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Evaluation of autochthonous *Saccharomyces bayanus* strains under stress conditions for making ice ciders

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ABSTRACT

The product diversification policy being carried out by the Asturian cider industry includes the development of beverages similar to so-called ice ciders. During apple juice fermentations, yeast cells are affected by several stress conditions. In this study, 74 *Saccharomyces bayanus* strains isolated from Asturian cider, have been analyzed in synthetic media for their growth ability, sugar fermentation capacity and acetic acid production under the stress conditions that occurr during the production of ice cider. According to the data obtained 23 strains high proliferation capabilities, sugar fermentation capacity and low acetic acid production have been differentiated. Ten strains were chosen to produce ice ciders from apple juice (31.8 ºBrix) at 12 °C. The products obtained were characterized by not having developed the malolactic conversion and their low contents of acetic acid, methanol and ethyl acetate. Significant differences among ciders were detected for alcoholic degree, total acidity, and contents of glycerol, pyruvic, fumaric and shikimic acids due to the yeast strain used. A Multiple Correspondence Analysis showed four strains associated with sensory quality variables. Our results open up the possibility of using autochthonous *S. bayanus* strains as starters in the making of Asturian ice ciders.

1. Introduction

Ice cider is obtained by the fermentation of apple juice with has high sugar content. The Quebec legislation provides that the sugar content of juice must be higher than 30 ºBrix and the finished product must have a minimum residual sugar content of 130 g L^{-1}, and an alcoholic degree between 7 and 13%. There are two methods to obtain a high-sugar content juice. The most commonly used is called cryo-concentration in which the fruit is pressed and the resulting juice is frozen in containers. The second technique, known as cryo-extraction consists of freezing picked apples and pressing until the juice is squeezed from them (Kirkey & Braden, 2014). The juice is then inoculated with yeasts, and fermentation is carried out at 15–17 °C for some months; the alcoholic fermentation typically ends while there is a still considerable content of residual sugars present (Nurgel, Pickering, & Inglis, 2004).

During the production of ice cider, yeasts are subjected to large hyperosmotic stress due to the high solutes concentrations. In wines, this stress has been associated with cell shrinkage, reduced peak cell concentration, yeast biomass accumulation throughout fermentation, and the production of high levels of glycerol and acetic acid in the final product (Pigeau & Inglis, 2005). In addition, as fermentation progresses the ethanol concentration increases and the cells are exposed to an increasingly toxic level of ethanol the effects of which on the physiology of yeast include growth inhibition, reduced cell size and viability, and increased membrane permeability (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). As a consequence of these stress conditions fermentations are often sluggish, taking months to reach the desired ethanol level, also resulting in high levels of volatile acidity (Kontkanen, Inglis, Pickering, & Reynolds, 2004).

The development of special sweet ciders similar to those recognized as ice ciders is part of the present policy for diversification of the Asturian cider making sector. In this sense, the use of

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selected local yeast strains as starters for the production of this kind of cider should ensure the capacity to detect and respond to the above mentioned stress conditions without significant viability losses, giving ciders their typical sensory characteristics (Bauer & Pretorius, 2000; Querol, Barrio, Huerta, & Ramón, 1992).

Saccharomyces bayanus has been found among the yeasts present in fermenting cider habitats (Pando, Querol, & Suárez, 2010; Suárez, Pando, Fernández, Querol, & Madrera, 2007a). Several studies have reported that *S. bayanus* is better suited to grow at lower temperatures than *S. cerevisiae* (López-Malo, Querol, & Guillamón, 2013; Belloch, Orlic, Barrio, & Querol, 2008; Kishimoto & Goto, 1995). *S. bayanus* and *S. kudriavzevii* are considered the most psychrotrophic species of the *Saccharomyces* genus (Belloch et al., 2008; Salvadó et al., 2011). Moreover, this cryotolerant species produces higher amounts of glycerol and less acetic acid than *S. cerevisiae*, this ability being strain-dependent (Bellon, Yang, Day, Inglis, & Chambers, 2015; Castellari et al., 1994; Masneuf-Pomarède, Bely, Marullo, Lonvaud-Funel, & Dubourdieu, 2010). These characteristics make *S. bayanus* strains suitable for the making of ice ciders.

The present study proposes a selection procedure of *S. bayanus* strains for the production of Asturian naturally sweet ciders. For this purpose, a screening study of seventy four strains was done based on their growth ability, sugar fermentation and acetic acid production under stress conditions. Subsequently, ten yeast strains were chosen for the production of ice cider and characterized on the basis of the chemical composition and sensory quality of the resulting ciders.

2. Material and methods

2.1. Yeast strains and inoculum preparation

Seventy four *S. bayanus* strains belonging to the SERIDA culture collection (CCAS) were used in this study (Table 1). They have been isolated from Asturian cellars, identified by RFLP analysis of the 5.8S-ITS ribosomal region and characterized by mtDNA restriction analysis (Pando et al., 2010; Suárez, Pando, González, & Querol, 2007b).

The cells, stored at −80 °C, were revived by streaking onto GPY agar (20 g L^{-1} glucose, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 20 g L^{-1} agar) and incubated at 30 °C for 48 h. One colony was used to inoculate 6 mL of GPY broth and incubated overnight at 30 °C on a shaker at 300 rpm. Next, the cultures were adjusted to an OD_{660} of 0.5 absorbance in a nanodrop 1000 (Thermo Scientific) by dilution with fresh GPY broth.

2.2. Yeast selection under stressful conditions

2.2.1. Synthetic media

Two synthetic media, MSH (102.37 g L^{-1} sucrose, 63.54 g L^{-1} glucose, 186.04 g L^{-1} fructose, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract) and SSH (30 g L^{-1} glucose, 105 g L^{-1} fructose, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 100 mlL^{-1} ethanol) were formulated to respectively represent a 32 ºBrix-apple juice and a cider with 9 ºBrix and 10% v/v ethanol. Both media were adjusted at pH 6.2 and solidified with agar (20 g L^{-1}).

Chalk Agar (CA) medium described by Lemaresquier et al. (1995) was modified to contain a concentration of sugars of 32 ºBrix as follows: 102.37 g L^{-1} sucrose, 63.54 g L^{-1} glucose, 186.04 g L^{-1} fructose, 3 g L^{-1} yeast extract, 3 g L^{-1} calcium carbonate, 15 g L^{-1} agar, (pH = 7.2).

Table 1
List of yeast strains used in this study.

Strain (CCAS number)	Cellar	City	Source
SB1, SB2, SB7	A	Villaviciosa	Fermenting must
C6, SB5	B	Siero	Fermenting must
SB3, SB4	C	Gijón	Fermenting must
SB8 to SB28	D	Villaviciosa	Fermenting must
SB29 to SB45, SB47 to SB49, SB51 to SB54, SB58 to SB66, SB70, SB73	E	Villaviciosa	Fermenting must
SB46, SB50, SB67, SB68, SB71, SB72	E	Villaviciosa	Apple juice
SB55 to SB57, SB69, SB74	E	Villaviciosa	Cider

CCAS: SERIDA culture collection.

2.2.2. Growth abilities

The inocula were 10-fold sequentially diluted in Ringer solution and 5 μL aliquots were spotted in a row onto MSH agar and SSH agar. A positive growth control, in which cells were not exposed to stress conditions was carried out on GPY agar at 30 °C. Growth was measured by comparing the number of colonies that appeared in different dilutions.

2.2.3. Sugar fermentation

The strains (200 μL) were inoculated in 10 mL of MSH and SSH broth with Durham tubes. Fermentation was observed by the presence of a gas bubble trapped inside the Durham tubes. Yeasts were classified according to the following scale: no bubble present, 0; presence of a small bubble, 1; bubble filled ¼ of Durham tube, 2; bubble filled ½ of the Durham tube, 3; bubble filled ¾ of the Durham tube, 4; bubble filled the Durham tube, 5.

2.2.4. Acetic acid production

This characteristic was tested by incubating 5 μL inoculum on modified-CA. The capacity of yeast to produce acetic acid was observed by the formation of a surrounding transparent halo (Suárez, Pando, Lastra, & Mangas, 2008). The yeasts were classified according to the following scale: halo <1 mm, 0 (no production); halo between 1 and 3 mm, 1 (low production); halo between 3 and 5 mm, 2 (medium production); halo >5 mm, 3 (high production).

All tests were carried out in duplicate at 12 °C over a period of 12 days and data were collected every three days.

2.2.5. Data analysis

Two cluster analyses were performed by using the V-PARVUS 2007 statistical package (Forina, Lanteri, Armanino, Casolino, & Casale, 2007). The first analysis was performed to evaluate the growth capacity of the 74 *S. bayanus* strains, by taking into account the highest dilutions at which colonies developed on MSH and SSH media at 6 and 9 days. A 74 × 74 similarity matrix, consisting of Ward's distances in which each observation was represented by a 4-dimensional vector, was used for hierarchical cluster analysis based on Ward's method (Murtagh & Legendre, 2014). The second analysis was performed to evaluate the ability of 48 pre-selected yeast strains to ferment sugars and to produce acetic acid, by taking into account the data obtained from the MSH and SSH media at 6, 9 and 12 days for sugar fermentation, and at 3, 6, 9 and 12 days for acetic acid production. A 48 × 48 similarity matrix, consisting of the Euclidean distances in which each observation was represented by a 10-dimensional vector, was used for the hierarchical cluster analysis based on the average linkage method.

2.3. Evaluation of yeasts for ice cider production

2.3.1. Fermentations

A natural apple juice (31.8 ºBrix) was obtained by pressing frozen whole apples (−8 °C) in the "El Gaitero" cellar (Villaviciosa, Asturias, Spain). The raw material consisted of a mixture of apple varieties belonging to the Protected Designation of Origin "Cider from Asturias".

The ten strains used in this experiment included the *S. bayanus* yeast referred to as C6, usually employed in our laboratory as a reference starter in the making of any kind of cider (Súarez et al., 2005; 2008). The inocula (paragraph 2.1) were pre-inoculated (2%, v/v) in 75 mL of sterilized apple juice (d = 1.045 gmL^{-1}) and shaken overnight at 30 °C. Next, the absorbance (OD_{660}) was adjusted to 0.5 and finally used to inoculate to 1.5% (v/v) of the natural apple juice. To avoid large differences in sugar concentration and temperature between the starter culture and the juice, a stepwise acclimatization was carried out by adding double volumes of juice every 30 min. The final fermentation volume was 750 mL. Two replicates per strain were done.

Fermentations were carried out at 12 °C in 1L capacity Erlenmeyer flasks closed with a Müller valve and monitored by measuring sugar utilization in terms of weight loss through time. After six months, one sample for microbiological analysis was taken, and the Erlenmeyer flasks were stored at 4 °C for 72 h to facilitate the separation of suspended solids. The final ciders obtained were racked in two bottles and preserved until analytical and sensory analysis, at −20 °C and 4 °C respectively.

2.3.2. Microbiological analysis

Several 1:10 (v/v) dilutions were performed in a Ringer's solution and plated for yeast counts in Wallerstein Laboratory Nutrient medium (Pallmann et al., 2001). The analyses of acetic and lactic acid bacteria were done following the methodology described by Cabranes, Mangas, and Blanco (1996).

2.3.3. Analytical methods

Oenological parameters (soluble solids content, alcoholic degree, total and volatile acidities) were determined according to accredited laboratory methods (ENAC 430/L930).

Sugars (sucrose, glucose, fructose), glycerol, sorbitol and organic acids (pyruvic, quinic, malic, shikimic, acetic, succinic and fumaric) were analyzed by HPLC according to the methods described elsewhere (Blanco, Gutiérrez, Mangas, & Noval, 1988; Picinelli et al., 2000).

Major volatile compounds (ethyl acetate, methanol, 1-propanol, *iso*-butanol, 1-butanol, amyl alcohols, acetoin and 2-phenylethanol) were determined by GC-FID. The samples (100 mL) were distilled by means of a direct-heating electronic distiller (Gibertini, Gomensoro, Madrid, Spain), 100 mL of distillate being collected. An aliquot of the distillate was filtered through a 0.22 μm PVDF (polyvinylidene difluoride) membrane filter (Teknokroma, Barcelona, Spain), injected onto the chromatographic system in the split mode (1:5), and analyzed according to the conditions described elsewhere Picinelli et al. (2000).

2.3.4. Sensory analysis

Sensory assessment of the resulting ciders was carried out to evaluate the ability of these yeast strains to produce good quality ice ciders. This analysis was performed by a panel of five people (three men and two women) belonging to the staff of the cellar and the laboratory, with previous experience in descriptive sensory analysis of cider. Two different steps were carried out. In the first, judges evaluated in one session a set of three chemically different samples (SB1, SB3 and SB53) to become familiar with this kind of cider, to reach a consensus in the sensory attributes, and to establish the sensory assessment procedure. In the second step, the samples were analyzed in two sessions (five ciders each), randomly served at 10–12 °C in standard cups (ISO 3591:1977). The evaluation protocol consisted of two phases: first impression, done immediately after serving, and second impression, after leaving the cider at rest in the cup, covered by a glass for 20 min. In both phases, panelists were asked to identify seven odor/aroma attributes (candy, apple-like, fruity, floral, butter, nutty and cocoa), three taste attributes (acidity, bitterness, astringency), and to assess the presence of odor/flavor (vinegar, glue, stale, leather, rancid, oxidized, watery) defects. The number of citations given to each attribute or defect was recorded to provide the respective citation frequencies. Finally, the judges gave an overall impression for the cider's quality by using a discontinuous five-point scale (5, excellent; 4, good; 3, fair; 2, unfair; 1, defective), and the median was calculated for further analyses.

2.3.5. Data analysis

An analysis of variance (ANOVA) was performed to evaluate the influence of yeast strains on the chemical composition of ciders, by using the SPSS program (SPSS, 1994).

The effect of yeast strains on the sensory characteristics of ciders was evaluated by Multiple Correspondence Analysis (MCA) (Greenacre & Blasius, 2006) with the V-PARVUS 2007 statistical program (Forina et al., 2007). Sensory variables were previously transformed to construct a (0, 1) data matrix as follows:

- For sensory attributes and defects binary values were obtained by assigning 1 to those presenting a number greater than or equal to 3 citations and 0 otherwise.
- For quality assessment, binary values were obtained by assigning 1 to those achieving a median value greater than or equal to 3 and 0 otherwise.

Variables with the same value for all of the 10 objects were removed. Seventeen variables were chosen, so a binary matrix dimension (10 × 34) was used for MCA analysis. Correspondence matrix was obtained from variance-covariance matrix of double profiles (y_{iv}) (i observation and v variable), where

$$Y_{iv=}\frac{x_{iv}}{\sqrt{\left(\sum_1^V x_{iv}\right)*\left(\sum_1^I x_{iv}\right)}}$$

(x_{iv} is the binary value for v variable and i observation; V = total number of categories; I = total number of observations).

3. Results and discussion

3.1. Yeast selection under stressful conditions

According to the patterns obtained by mitochondrial DNA restriction analysis all 74 strains were found to be different from each other (data not shown). Criteria used for yeasts selection were the following: growth ability, sugar fermentation capacity and low production of acetic acid.

The growth abilities were evaluated by the appearance of colonies in different dilutions (Table 2). After three days under conditions of no stress (GPY media), the strains showed growth in the 10^6–10^7-fold dilutions, whereas under stress conditions (MSH and

Table 2
Number of strains showing colony development in the highest dilution at high sugar (MSH) and ethanol (SSH) concentration.

Medium	GPY (30 °C)				MSH (12 °C)				SSH (12 °C)			
Dilution/Days	3	6	9	12	3	6	9	12	3	6	9	12
10^{-1}					65	7	0		74	18	5	3
10^{-2}					7	2	0		0	21	2	2
10^{-3}					1	5	4		0	23	1	0
10^{-4}					0	9	0	2	0	9	8	1
10^{-5}	1	1	1	1	1	14	9	8	0	0	20	8
10^{-6}	16	16	16	16	0	21	22	24	0	3	16	22
10^{-7}	57	57	57	57	0	16	39	40	0	0	22	38

SSH medium) the presence of small colonies only in the 10-fold dilution for most of the strains (87–100%) was detected.

The stress conditions could have caused an arrest of the cell cycle and the protein synthesis. The increase in the external osmolarity induces a delay of the G1 and G2 phases of the cell cycle (Alexander et al., 2001). However, the cell often adapts over time to the new conditions, and alterations in the genomic transcription levels subside. The response to environmental stress is proportional to the seriousness of the environmental impact (Gasch & Werner-Washburne, 2002). In our study, the colony development observed at 6 and 9 days showed that yeasts experienced more stress in the SSH agar than in the MSH. On the 12th day, most of the yeasts had been already adapted to the stress conditions of the media, a resumption of growth and cell proliferation being observed.

Cluster analysis showed nine natural groups with 75% similarity (Fig. 1). All the indigenous strains with low cell proliferation in the MSH agar were clustered in the groups VI, VII, VIII and IX. In addition, five strains (SB10, SB21, SB24, SB28 and SB73) were able to grow only in the 10^{-1} and 10^{-2} dilutions in the SSH agar and therefore, the level of ethanol was the most limiting factor for these yeasts to develop.

The first criterion used in the pre-selection of yeast strains led to the elimination of 26 yeasts (groups: VI-IX) because of their unsuitability to grow under the conditions of stress that occurr during the making of ciders.

Fermentation tests carried out on the remaining 48 strains also showed the adaptation of yeasts to stress conditions (Table 3). On the third day, no visible gas production was observed. Throughout the experimental period, bubble size grew in the MSH broth but the same was not seen in the SSH broth. In the last medium eight strains (SB17, SB23, SB26, SB30, SB31, SB34, SB51 and SB65) did not produce bubbles on the 12th day, which would mean that they either do not ferment sugars or do so slowly that the presence of carbon dioxide could not be detected in the Durham tubes (Van Dijken, Van den Bosch, Hermans, Rodrigues de Miranda, & Scheffers, 1986).

Regarding the production of acetic acid, yeasts began to display greater differences after 6 days. It is worth noting that 96% of the indigenous *S. bayanus* strains were classified as low or middle producers and no one of them as a high producer of acetic acid. It has been reported that *S. bayanus* produce reduced levels of volatile acidity in wine fermentation (Eglinton et al., 2000). In this sense Bellon et al. (2015) showed that the use of hybrid strains of *S. cerevisiae* x *S. bayanus* instead of *S. cerevisiae* reduced the acetic acid concentration in fermentations of Riesling ice wines.

A new cluster analysis was performed by taking into account the sugar fermentation and acetic acid production capacities of the 48 pre-selected yeast strains (Fig. 2). At 65% similarity, the strains were grouped into seven clusters. The groups referred to as VI and VII included eight strains that after 9 days of incubation in the MSH

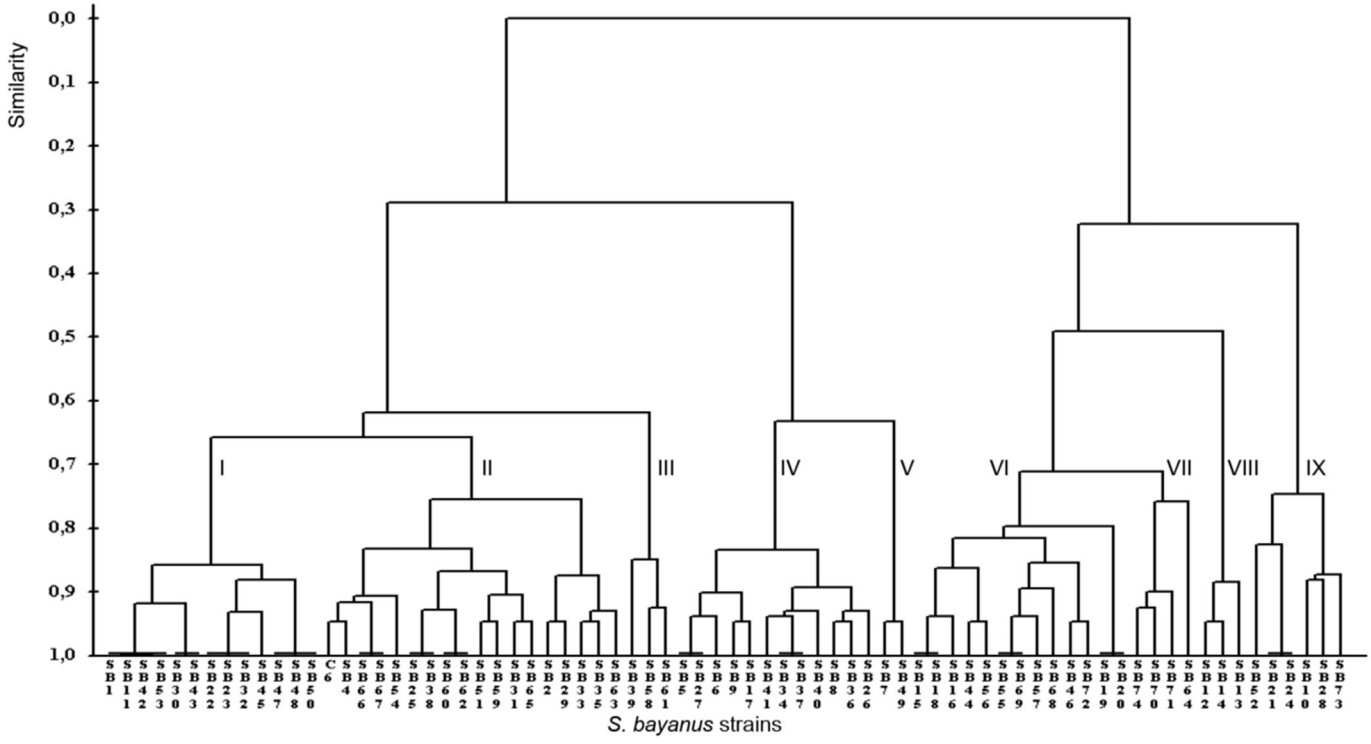


Fig. 1. Dendrogram generated by applying Ward's hierarchical method to the growth ability of the *S. bayanus* strains.

Table 3
Number of yeasts classified according to their ability to ferment sugars and acetic acid production over 12 days.

Medium	MSH				SSH				Medium	modified-CA			
Scale classification for gas production/Days	3	6	9	12	3	6	9	12	Scale classification for acetic acid production/Days	3	6	9	12
0	48	42	10	0	48	28	17	8	0	46	4	2	2
1	0	5	2	1	0	18	20	19	1	2	40	35	24
2	0	1	3	0	0	1	5	3	2	0	4	11	22
3	0	0	7	2	0	0	0	3	3	0	0	0	0
4	0	0	9	0	0	0	1	6					
5	0	0	17	45	0	1	5	9					

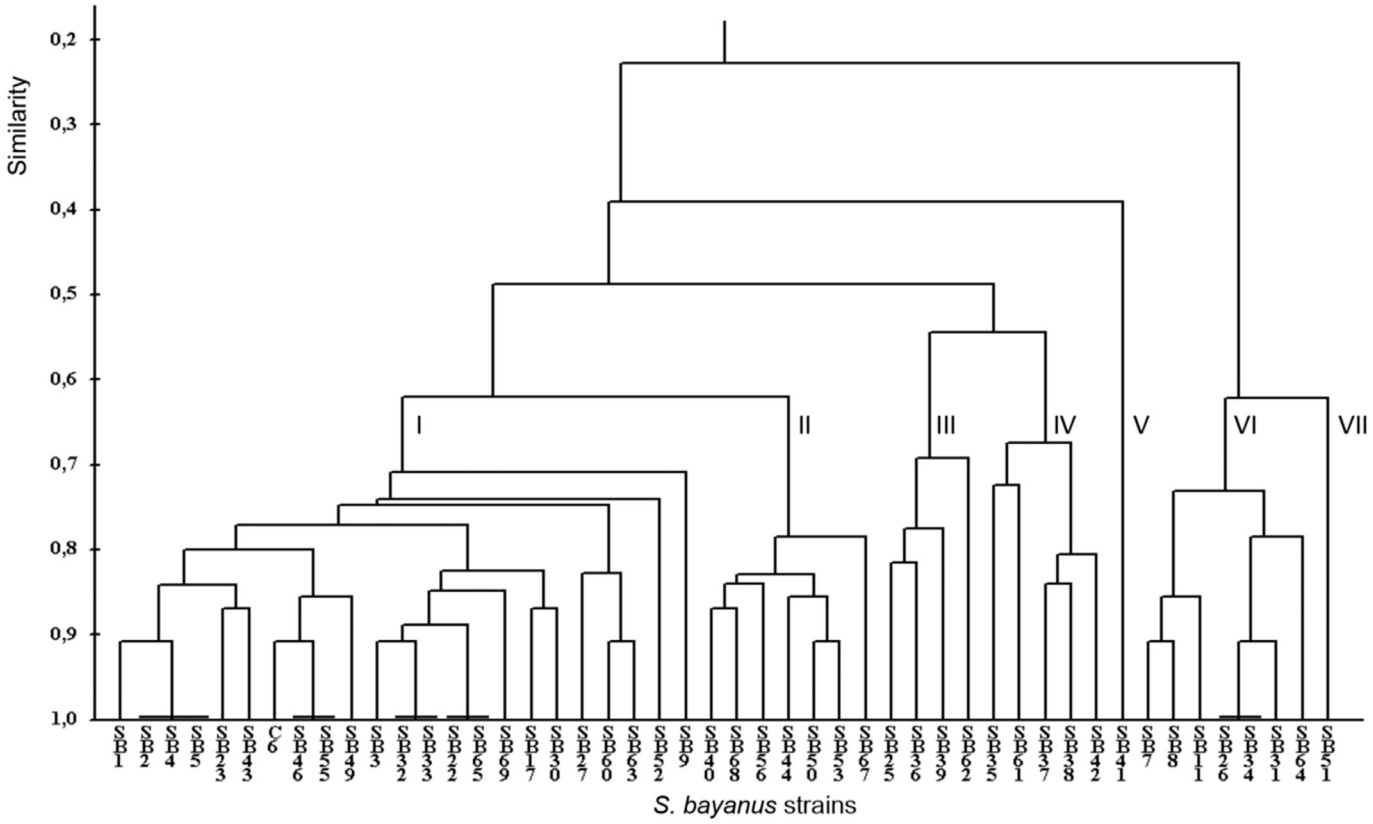


Fig. 2. Dendrogram generated by applying the average linkage method to the sugar fermentation and acetic acid production capacities of the pre-selected yeast strains.

medium had not yet produced any gas. Therefore, on the basis of the capacity to ferment sugars, these eight strains were discarded. Among the remaining 40 strains, 23 were classified, on the basis of the size of the transparent halo in the modified-CA medium, as low or non-producers of acetic acid. According to previous experience of our research group, the above classified yeast strains produced concentrations of acetic acid lower than 0.4 g L^{-1} (Suárez et al., 2008). Taking into account the production of low contents of acetic acid as the third selection criterion, ten autochthonous yeasts were finally chosen for further characterization.

3.2. Evaluation of yeasts for ice cider production

The performance of the ten yeast strains selected to carry out the fermentation was assessed in small scale experiments, by using an apple juice (32 °Brix) obtained by cryo-extraction. The assays were concluded in six months in order to achieve ethanol concentrations above 10% (v/v).

The microbiological and analytical composition of the juice and ciders are shown in Table 4. At the end of fermentation the yeast concentration was greater than 10^4 cfu mL^{-1} and none of the units

Table 4
Microbiological and analytical composition of enriched juice and ice ciders made with ten *S. bayanus* strains. Values for ciders are the mean of two experimental units.

	Juice	Significance	C6	SB1	SB2	SB3	SB4	SB9	SB22	SB23	SB42	SB53
Yeast count (cfu mL^{-1})	1.47E+04	n.a.	1.21E+04	1.04E+04	1.78E+05	2.40E+06	4.78E+04	9.62E+04	2,09E+04	1.14E+06	5.53E+04	1.01E+06
Bacteria count (cfu mL^{-1})	7.49E+03	n.a.	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Alcoholic degree (% v/v)	n.a.	*	10.88 a,b	13.13 d	11.11 a,b	10.98 a,b	10.71 a	10.65 a	11.61 a,b,c	10.74 a	12.09 b,c,d	12.49 c,d
°Brix	31.80		13.2 a	11.8 a	13.0 a	15.0 a	13.4 a	13.6 a	11.7 a	13.4 a	10.9 a	10.3 a
Volatile acidity (g L^{-1} acetic acid)	n.a.		0.48 a	0.44 a	0.54 a	0.41 a	0.42 a	0.49 a	0.42 a	0.58 a	0.58 a	0.47 a
Total acidity (g L^{-1}sulphuric acid)	14.06	*	13.72 b	13.34 b	13.71 b	13.37 b	13.57 b	13.44 b	13.40 b	13.66 b	13.33 b	12.62 a
Sucrose (g L^{-1})	37.08		0.8 a,b	0.8 b	0.8 a,b	0.7 a,b	0.7 a,b	0.7 a	0.8 a,b	0.7 a,b	0.8 a,b	0.7 a,b
Glucose (g L^{-1})	49.11		12.8 b	6.6 a,b	7.3 a,b	12.8 b	10.1 a,b	12.9 b	6.0 a,b	8.9 a,b	3.3 a	3.3 a
Fructose (g L^{-1})	146.73		66.0 a	64.5 a	72.2 a	80.0 a	70.3 a	69.7 a	56.4 a	69.2 a	60.3 a	56.5 a
Glycerol (g L^{-1})	n.d.	*	8.5 c,d	8.4 c,d	8.0 a,b,c	8.1 b,c	8.8 d	7.7 a,b	7.7 a,b	7.6 a,b	7.5 a	7.8 a,b
Sorbitol (g L^{-1})	19.24		18.7 b	19.2 b	19.1 b	18.8 b	18.9 b	18.8 b	17.3 a	18.4 b	18.6 b	18.7 b
Piruvic acid (g L^{-1})	n.d.	*	58.2 d	0.0 a	0.0 a	0.0 a	42.4 c,d	53.4 d	33.7 b,c	29.9 b,c	21.8 b	19.9 b
Acetic acid (g L^{-1})	n.d.		0.3 a	0.3 a	0.3 a	0.2 a	0.3 a	0.3 a	0.3 a	0.4 a	0.4 a	0.3 a
Malic acid ((g L^{-1})	19.09		17.2 c,d	16.1 a	16.8 b,c,d	17.4 d	17.1 c,d	17.2 c,d	16.6 a,b,c	17.1 c,d	16.8 a,b,c,d	16.3 a,b
Quinic acid (g L^{-1})	n.d.		0.6 a	0.7 b,c	0.7 c	0.7 b,c	0.6 a	0.7 b,c	0.7 b,c	0.7 b,c	0.7 b,c	0.7 b,c
Succinic acid (g L^{-1})	n.d		0.6 a	1.0 a	0.7 a	0.5 a	0.6 a	0.5 a	0.8 a	0.8 a	0.8 a	0.6 a
Fumaric acid (mg L^{-1})	n.d.	*	15.6 a,b	17.1 b	15.7 a,b	19.4 c	15.1 a	15.6 a,b	15.7 a,b	15.5 a,b	17.0 a,b	15.7 a,b
Shikimic acid (mg L^{-1})	34.99	*	49 b	51 b,c	49 b	46 a	51 b,c	46 a	54 c	49 b	49 b	43 a
Ratio sugar/total acidity			5.8	5.4	5.8	7.0	6.0	6.2	4.7	5.7	4.8	4.8
Ratio acetic acid/glycerol			0.03	0.03	0.03	0.02	0.03	0.04	0.04	0.05	0.05	0.04

n.a.: unanalyzed; n.d.: not detected; *$P < 0.05$ and a, b, c and d are Duncan's test letters, mean values in the row with the same letter indicate no significant differences between them.

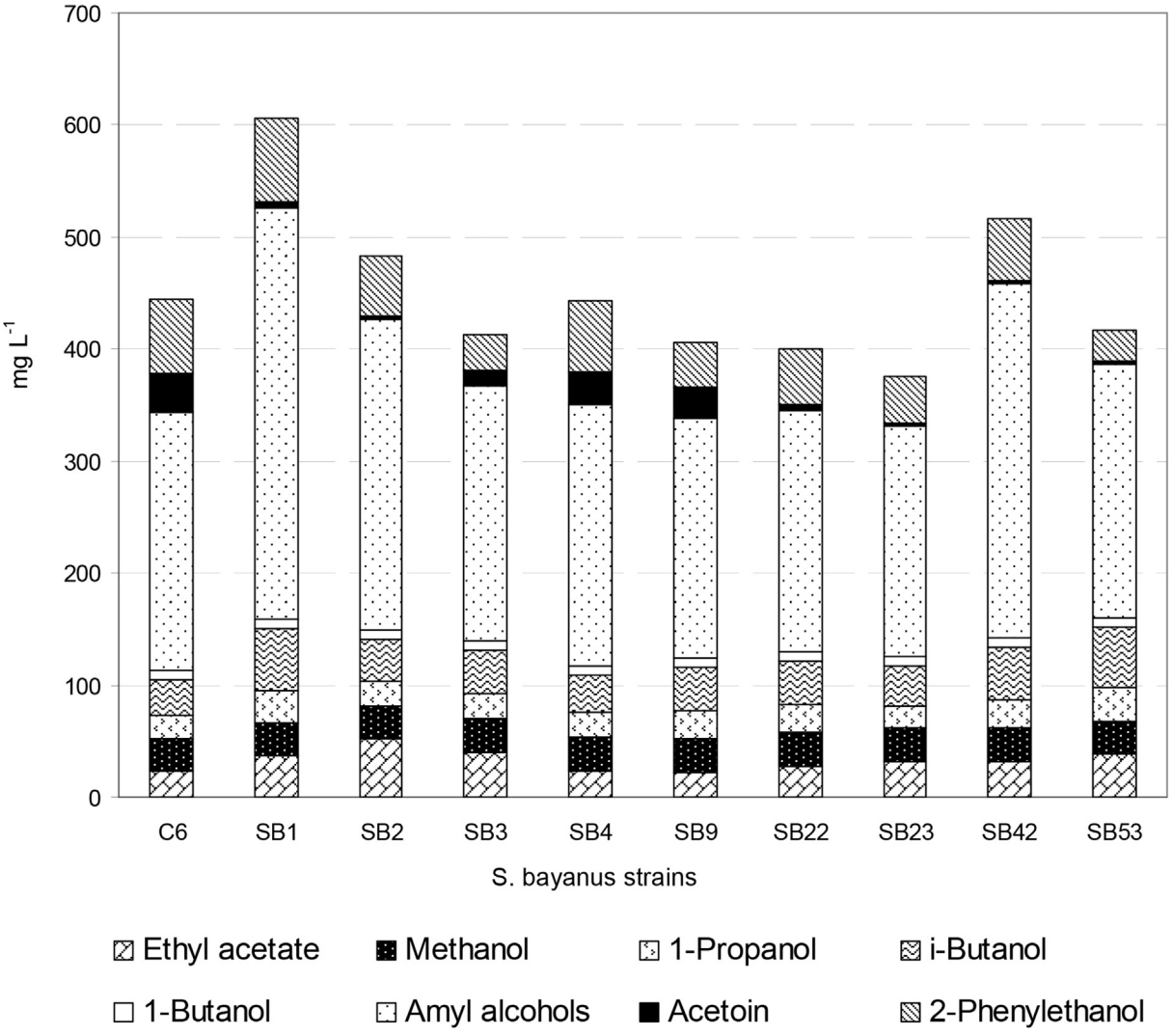


Fig. 3. Concentration of major volatile compounds in ice ciders made with ten selected *S. bayanus* strains.

Table 5
Sensory description and quality assessment of the ice ciders.

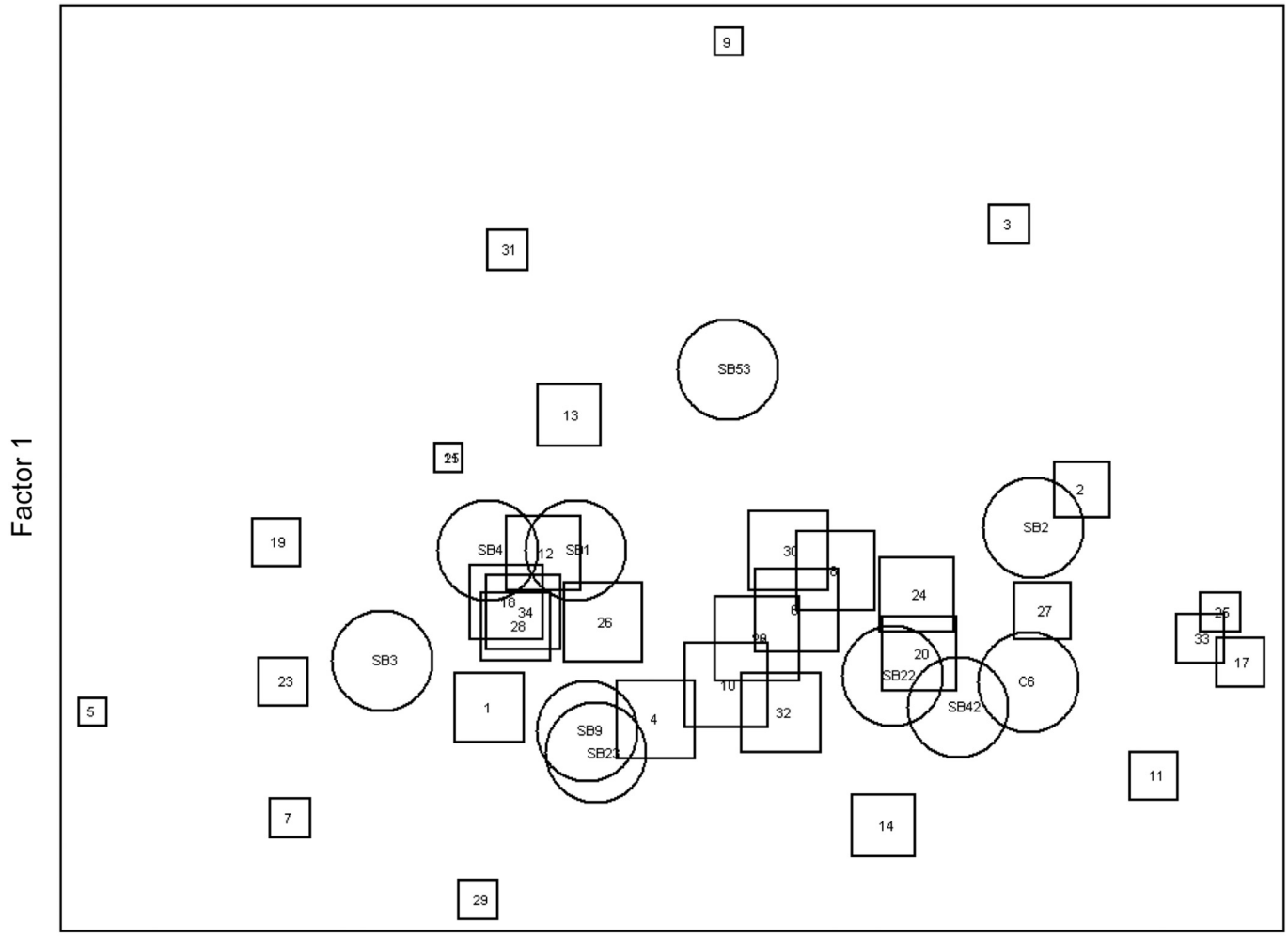
Liquor-ciders		C6	SB1	SB2	SB3	SB4	SB9	SB22	SB23	SB42	SB53
First impression	Odor/Aroma										
	Defective		X		X	X	X		X	X	
	Not defective	X		X				X			X
	Main attributes	Butter	Vinegar	Fruity	Vinegar	Rancid	Rancid	Candy	Vinegar	Fruity	Fruity
	Taste										
	Defective				X				X		
	Not defective	X	X	X		X	X	X		X	X
	Balanced	X						X		X	
	Unbalanced		X	X	X	X	X		X		X
	Main attributes	Acidic	Bitter	Bitter	Bitter	Bitter	Acidic	Bitter	Acidic	Bitter	Bitter
Second impression	Odor/Aroma										
	Defective		X		X	X					
	Not defective	X		X			X	X	X	X	X
	Main attributes	Fruity	Fruity	Fruity	Oxidized	Rancid	Fruity	Fruity	Fruity	Fruity	Alcoholic
	Taste										
	Defective				X	X	X				
	Not defective	X	X	X				X	X	X	X
	Balanced	X	X	X						X	
	Unbalanced				X	X	X	X	X		X
	Main attributes	Bitter	Bitter	Bitter	Bitter	Bitter	Acidic	Bitter	Acidic	Bitter	Bitter

Sensory terms marked with "X" were identified by at least 3 panelists.

presented bacterial contamination.

The ethanol concentration of the naturally sweet ciders, ranging between 11 and 13% (v/v), was significantly affected by yeast strains ($P < 0.05$). Total acidity was very high in all the ciders because the freeze concentration process concentrated both acids and sugars in nearly the same proportion. It is noticeable that malolactic conversion did not occur, since a high concentration of malic acid was still detected at the end of the fermentative process. For all of strains the sucrose conversion percentage was close to 98%, with greater variation being found for glucose (73–96%) and fructose (53–71%). Differences in the time needed to totally consume glucose and fructose have been described at low temperature in *S. bayanus* strains (Tronchoni, Gamero, Arroyo-López, Barrio, & Querol, 2009). Glycerol and pyruvic acid formed in the glycerol-pyruvic fermentation showed significant differences between strains in terms of balancing the internal and external osmotic pressure. As can be seen in Table 4, fumaric and shikimic acids are synthetized by yeasts. Fumaric acid comes from the Krebs'cycle, while shikimic acid is a very important molecule for synthetizing aromatic aminoacids. Significant differences ($p < 0.05$) among strains were detected for both acids.

In Fig. 3 the concentrations of the major volatile compounds analyzed in these ciders are represented. As seen, fusel alcohols (amylics, 2-phenylethanol and *iso*-butanol) were the main components, together with ethyl acetate, in agreement with the usual profiles found in Asturian ciders (Picinelli Lobo, Antón-Díaz, Mangas Alonso, & Suárez Valles, 2016). The contents of 1-butanol and methanol were the same in all ciders, as they come from the



○ Yeast strains; □ Sensory variables; 1-18: First impression; 1: defective odor; 2: not defective odor; 3: fruity odor; 4: no fruity odor; 5: vinegar odor; 6: no vinegar odor; 7: defective flavor; 8: not defective flavor; 9: fruity flavor; 10: no fruity flavor; 11: balanced flavor; 12: unbalanced flavor; 13: bitter; 14: no bitter; 15: watery; 16: no watery; 17: positive assessment; 18: negative assessment. 19-34: Second impression; 19: defective odor; 20: not defective odor; 21: fruity odor; 22: no fruity odor; 23: defective flavor; 24: not defective flavor; 25: fruity flavor; 26: no fruity flavor, 27: balanced flavor; 28: unbalanced flavor; 29: acid; 30: no acid; 31: bitter; 32: no bitter; 33: positive assessment; 34: negative assessment.

Fig. 4. Projection of ice ciders (10) and sensory variables (34) on the first two factors obtained by Multiple Correspondence Analysis.

raw material. Methanol is cleaved from pectins and therefore, its concentration is influenced by apple maturity and the extraction methods while 1-butanol is a varietal component.

Major volatile compounds may play an important role in the quality of fermented beverages, in particular those reported as active odorants, such as amyl alcohols and 2-phenylethanol (Antón, Suárez Valles, García Hevia, & Picinelli Lobo, 2014). Great attention is usually given to volatile acidity and to the concentration of ethyl acetate, in terms of quality control of alcoholic beverages. The formation of higher contents of acetic acid in ice ciders compared with conventional ones could be expected; this fact has been explained in wines as a response of yeasts to the hyperosmotic stress caused by the presence of elevated levels of sugars (Pigeau & Inglis, 2005). Conversely, these experimental ciders reached values for volatile acidity up to 0.58 g acetic acid L^{-1} (Table 4), well below the Spanish legal limit for this parameter for cider (2.20 g acetic acid L^{-1}). Regarding the ethyl acetate contents, these ranged between 22 and 50 mg/L^{-1}, similar to those reported for young Spanish ciders (Picinelli Lobo et al., 2016). These results reinforce the ability of the selected yeast strains to ferment in stressful conditions.

From the sensory point of view, the ciders referred to as C6, SB2, SB22, and SB53 were always positively assessed (not defective), while the opposite was observed for cider made with the SB3 strain. In general terms, all ciders were described as lacking sweetness, which might be justified by the low sugars/total acidity ratios reached in these samples, ranging between 4.7 and 7.0 (Table 4), which is less than half of that described in ice wines (Nurgel et al., 2004). However, it is not easy to explain the influence of chemical composition on the sensory profiles of ice ciders because of the complexity of this particular matrix. For instance, bitter and acidic were the main descriptors for cider taste, these attributes were assessed as balanced in the case of samples referred to as C6, SB2, SB22 and SB42 (Table 5), although there were not significant differences in the total acidity of most of the ciders (Table 4). Likewise, a vinegar odor was identified in ciders referred to as SB1, SB3 and SB23 while no significant differences were found for volatile acidity. This fact was even more difficult to understand taking into account previous results on the relationship between volatile acidity and the evaluation of the vinegar attribute in ciders (Antón et al., 2014) and wines (Cliff & Pickering, 2006). The latter reported that the odor detection threshold for acetic acid in ice wines was 3-fold that of table ones, suggesting that the higher viscosity of the ice wines could modify its perception.

A Multiple Correspondence Analysis was performed to establish relationships among sensory variables and ciders. Five factors that accounted for 86% of the variance were computed considering the variance of each was greater than 1/Q (Q: number of binary variables, equals to 17). Fig. 4 displays the projection of the observations and sensory variables onto the plane formed by the two first factors (48% of variance explained). The size of variables in the projection (square symbol) is related to the frequency computed in the binary matrix. As can be seen, ciders were structured into two groups. The first one, formed by ciders SB1, SB3, SB4, SB9 and SB23, was associated with sensory defective attributes while the second group, which included samples C6, SB2, SB22 and SB42, was associated with sensory quality variables. The cider referred to as SB53, linked to bitter notes, was excluded from the above groups.

4. Conclusions

The results of this paper demonstrate that *S.bayanus* strains can be used as starters in ice cider-making. Through simple screening tests we have been able to select *S. bayanus* strains producing ciders with high alcohol and low volatile acidity. Sensory and analytical results showed the potential of autochthonous strains for the production of naturally sweet ciders.

Further research is needed to improve the quality of these Asturian ciders, with the main target of improvement being the residual sugars/total acidity ratio.

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