of enzymatic and antagonist activities, due to the fact that they exhibited similar enological features. The strain *S. uvarum* NPCC 1314 isolated from *chicha* and located in cluster II (Fig. 1), was also selected by its enzyme profile as well as its intermediate physicochemical and kinetic characteristics compared to the other strains of the same origin.

3.2. Scaling fermentation (1 L) with selected strains

3.2.1. Fermentation kinetics

Scaled-up fermentations (1 L) were carried out at 25 °C using two different apple juices: Granny Smith (GS) and Royal Gala (RG). All fermentations were inoculated individually with the three yeast strains previously selected and the fermentations monitored by °Brix measures along the process.

According to the data obtained from °Brix measures during the complete processes, all fermentations were completed in 15 days. The °Brix decrease curves were fitted to an exponential decay model, obtaining R^2 values of 99.2–99.8%. The most important difference in kinetic parameters among strains was the substrate consumption rate (k). In RG juice, both *S. uvarum* strains (NPCC 1290 and 1314) showed significantly higher k value than the rest, while *S. cerevisiae* NPCC 1178 showed the lowest one. Contrarily, in GS juice, *S. eubayanus* was the strain with the significantly lowest k value (Table 3).

3.2.2. Chemical composition of ciders

Regarding general chemical composition of the ciders fermented with the selected yeast strains, no significant differences were observed in concentrations of most parameters including residual sugars (glucose and fructose), acetaldehyde and ethanol (Table 4). The exceptions were the high levels of residual fructose observed in GS must fermented with *S. eubayanus*, the high glycerol values obtained with *S. uvarum* (for the

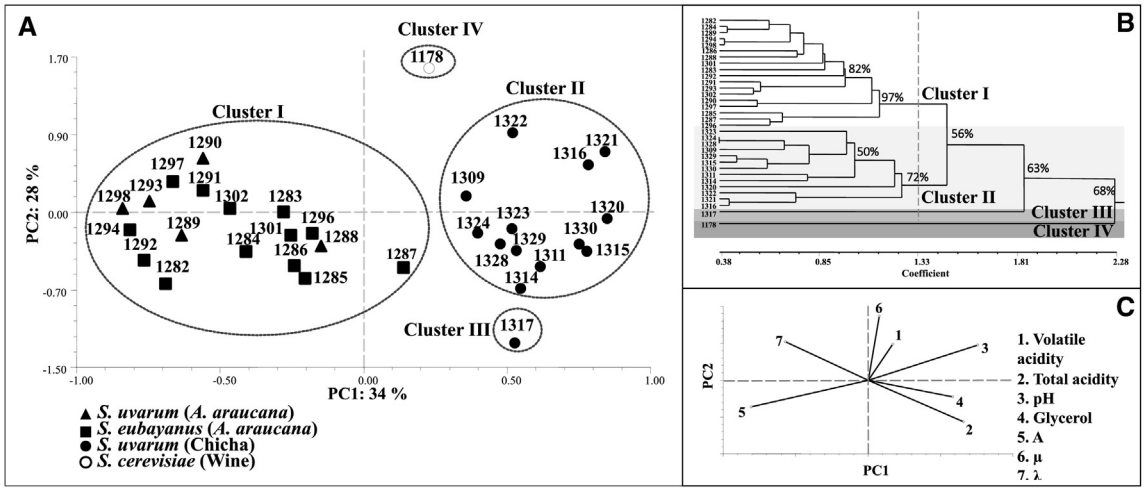

Fig. 1. (A) Principal Components Analysis (PCA) and (B) UPGMA cluster obtained from both physicochemical and kinetics parameters of microfermentation trials. (C) Projection of the eigenvectors on the plane PC1-PC2; the length of each eigenvector is directly proportional to the percentage of variability of the involved parameter explained by the first two principal components.

Table 2
Enzymatic and antagonist activities of *S. uvarum* and *S. eubayanus* strains.

Species	Origin	Strain (NPCC number)[a]	Enzymatic activities (substrate)[b]					Antagonist activity[c]	
			Protease (skim milk)	Pectinase (citric pectin)	ßGl (pNPG)	ßGl (esculin)	ßXy (pNPX)	*Cg*	*Sc*
S. eubayanus	*A. araucana*	1282	−	+	+	+	+	−	−
		1283, 1284, 1286, 1287, 1296	−	+	+	−	+	−	−
		1285	−	+	+	−	+	−	+
		1291, 1292, 1294	−	+	+	+	+	−	+
		1297, 1302	−	+	+	−	+	+	+
		1301	−	−	+	+	+	−	−
S. uvarum		1289	+	+	+	−	+	−	−
		1288, 1290	+	+	+	+	+	−	−
		1293,1298	+	−	+	−	+	−	−
	Chicha	1309	−	+	+	+	+	−	+
		1316	+	+	+	−	+	−	−
		1320, 1321, 1330	+	+	+	+	+	−	−
		1317, 1328, 1329	+	−	+	+	+	−	−
		1311, 1314, 1324	+	+	+	+	+	−	+
		1323	+	+	+	−	+	−	+
		1315	+	+	+	+	−	−	−
		1322	−	+	+	+	−	−	−

[a] NPCC: North Patagonian Culture Collection, Neuquén, Argentina.
[b] βGl: β-glucosidase, βXy: β-xylosidase, pNPG: *p*-nitro-phenyl-glucoside, pNPX: *p*-nitro-phenyl-xyloside.
[c] *Cg*: *Candida glabrata* sensitive control strain; *Sc*: *Saccharomyces cerevisiae* sensitive control strain.

two apple musts employed and the two strains evaluated) and *S. eubayanus* (only in RG juice) and the higher amount of ethanol produced by *S. cerevisiae* NPCC 1178 with regards to that produced by *S. uvarum* NPCC 1314 in GS must (Table 4).

The effect of the inoculated yeast strain was also evident in the composition of the organic acids in the base ciders obtained. Shikimic acid concentrations were always higher in RG ciders than in GS ones, independently from the strain used. Interestingly, both RG and GS musts juices fermented with the two *S. uvarum* strains evidenced the highest concentrations of this acid (Table 4).

Independently from the apple must evaluated, all non-*S. cerevisiae* yeast strains produced the highest concentrations of total higher alcohols in the ciders. These differences with regards to *S. cerevisiae* were particularly due to the elevated concentrations of isoamyl alcohol and 2-phenylethanol (Table 4). In particular, the amount of 2-phenylethanol produced by *S. eubayanus* and *S. uvarum* was at least twice than the amount produced by *S. cerevisiae*.

The highest amount of total esters was significantly detected in the ciders fermented by *S. uvarum* NPCC 1314, isolated from apple chicha. This difference was, in part, due to the elevated concentration of ethyl acetate produced by this strain (twice or more times higher than the rest). However, this strain also produced high concentrations of other esters as 2-phenylethyl acetate, isobutyl acetate, hexyl acetate, ethyl octanoate and ethyl decanoate. The last three esters were particularly abundant in RG cider (Table 4).

Among minor compounds including terpenes and volatile phenols, significant differences were only observed in the concentrations of geraniol and 4-ethylguaiacol (Table 4).

4. Discussion

The yeast species *S. cerevisiae*, is a well-known microorganism in bread, wine and beer elaboration processes. Features like its capacity to survive and grow at high sugar and ethanol concentrations as well as its worldwide geographic distribution have been where the cause for the high number of investigations on this species and its status of model for biological studies in eukaryotes (Moreno Arribas and Polo, 2005).

Other lesser-known species in the *Saccharomyces* genus can also be found in fermentation processes. These species can possess similar or superior industrial characteristics than *S. cerevisiae* or, at least, they can be better adapted to processes with particular characteristics. This is the case of the species *S. uvarum* (Almeida et al., 2014Sipiczki, 2008), generally associated with fermentation processes carried out at low temperatures (Sipiczki, 2002). Although several studies on this species have been carried out during the last 15 years, only a few of them have deepened its main metabolic particularities and biotechnological potential. The main reason for this phenomenon could be the relatively recent acceptance of *S. uvarum* as an individual species, separated from *S. eubayanus* and the group of hybrids *S. bayanus* (Rainieri et al., 1999,

Table 3
Kinetic parameters obtained for 1 L fermentations using the Exponential decay function (Arroyo-López et al., 2008) in two different apple musts.

Kinetic parameters	Royal Gala apple must				Granny Smith apple must			
	S. cerevisiae NPCC 1178	*S. eubayanus* NPCC 1292	*S. uvarum* NPCC 1290	*S. uvarum* NPCC 1314	*S. cerevisiae* NPCC 1178	*S. eubayanus* NPCC 1292	*S. uvarum* NPCC 1290	*S. uvarum* NPCC 1314
D (°Brix)	0.02 ± 0.01	0.23 ± 0.02	0.08 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.45 ± 0.07	0.16 ± 0.08	0.02 ± 0.01
mS	1.00 ± 0.04	0.85 ± 0.01	0.11 ± 0.01	0.05 ± 0.00	3.5 ± 0.34[a]	7.01 ± 0.97[b]	3.2 ± 0.98[a]	2.47 ± 0.52[a]
K (°Brix h^{-1})	7.83 ± 0.07	7.66 ± 0.17	7.91 ± 0.03	7.98 ± 0.01	7.44 ± 0.01[b]	6.62 ± 0.30[ab]	7.24 ± 0.01[ab]	6.32 ± 0.44[a]

D is a specific value when $t \rightarrow \infty$, S is the estimated value of change, and K is the kinetic constant (°Brix h^{-1}).
Fit of model with R2 between 99.2 and 99.8%. Significant coefficients (p-value ≤ 0.050) by apple must. Different superscript letters in the same row indicate significant differences between values obtained for the same apple must.

Table 4
Chemical composition of ciders (1 L) fermented using different yeast strains and apple musts.

COMPOUND	Royal Gala apple must				Granny Smith apple must			
	S. cerevisiae NPCC 1178	*S. eubayanus* NPCC 1292	*S. uvarum* NPCC 1290	*S. uvarum* NPCC 1314	*S. cerevisiae* NPCC 1178	*S. eubayanus* NPCC 1292	*S. uvarum* NPCC 1290	*S. uvarum* NPCC 1314
General compounds								
Glucose (g L^{-1})	0.02 ± 0.01	0.23 ± 0.02	0.08 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.45 ± 0.07	0.16 ± 0.08	0.02 ± 0.01
Fructose (g L^{-1})	1.00 ± 0.04	0.85 ± 0.01	0.11 ± 0.01	0.05 ± 0.00	3.5 ± 0.34^{a}	7.01 ± 0.97^{b}	3.2 ± 0.98^{a}	2.47 ± 0.52^{a}
Ethanol (% *v*/v)	7.83 ± 0.07	7.66 ± 0.17	7.91 ± 0.03	7.98 ± 0.01	7.44 ± 0.01^{b}	6.62 ± 0.30^{ab}	7.24 ± 0.01^{ab}	6.32 ± 0.44^{a}
Glycerol (g L^{-1})	3.73 ± 0.21^{a}	4.37 ± 0.39^{ab}	4.76 ± 0.05^{c}	4.15 ± 0.45^{bc}	3.48 ± 0.28	3.55 ± 0.33	4.17 ± 0.26	4.29 ± 0.39
Acetaldehyde (g L^{-1})	17.15 ± 2.05	9.85 ± 0.77	12.1 ± 6.08	12.05 ± 0.35	16.30 ± 2.26	15.15 ± 4.45	12.50 ± 0.71	8.80 ± 0.56
Free SO_2	35.0 ± 4.2	45.0 ± 0.1	35.0 ± 0.2	43.0 ± 0.2	11.5 ± 2.1	13.0 ± 0.1	13.0 ± 0.1	13.0 ± 0.01
Total SO_2	73.5 ± 4.9^{a}	101.5 ± 2.1^{b}	80.0 ± 4.2^{a}	80.0 ± 4.2^{a}	38.0 ± 0.1	33.5 ± 2.1	30.5 ± 2.1	32.0 ± 0.1
pH	3.46 ± 0.01	3.33 ± 0.01	3.38 ± 0.05	3.44 ± 0.06	3.29 ± 0.38	3.14 ± 0.03	3.31 ± 0.09	3.21 ± 0.01
Organic acids								
Acetic acid (g L^{-1})	0.20 ± 0.02	0.10 ± 0.03	0.10 ± 0.01	0.20 ± 0.02	0.10 ± 0	0.10 ± 0.02	0.10 ± 0.01	0.15 ± 0.07
Lactic acid (g L^{-1})	0.10 ± 0.01	0.10 ± 0.01	0.30 ± 0.01	0.20 ± 0.01	ND	ND	ND	ND
Malic acid (g L^{-1})	6.75 ± 0.07	6.5 ± 0.1	6.95 ± 0.21	6.85 ± 0.07	7.60 ± 0.42^{a}	9.50 ± 0.14^{b}	9.25 ± 0.07^{b}	8.85 ± 0.35^{b}
Shikimic acid (mg L^{-1})	9.05 ± 0.21^{ab}	5.7 ± 0.42^{a}	13.05 ± 0.07^{b}	13.30 ± 2.40^{b}	3.00 ± 0.01^{a}	3.00 ± 0.01^{a}	4.60 ± 0.56^{b}	6.35 ± 0.21^{c}
Total organic acids (g L^{-1})	7.06 ± 0.09	6.71 ± 0.11	7.36 ± 0,23	7.26 ± 0.10	7.70 ± 0.42 ^{a}	9.60 ± 0,16 ^{b}	9.35 ± 0,08 ^{ab}	9.00 ± 0.42 ^{ab}
Higher alcohols								
1-Propanol	12.28 ± 0.58	10.97 ± 0.15	14.71 ± 1.11	15.14 ± 1.86	10.62 ± 1.08	8.68 ± 0.44	10.03 ± 0.13	6.71 ± 4.16
1-Butanol	2.17 ± 0.22	2.14 ± 0.04	2.31 ± 0.13	2.29 ± 0.03	1.52 ± 0.68	1.23 ± 0.13	1.22 ± 0.07	1.11 ± 0.06
1-Hexanol	1.35 ± 0.07	1.26 ± 0.01	1.33 ± 0.15	1.39 ± 0.08	0.95 ± 0.09	1.39 ± 0.46	0.97 ± 0.07	1.27 ± 0.40
2-Phenylethanol	3.09 ± 0.31^{a}	9.31 ± 0.52^{b}	10.88 ± 1.83^{b}	7.97 ± 2.28^{ab}	4.07 ± 0.11^{a}	12.77 ± 0.27^{b}	15.91 ± 0.85^{b}	11.28 ± 0.26^{b}
Benzyl alcohol	0.17 ± 0.01^{b}	0.07 ± 0.01^{a}	0.13 ± 0.01^{ab}	0.11 ± 0.03^{ab}	0.03 ± 6E^{-3ab}	0.02 ± 0.01^{a}	0.02 ± 3E^{-3ab}	0.03 ± 1E^{-4b}
Isoamyl alcohol	91.2 ± 8.63^{a}	133.70 ± 4.24^{ab}	165.35 ± 17.46^{b}	119.05 ± 15.91^{ab}	108.05 ± 5.30^{a}	165.85 ± 7.28^{b}	163.50 ± 9.05^{b}	165.30 ± 14.28^{b}
Isobutyl alcohol	24.70 ± 1.13^{a}	20.3 ± 1.41^{a}	30.95 ± 1.34^{b}	23.75 ± 1.63^{a}	16.00 ± 0.01	19.81 ± 1.84	18.3 ± 0.14	22.45 ± 6.86
Total higher alcohols	134.96 ± 10.94^{a}	177.75 ± 5.33^{b}	225.67 ± 19.25^{b}	169.71 ± 18.05^{ab}	141.25 ± 3.33^{a}	209.75 ± 8.74^{b}	209.96 ± 10.33^{b}	208.17 ± 17.05^{b}
Esters								
Ethyl acetate	6.37 ± 1.51^{a}	6.00 ± 0.11^{a}	7.09 ± 0.71^{a}	14.11 ± 1.63^{b}	7.49 ± 0.36	7.32 ± 1.02	8.19 ± 0.10	29.63 ± 10.93
2-Phenylethyl acetate	0.041 ± 0.001	0.094 ± 0.005	0.084 ± 0.024	0.226 ± 0.095	0.013 ± 0.003^{a}	0.11 ± 0.01^{a}	0.21 ± 0.03^{ab}	0.40 ± 0.12^{b}
Benzyl acetate (μg L^{-1})	1.40 ± 0.01^{ab}	0.75 ± 0.03^{a}	0.86 ± 0.07^{a}	2.0 ± 0.2^{b}	2.0 ± 0.9	0.60 ± 0.07	0.40 ± 0.03	0.80 ± 0.07
Hexyl acetate (μg L^{-1})	13.0 ± 6.0	7.0 ± 0.7	4.5 ± 0.7	22.0 ± 6.0	4.0 ± 1.0	3.0 ± 0.7	10.0 ± 4.0	4.0 ± 1.9
Isoamyl acetate	0.095 ± 0.007^{b}	0.025 ± 0.001^{a}	0.025 ± 0.001^{a}	0.11 ± 0.014^{b}	0.060 ± 0.001	0.05 ± 0.007	0.05 ± 0.01	0.06 ± 0.02
Isobutyl acetate (μg L^{-1})	5.0 ± 1.0^{a}	5.0 ± 0.7^{a}	5.0 ± 0.3^{a}	7.0 ± 0.5^{b}	4.0 ± 0.2	5.0 ± 0.1	5.0 ± 0.3	7.0 ± 2.0
Ethyl butyrate	0.041 ± 0.001	0.046 ± 0.002	0.048 ± 0.001	0.045 ± 0.005	0.028 ± 0.005^{a}	0.036 ± 0.002^{b}	0.042 ± 0.002^{b}	0.039 ± 0.008^{b}
Ethyl decanoate (μg L^{-1})	13.0 ± 3.0^{cd}	2.0 ± 0.1^{a}	7.0 ± 1.0^{ab}	21.0 ± 0.3^{d}	13.0 ± 0.8^{b}	3.0 ± 0.7^{a}	15.0 ± 0.8^{b}	10.0 ± 0.4^{ab}
Ethyl hexanoate	0.047 ± 0.006^{ab}	0.032 ± 0.001^{a}	0.045 ± 0.003^{ab}	0.056 ± 0.004^{b}	0.042 ± 0.003	0.019 ± 0.001	0.040 ± 0.012	0.039 ± 0.001
Ethyl lactate	1.41 ± 0.08^{a}	1.78 ± 0.08^{ab}	3.32 ± 0.45^{b}	2.41 ± 0.77^{ab}	1.11 ± 0.28	1.72 ± 0.65	1.49 ± 0.03	1.88 ± 0.02
Ethyl octanoate (μg L^{-1})	48.0 ± 14.0	14.0 ± 1.0	30.0 ± 12.0	73.0 ± 6.0	25.0 ± 3.0^{c}	2.0 ± 0.2^{a}	15.0 ± 0.6^{b}	12.0 ± 2.0^{b}
Diethyl succinate	0.015 ± 0.003	0.020 ± 0.003	0.036 ± 0.011	0.044 ± 0.009	0.018 ± 0.004	0.031 ± 0.001	0.047 ± 0.007	0.021 ± 0.024
Total esters	8.11 ± 1.54^{a}	8.03 ± 0.02^{a}	10.71 ± 1.09^{a}	17.13 ± 0.74^{b}	8.82 ± 0.07^{a}	9.30 ± 1.65^{a}	10.11 ± 0.09^{a}	32.11 ± 6.02^{b}
Terpenes (μg L^{-1})								
4-Terpineol	0.70 ± 0.05	0.50 ± 0.05	0.70 ± 0.09	0.70 ± 0.10	0.70 ± 0.04	0.60 ± 0.10	3.00 ± 2.00	0.60 ± 0.04
Limonene	0.035 ± 0.003	0.018 ± 0.002	0.040 ± 0.009	ND	0.040 ± 0.002	0.188 ± 0.020	ND	0.018 ± 0.002
Linalool	11.0 ± 1.0	7.0 ± 1.0	8.0 ± 1.0	10.0 ± 1.0	6.0 ± 0.7	4.0 ± 3.0	8.0 ± 1.0	4.0 ± 3.0
Nerol	ND	ND	ND	ND	1.0 ± 0.1	0.5 ± 0.1	1.0 ± 0.5	1.0 ± 0.8
Geraniol	10.0 ± 1.0^{c}	6.0 ± 0.3^{b}	3.0 ± 0.7^{a}	10.0 ± 1.0^{c}	10.0 ± 1.0	5.0 ± 1.0	9.0 ± 0.4	3.0 ± 1.0
Total terpenes	21.7 ± 2.0^{b}	13.5 ± 1.0^{a}	11.7 ± 1.0^{a}	21.7 ± 2.0^{b}	17.7 ± 1.0^{b}	10.3 ± 1.0^{a}	21.0 ± 1.0^{b}	8.6 ± 0.1^{a}
Volatile phenols (μg L^{-1})								
4-Ethylphenol	6.0 ± 0.4	4.0 ± 1.0	5.0 ± 0.4	6.0 ± 0.8	5.0 ± 2.0	1.0 ± 0.2	1.0 ± 0.1	2.0 ± 0.2
4-Ethylguaiacol	5.0 ± 0.1^{b}	3.0 ± 0.3^{a}	4.0 ± 0.4^{ab}	5.0 ± 0.3^{b}	1.0 ± 0.7^{b}	0.4 ± 0.1^{a}	0.3 ± 0.1^{a}	0.4 ± 0.2^{a}
Total volatile phenols	11.0 ± 0.5	7.0 ± 1.3	9.0 ± 0.8	11.0 ± 1.1	6.0 ± 2.7	1.4 ± 0.3	1.3 ± 0.2	2.4 ± 0.4

Values are expressed in mg L^{-1} except in the indicated cases. ND: not detected.
Different superscript letters in the same row indicate significant differences between values obtained for the same apple juice (ANOVA and Tukey test, $n = 2$).

Nguyen et al., 2000; Libkind et al., 2011). With regards to *S. eubayanus* its recent discovery and the low number of strains available worldwide are the reason for the lack of studies. Only a few reports related mainly to its diversity and biogeography can be found (Bing et al., 2014; Gayevskiy and Goddard, 2016; Hittinger, 2013; Peris et al., 2014; Peris et al., 2016; Rodríguez et al., 2014), with almost no information available about its technologically relevant features.

In this work we evaluated the potentiality of a set of strains belonging to *S. uvarum* and *S. eubayanus* species isolated from different habitats (Table 1). From a geographic point of view, the sampling areas are relatively close and show similar climatic features; however, the main differences are related to the specific ecological habitat from which the strains were obtained. This different origin clearly influenced the metabolic features of the strains, as it could be revealed in this work. Ciders obtained after fermentations (50 mL) with strains from natural habitats (both *S. uvarum* and *S. eubayanus*) cluster together in the PCA carried out on the basis of the kinetic features and the basic chemical composition analyzed. Besides, these features were different from those obtained in fermentations carried out with *S. cerevisiae*. Although several research publications have make emphasis on the fructophilic

capacity of *S. uvarum*, the concentration of residual fructose in the ciders elaborated with this species, and also with its sibling species *S. eubayanus*, were not different from that obtained with *S. cerevisiae*. Contrarily, in 1 L fermentations using GS apple must, the highest residual fructose concentrations were obtained with *S. eubayanus*. Nevertheless, the lower λ value in micro-fermentations conducted with *S. uvarum* and *S. eubayanus* with regard to *S. cerevisiae*, could be related to the need for the adaption of this last species to an initial higher amount of fructose than glucose typical in apple musts, but not in grape must. Different explanations for an improved fructose utilization have been mentioned for different *Saccharomyces* species. In *S. cerevisiae*, the high fructose utilization was proposed to be due to the presence of a mutated hexose transporter (Guillaume et al., 2007). In the species *S. bayanus* and *S. pastorianus*, the existence of fructose/H^+ symport in coexistence with the facilitated diffusion system for hexoses was proposed as a cause of the good fructose transport (Gonçalves et al., 2000). Interestingly, Tronchoni et al. (2009) evidenced that *S. uvarum*, as well as *S. kudriavzevii*, showed a slight preference for fructose with regards to *S. cerevisiae*, at the beginning of fermentation. This preference could also be happening in our experiments, as a possible explanation for the lower λ of *S. uvarum* and *S. eubayanus*.

According to bibliographic reports in wine, *S. uvarum* produces lower ethanol and higher glycerol concentrations than *S. cerevisiae* (Castellari et al., 1994; Magyar and Tóth, 2011; Sipiczki, 2002). No information about *S. eubayanus* are reported but, assuming its genetic relationship with *S. uvarum*, *S. bayanus* (hybrid between *S. uvarum* and *S. eubayanus*) and *S. pastorianus* (hybrid between *S. eubayanus* and *S. cerevisiae*), it is likely to find similar metabolic features between *S. uvarum* and *S. eubayanus*. In this work, significant differences in ethanol concentration were found in both microfermentations (50 mL) and 1 L fermentations (GS must). In the case of microfermentations, *S. uvarum* strains from *chicha* produced the lowest levels of ethanol together with *S. cerevisiae;* however, in 1 L fermentations in GS must a lower production of this compound was observed for the three non-*S. cerevisiae* strains. Ethanol is an important flavour component and a product of the Embden-Meyerhof-Parnas glycolytic pathway whose main function is the production of energy. This pathway also gives rise to pyruvic acid, a precursor to acetic acid and a series of other molecules that are related to the aromatic complexity of fermented beverages. Glycerol is also a product derived from the glycolytic pathway and is primarily formed in the early phase of fermentation as a response to osmotic stress (Nevoigt and Stahl, 1997) and to maintain the redox balance in the cell altered due to an excess of cytoplasmic NADH typical from the fermentation process (Albers et al., 1998, Valadi et al., 2004). It has been also associated with a yeast adaptation to low-temperature growth (Izawa et al., 2004). In fermented beverages, this metabolite contributes positively to quality by providing slight sweetness, smoothness and fullness and reducing wine astringency (Ishikawa and Noble, 1995; Remize et al., 2000; Swiegers et al., 2005). The production of high levels of glycerol has been proposed as a typical feature of *S. uvarum* (Antonelli et al., 1999; Bertolini et al., 1996; Castellari et al., 1994; Gamero et al., 2013; Giudici et al., 1995; López-Malo et al., 2013; Rainieri et al., 1999). However, some authors have found that this property is highly variable among *S. uvarum* strains (Magyar and Tóth, 2011). Moreover, using synthetic medium, Masneuf-Pomarède et al. (2010) found high intraspecific variation of glycerol production within *S. uvarum*, with no significant interspecific difference compared to *S. cerevisiae*. Our results partially confirm these reports; evidencing a high variation in the production of this compound among strains, independently from their origin. A similar behaviour was observed here for the first time among strains of *S. eubayanus*, some of them producing the highest and other producing the lowest glycerol concentrations among analyzed ciders. Regarding the balance between ethanol and glycerol in the fermented products, the preselected *S. eubayanus* and *S. uvarum* strains produced higher amounts of glycerol than *S. cerevisiae*, making these strains enologically interesting. A potential explanation for a high glycerol production without decrease of ethanol generation is the synthesis of low concentrations of acetic acid – as it can be experimentally evidenced in the lowest volatile acidity levels in non-*S. cerevisiae* fermentations at both 50 mL and 1 L volumes – in order to maintain the mass balance in the cell.

Pyruvic acid produced during glycolysis is also the substrate for the synthesis of higher keto acids, key compounds in the production of the majority of alcohols, acids and esters. Among esters, one of the most significant compounds that affect flavour in fermented beverages is ethyl acetate. As ethanol is the dominant alcohol in cider, ethyl acetate, produced from acetyl-CoA and ethanol, is the most abundant ester. Ethyl acetate together with acetic acid participates in the sensation of sourness and roughness this beverage and they are not desired compounds at high concentrations (Whiting, 1976). Although in this work the concentration of acetic acid was low particularly in fermentations carried out with *S. uvarum* and *S. eubayanus*, the production of ethyl acetate was always the highest (twice or three times higher than the rest for RG and GS musts, respectively) in ciders elaborated with *S. uvarum* NPCC 1314 isolated from fermentative environment. This strain was also the highest producer of other esters like 2-phenylethyl acetate, isobutyl acetate, ethyl octanoate and ethyl decanoate. All this esters have been related to desired fruity and flowery notes in fermented beverages: isobutyl acetate is associated with fruity flavor, ethyl octanoate with apple-like notes, ethyl decanoate with pear-like notes and 2-phenylethyl acetate with flowery flavor (Huang et al., 2001; Lambrechts and Pretorius, 2000). According to these characteristics, ciders obtained with *S. uvarum* 1314 showed the best combination of features.

The nature and concentration of organic acids are also important factors influencing the organoleptic characteristics of fruit and vegetables, i.e. their flavour. Besides, organic acids may have a protective role against various diseases due to their antioxidant activity (Silva et al., 2004). The major organic acid in apple ciders is L(−)-malic, but cider apple varieties commonly contain other acids like shikimic, quinic, chlorogenic and p-coumarylquinic acids (Beech, 1972). Quinic and shikimic acids are extremely important in the flavour balance of ciders, directly affecting the acidity and, as precursors, also affecting the phenolic fraction. Our results indicate that malic acid was the most abundant acids in the analyzed ciders; however, important differences in shikimic acid were also evidenced among treatments. Malic acid content was significantly higher in ciders fermented with both *S. uvarum* and *S. eubayanus* using GS apple must; this phenomenon could be related with the described capacity of *S. uvarum*, probably present in *S. eubayanus* too, for synthesizing malic acid instead of degrading it (Castellari et al., 1994; Giudici et al., 1995). Interestingly, the levels of shikimic acid were significantly higher only in fermentations carried out with *S. uvarum* independently from the origin (natural source or fermentation). It is known that shikimic acid, formed from phosphoenol pyruvate and erythrose-4-phosphate, is involved in the synthesis of the amino acids phenylalanine, tyrosine and tryptophan, and phenylalanine is the precursor for the synthesis of 2-phenylethanol and 2-phenylethyl acetate (Dietrich and Pour-Nikfardjam, 2009). Both the higher alcohol 2-phenylethanol and the ester 2-phenylethyl acetate were found, coincidently, in the significantly highest concentrations in ciders elaborated with the cryotolerant strains. Previously reported data have indicated that the production of these compounds is a typical trait of *S. uvarum* (Antonelli et al., 1999; Gamero et al., 2013; Masneuf-Pomarède et al., 2010; Stribny et al., 2015). Altogether, our results could indicate that this metabolic pathway could be an important difference between the three species studied in this work.

Together with the 2-phenylethanol, the isoamyl alcohol - and consequently the total amount of higher alcohols - was present in significantly higher proportion in ciders fermented with the cryotolerant (both *S.*

eubayanus and *S. uvarum*) yeasts than in *S. cerevisiae* fermented ones. The higher alcohols are quantitatively the major volatile components in cider (Herrero et al., 2006). They are formed as by-products of both anabolic (Genevois and Lafon pathway) and catabolic metabolism (Ehrlich pathway) (Mangas et al., 1994), and together with the glycerol synthesis pathway, allow the re-equilibration of the redox balance involving NAD+/NADH cofactors. Some higher alcohols, particularly isoamyl alcohol, contribute to unpleasant flavour at elevated concentrations (Rous et al., 1983), but 2-phenylethanol is responsible for the aroma of rose petals and honey and is deemed one of the most important aromatic alcohols contributing to flavour in fermented beverages. It has been previously reported that one of the specific enological properties of *S. uvarum* is the high production of total higher alcohols and specifically 2-phenylethanol (Demuyter et al., 2004; Coloretti et al., 2006; Gangl et al., 2009; Masneuf-Pomarède et al., 2010); however, our work is the first report about the same capacity for *S. eubayanus*. The ability to produce high levels of 2-phenylethyl alcohol has been related to a higher capacity to utilize the assimilable nitrogen in must, a feature that could be related to the yeast species (Mendes-Ferreira et al., 2009; Torrea et al., 2003) and that is being currently studied in our laboratory.

Finally, we included in this analysis the evaluation of the analyzed yeasts to produce a set of enzymatic activities related to the improvement of aroma or stabilization of ciders including pectinases, proteases and glycosidases (Günata et al., 1988; Hsu et al., 1989; Paillard, 1990; Fleet, 2008; Ugliano, 2009; Pando Bedriñana et al., 2012; Martín and Morata de Ambrosini, 2014). It is known that *S. cerevisiae* is not a good producer of extracellular enzymes, however, only a few (*S. uvarum*) or none (*S. eubayanus*) bibliographic reports have evaluated these activities in the cryotolerant species studied here. Our qualitative enzymatic screening evidenced the four activities tested in these cryotolerant species with the only exception of the protease activity absent in *S. eubayanus*. On the other hand, it is interesting to highlight the importance of using at least two types of substrates for βGl screening studies in order to minimize the risk of obtaining false-negative results caused by the specific substrate selectivity of the enzyme as reported by Hernández et al. (2003). The antagonistic activity of a yeast that will be used as a starter culture is also considered a relevant technological attribute (Rainieri and Pretorius, 2000). Strains possessing this characteristic will be able to inhibit the growth of wild *S. cerevisiae* yeasts present during natural fermentations, improving their implantation capacity. In this work, antagonist activity particularly effective against *S. cerevisiae*, was observed for the first time for *S. eubayanus*, which constitute a relevant additional feature to be considered in cidermaking.

5. Conclusions

In this work, we evidenced for the first time that, regarding enological features of the *S. uvarum* and *S. eubayanus*, the source of the strains is as relevant as the phylogenetic relationships among them. *S. uvarum* and *S. eubayanus* from *A. araucana* showed similar physicochemical features and different from those showed by *S. uvarum* from fermented beverages, although they were isolated from close geographic areas in Patagonia. We confirm the proposed metabolic and enological features of *S. uvarum*, evidenced in strains from different origins and characterized by a high glycerol production, and high 2-phenylethanol and its acetate. Finally, we demonstrate that *S. eubayanus* shows similar physiological characteristics to *S. uvarum* and demonstrated for the first time the capacity of this species to ferment apple juices producing ciders without defects as well as to produce antagonist molecules active again *S. cerevisiae*. Three strains with an excellent combination of properties including two *S. uvarum* and one *S. eubayanus* were selected to be used in cidermaking.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2017.02.018.

Acknowledgements

This work was supported by Grants PICT 2011-1738 from ANPCyT and PI04-A128 from Universidad Nacional del Comahue (Argentina) to C.L. Authors want to thank Lucía González for her technical assistance. MGF thanks to CONICET for her PhD fellowship.

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