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Optimization of fermentation-relevant factors: A strategy to reduce ethanol in red wine by sequential culture of native yeasts

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ABSTRACT

Current consumer preferences are determined by well-structured, full-bodied wines with a rich flavor and with reduced alcohol levels. One of the strategies for obtaining wines with reduced ethanol content is sequential inoculation of non-*Saccharomyces* and *Saccharomyces cerevisiae* yeasts. However, different factors affect the production of metabolites like ethanol, glycerol and acetic acid by inoculated yeasts. In order to obtain low alcohol wines without quality loss, the aims of our study were: i) to determine optimum conditions (fermentation temperature and time of permanence and initial inoculum size of the non-*Saccharomyces* population at the beginning of the process, prior to inoculation with *S. cerevisiae*); ii) to validate the optimized factors; and iii) to assess sensory quality of the wines obtained after validation. Two combinations of yeasts were used in this study: *Hanseniaspora uvarum* BHu9/*S. cerevisiae* BSc114 and *Candida membranaefaciens* BCm71/*S. cerevisiae* BSc114. Optimization of three fermentation factors that affect to non-*Saccharomyces* yeasts prior to *S. cerevisiae* inoculation was carried out using a Box-Behnken experimental design. Applying the models constructed by Response Surface Methodology, the lowest ethanol production by *H. uvarum* BHu9/*S. cerevisiae* BSc114 co-culture was obtained when *H. uvarum* BHu9 was inoculated 48 h 37 min prior to *S. cerevisiae* inoculation, at a fermentation temperature of 25 °C and at an initial inoculum size of 5×10^6 cells/mL. Lowest alcohol production with *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 was observed when *C. membranaefaciens* BCm71 was inoculated 24 h 15 min prior to *S. cerevisiae* at a fermentation temperature of 24.94 °C and at an initial inoculum size of 2.72×10^6 cells/mL. The optimized conditions of the two co-cultures were subsequently submitted to lab-scale validation. Both proposed strategies yielded ethanol levels that were significantly lower than control cultures (*S. cerevisiae*). Wines fermented with non-*Saccharomyces*/*Saccharomyces* co-cultures under optimized conditions were also associated with higher aromatic complexity characterized by the presence of red fruit aromas, whereas wines obtained with *S. cerevisiae* BSc114 were described by parameters linked with high ethanol levels.

1. Introduction

Spontaneous alcohol fermentation is a process carried out by a complex group of diverse yeast species associated with the grape and enological environment, and generally classified into two groups: non-*Saccharomyces* and *Saccharomyces* yeasts. The development of non-*Saccharomyces* yeasts during the early stage of winemaking is still considered an uncontrollable risk by some scientists and enologists, whereas for others, their participation is a way to enhance the wine quality (Albertin et al., 2017). Numerous researchers have reported exhaustive and appropriate screening methods to select non-*Saccharomyces* yeasts that positively affect the winemaking process (Comitini et al., 2011; Mestre et al., 2017; Varela, 2016; Viana et al., 2008).

Current consumer preferences are determined by well-structured, full-bodied wines with a rich flavor and with reduced alcohol levels. However, successful accomplishment of this set of traits is rather difficult due to imbalance between sugar accumulation and phenolic maturity of the berries (which require late harvest in order to guarantee proper aromatic and phenolic maturity) (Goold et al., 2017). Diverse strategies (viticultural, pre-fermentation, microbiological and post

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fermentation practices) have been proposed to obtain wine with reduced ethanol content, and the use of native non-*Saccharomyces* yeasts could be emphasized (Contreras et al., 2014). Some authors have reported a reduction in ethanol concentration using non-*Saccharomyces* yeasts in co-cultures with *S. cerevisiae* (compared to the ethanol concentration obtained with a single *S. cerevisiae* inoculum) (Ciani et al., 2014; Contreras et al., 2014, 2015; Englezos et al., 2016; Varela, 2016). In a previous study, we evaluated 114 non-*Saccharomyces* yeasts to determine their respiratory, fermentation and physiological characteristics in order to select yeast isolates as candidates for the design of sequential co-culture for production of wine with reduced alcohol content (Mestre et al., 2017). However, many factors are involved in the yeast-yeast interactions and metabolite production of the inoculated yeasts. Optimization of some factors to favor the desired metabolic activity of the selected non-*Saccharomyces* yeasts is very important to obtain good results.

It is well known that different factors can affect the course of the fermentation process, affecting the performance and adaptation of each specific yeast population. Temperature is one of the main variables that directly affects microorganism growth and membrane composition (Charoenchai et al., 1998), and consequently it defines the final wine composition (Torija et al., 2003). A number of researchers have emphasized that low fermentation temperatures (10–15 °C) increase tolerance of certain non-*Saccharomyces* yeasts to ethanol and high sugar concentrations, and therefore they can remain longer in the process (Gao and Fleet, 1988; Tofalo et al., 2012; Zott et al., 2008).

The use of different yeast starter species under co-culture conditions needs to be carefully monitored and analyzed prior to its application in winemaking.

In order to promote the impact of non-*Saccharomyces* populations on the final wine composition, several authors have shown that by increasing the inoculation ratio of the non-*Saccharomyces* (NS) population (like NS:*S. cerevisiae* 10:1, 100:1 or 1000:1) they could outgrow *S. cerevisiae* populations, which would prolong persistence of NS populations (Comitini et al., 2011; Domizio et al., 2011; Pérez-Nevado et al., 2006). Another strategy is the use of sequential inoculation, which delays *S. cerevisiae* starter development. This approach is supposed to mimic spontaneous fermentation, and it would allow more interaction between NS and *S. cerevisiae* yeast populations (Ciani et al., 2016). Therefore, the concentration and viability of non-*Saccharomyces* inocula at the beginning of the alcoholic fermentation as well as the period of time that non-*Saccharomyces* yeasts remain alone (in absence of *S. cerevisiae*) is crucial to their implantation and persistence in the process.

Response Surface Methodology (RSM) is a very useful statistical and mathematical tool to optimize a response (output variable) which is influenced by several independent variables (input variables) (Behera et al., 2018). Several researchers have studied optimization of factors that affect winemaking such as pH, temperature, ammonium and glucose concentrations, inoculum size, and inoculation strategy (Arroyo-López et al., 2009; D'amato et al., 2006; Englezos et al., 2016; Gosh et al., 2012). However, more information is necessary to better understand NS yeasts during winemaking in order to reduce ethanol in wines. In this context, we proposed optimizing three controllable factors that directly affect the performance of non-*Saccharomyces* yeasts during the fermentation using a Box-Behnken experimental design (BB). This method is suitable for exploration of quadratic response surfaces and it generates a second degree polynomial model, which is used to optimize a process using a small number of experimental runs (Amenaghawon et al., 2013). In order to obtain wines with reduced ethanol without loss of quality, the aims of our study were: i) to determine the optimum conditions of the time of permanence of non-*Saccharomyces*, fermentation temperature and the non-*Saccharomyces* inoculum size at the beginning of the process prior to *S. cerevisiae* inoculation; ii) to validate the optimized factors; and iii) to assess sensory quality of the wines obtained after validation.

2. Materials and methods

2.1. Microorganisms

Hanseniaspora uvarum BHu9, *Candida membranaefaciens* BCm71 and *Saccharomyces cerevisiae* BSc114 were used in this study. These yeasts have been previously isolated from oenological environments and molecularly identified by our research group (Maturano et al., 2015). Selection of the yeasts was based on their fermentative performance and respiratory characteristics to be employed in sequential inoculation to obtain wines with reduced ethanol content (Mestre et al., 2017). Strains were stored and cryogenically preserved at −80 °C in the Culture Collection of Autochthonous Microorganisms (Institute of Biotechnology, School of Engineering-UNSJ, San Juan, Argentina).

2.2. Yeast inoculum preparation

Each strain was grown on YEPD-agar (g/L): Yeast extract 10, peptone 20, glucose 20, agar-agar 20, and incubated at 25 °C. After 48 h, entire plates were collected and inoculated in YEPD-broth at 25 °C for 4 h under aerobic conditions (130 rpm). After that, the pre-inoculum was transferred to the grape must (13 °Brix, pH 3.8, supplemented with 0.1% yeast extract and 0.4% peptone) and kept at 25 °C during 12 h under aerobic conditions (130 rpm). Cell counts were carried out with an improved Neubauer counting chamber. The pre-adaptation in YEPD broth, was carried out in order to reduce the lag-stage in the grape must, which would mean that strains started growing exponentially in the grape juice.

2.3. Experimental design

A Box–Behnken (BB) experimental design was employed to assess the effects of three independent variables at three different levels for ethanol reduction (Table 1). The selection of the variables and their levels was established into a range of real conditions employed in winemaking. BB designs were independently assayed for the following yeast combinations: BHu9/BSc114 and BCm71/BSc114. Time of permanence of non-*Saccharomyces* (NS) prior to *S. cerevisiae* BSc114 inoculation (X_1), temperature of must prior to *S. cerevisiae* BSc114 inoculation (X_2) and initial inoculum size of NS yeasts (X_3) were determined. Minimum, medium and maximum time of permanence, temperature and inoculation size assayed were: 24, 48 and 72 h; 15, 20 and 25 °C; and 1, 3 and 5 × 10^6 cells/mL, respectively. Then, each experiment was inoculated with 2 × 10^6 cells/mL of *S. cerevisiae* BSc114 in order to complete alcoholic fermentation under static conditions at 25 °C ± 2.

Ethanol production was selected as response variable, while glycerol and acetic acid were used as quality parameters. A total of 15 experiments, including three replicates of the center point, were carried out in 75 mL of sterile grape must at 21 °Brix (pH was adjusted to 3.8 with tartaric acid, total acidity 5.5 g/L). All fermentations were conducted under static conditions and monitored through the release of CO_2 by measuring daily weight loss. Gentle manual agitation of the Erlenmeyer flask was performed every 24 h for 5 s to assure oxygen availability in the fermentation medium.

Table 1
Summary of the three parameters evaluated according to the Box-Behnken design for two yeast combinations: BHu9/BSc114 and BCm71/BSc114.

Levels	X_1 - time of permanence of NS yeasts (h)	X_2 - temperature (°C)	X_3 – inoculum size of NS yeasts (×10^6 cells/mL)
Minimum	24	15	1
Medium	48	20	3
Maximum	72	25	5

Yeast population dynamics were analyzed throughout the fermentation process. Must and wine samples were taken at different stages: 1, 2, 3, 4, 5, 6, 7, 10, 14 and 22 days. Decimal dilutions in sterile physiologic solution (0.85% NaCl) were performed, spread onto Wallerstein Laboratory Nutrient (WLN) Agar medium (Oxoid, Hampshire, UK) and incubated during 4 days at 28 °C. Total yeast colonies were counted and examined daily until the *Saccharomyces* and non-*Saccharomyces* yeasts could be differentiated morphologically according to Pallmann et al. (2001): *H. uvarum* BHu9 (green colonies), *C. membranaefaciens* BCm71 (light blue colonies) and *S. cerevisiae* BSc114 (white colonies) (Fig. S1 a, b, c and d, Supplementary data).

2.4. Validation model

Lab-scale validation was conducted using the optimized factors previously determined by RSM analysis. *Vitis vinifera* L. cv. Malbec grapes were harvested from a vineyard located in Cañada Honda (San Juan, Argentina) during the 2017 season. After crushing, 3 L of juice was distributed in 5 L vessels and sterilized during 15 min at 70 °C. The must juice had the following composition: 238.2 g/L of sugar (glucose and fructose), pH 3.8, titratable acidity 5.3 g/L and 175 mg/L yeast assimilable nitrogen (YAN). Musts were supplemented with nitrogen by adding 20 mg/L of diammonium phosphate after 48 h and in the middle of the fermentation (when 5% weight loss was verified). These supplementations were selected based on nitrogen uptake previously assayed on selected yeasts (data not shown).

H. uvarum BHu9/*S. cerevisiae* BSc114 (T1) and *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 (T2) were evaluated in sequential treatments with optimized factors according to RSM. Monoculture fermentation with *S. cerevisiae* BSc114 was used as control (T3). All treatments were performed under static conditions and monitored through the release of CO_2, by measuring daily weight loss until constant weight. Moreover, punching down was carried each 24 h in order to kept the dissolved oxygen levels throughout the process. In order to know the *Saccharomyces* and non-*Saccharomyces* populations, samples were collected periodically. Dilutions of 10^{-3} to 10^{-5} were spread onto WLN agar medium and incubated during 7 days at 28 °C. Three types of colonies could be morphologically differentiated according to Pallmann et al. (2001).

After the sugar was completely consumed, fermentations were stopped by adding 50 mg/L of SO_2. The wines were chemically stabilized, filtered, bottled and conserved at 16 °C until chemical and sensorial analyses.

2.5. Chemical analyses

Ethanol concentration (% v/v), pH, residual sugars, glycerol, titratable acidity and acetic acid (g/L) were determined using an ALPHA FT-IR Wine Analyzer (Bruker Optics, Ettlingen, Germany).

2.6. Sensorial analysis

Wines were evaluated after 4 months of bottle aging. They were analyzed by descriptive analysis following previously published guidelines (Lawless and Heymann, 2010). A trained panel of 7 individuals (5 males and 2 females between 35 and 50 years old) from the Wine Sensorial Analysis Department at Instituto Nacional de Vitivinicultura (Mendoza, Argentina) tasted the wines. All treatments were tasted blindly and in duplicate. A constant volume of 30 mL of each wine at room temperature was evaluated and cold water was used for palate cleansing. A total of 19 sensorial attributes were evaluated: three color/appearance descriptors (color intensity, red and brown color), eight aroma descriptors (mineral note, plum, cherry, blackberry, strawberry, violets, chili pepper, vanilla) and eight taste parameters (acidity, sweetness, astringency, hotness, bitterness, structure, aromatic persistence and varietal typicity). The intensity of each attribute was assessed using a non-structured scale from 0 to 5, where 0 indicates that the descriptor was not perceived and values between 1 and 5 indicate that the intensity of the descriptors was very low to very high.

2.7. Data analysis

Each assay was performed independently in triplicate and the results are represented as the average of three determinations with the corresponding standard deviation (± SD). Differences between measurements were determined using one-way analysis of variance (ANOVA). In order to simplify the interpretation of the sensorial analysis, principal components analysis (PCA) was applied using the Infostat statistical package (version 2012, Cordoba, Argentina).

The design and statistical analysis of RSM was generated with Stat-Ease software (Design-Expert 7.0.0, Minneapolis, USA). Analysis of Variance (ANOVA) was carried out to estimate statistically significant factors and the quality of the model equation was expressed as (R^2) using ethanol values.

3. Results

3.1. Effects of fermentation - relevant factors

In order to reduce the ethanol concentration in wine, optimization of three fermentation factors (time of permanence prior to addition of *S. cerevisiae* BSc144 (X_1), temperature (X_2) and inoculation size at the beginning of the process (X_3)) were evaluated in two non-*Saccharomyces*/*Saccharomyces* yeast combinations (*H. uvarum* BHu9/*S. cerevisiae* BSc114 and *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114) using a Box-Behnken experimental design. Table 2 shows the final values of ethanol, glycerol and acetic acid of the co-cultures NS/*Saccharomyces* and their respective controls.

Tables S1 and S2 of the Supplementary data show the coefficients estimated for both co-cultures and their significances. Two second-order polynomial equations were obtained for ethanol production. Eq1 and Eq2 (expressed in actual values) represent *H. uvarum* BHu9/*S. cerevisiae* BSc114 and *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 yeast combination models, respectively. In order to reduce the complexity of the equations only significant coefficients were used. Both models had a non-significant lack of fit ($p > 0.05$). The variability in response, measured through the R^2 coefficient, was 89% for *H. uvarum* BHu9/*S. cerevisiae* BSc114 and 98% for *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114, indicating that both models are acceptable predictors (Tables S1 and S2, Supplementary data).

According to our results, in both cases ethanol production was significantly influenced by the time of permanence of non-*Saccharomyces* prior to addition of *S. cerevisiae* to the fermentation. Addition of *S. cerevisiae* BSc114 48 h after inoculation of *H. uvarum* BHu9 showed lowest ethanol levels (Fig. 1a). When *C. membranaefaciens* BCm71 remained 24 h as a monoculture, lowest ethanol values were registered (Fig. 1d). The temperature also significantly affected ethanol production in fermentations with *C. membranaefaciens* BCm71. In this case, higher temperatures favored the reduction of ethanol in wines (Fig. 1e). In the fermentations with *H. uvarum* BHu9, the temperature did not significantly affect ethanol production (Fig. 1b). However, it can be observed that the lowest ethanol yields were registered when *H. uvarum* was grown at 25 °C. The inoculum size of non-*Saccharomyces* had no effect on the ethanol levels for either yeast combination (Fig. 1c and f). However, the time of permanence of non-*Saccharomyces* prior to addition of *S. cerevisiae* showed significant interaction with the inoculum size of non-*Saccharomyces* yeasts in both co-cultures ($p < 0.05$) (Tables S1 and S2, Supplementary data).

The resulting response surfaces show the effect of time of permanence of non-*Saccharomyces*, fermentation temperature and non-*Saccharomyces* inoculum size on ethanol levels of the two yeast co-cultures (Fig. 2; left and right panel). The response surface models

Table 2
Experimental runs included in the Box-Behnken design and permanence of NS yeasts, ethanol, glycerol and acetic acid values registered for *Candida membranaefaciens* BCm71, *Hanseniaspora uvarum* BHu9 and *Saccharomyces cerevisiae* BSc114under pure and co-culture conditions.

Run	Factor			BCm71/BSc114				BHu9/BSc114			
	X_1	X_2	X_3	Ethanol (% v/v)	Glycerol (g/L)	Acetic acid (g/L)	P NS (days)	Ethanol (% v/v)	Glycerol (g/L)	Acetic acid (g/L)	P NS (days)
1	48	20	3	11.83 ± 1.07	7.7 ± 0,87	0.53 ± 0.12	4	10.03 ± 0.4	9.35 ± 1.06	0.63 ± 0.08	6
2	72	25	3	10.83 ± 1.53	8.86 ± 1,83	0.61 ± 0.12	5	10.02 ± 0.32	11 ± 0	0.79 ± 0	5
3	24	15	3	10.6 ± 1.45	9.7 ± 0,66	0.64 ± 0.06	2	9.97 ± 0.23	9.95 ± 1.06	0.61 ± 0.01	4
4	48	25	1	10.13 ± 1.53	9.7 ± 0,17	0.6 ± 0.08	5	9.81 ± 0.35	8.6 ± 1.41	0.545 ± 0.01	5
5	72	15	3	11.3 ± 0.95	8.63 ± 1,59	0.64 ± 0.13	5	10.47 ± 0.20	9.35 ± 0.92	0.635 ± 0.04	6
6	48	15	1	10.53 ± 1.19	8.13 ± 0,60	0.65 ± 0.11	3	9.75 ± 0.58	9.2 ± 1.13	0.565 ± 0.06	5
7	48	20	3	9.53 ± 2.01	8.26 ± 0,65	0.69 ± 0.13	4	10.88 ± 0.10	9.5 ± 0.57	0.625 ± 0.04	6
8	24	25	3	9.4 ± 1.47	8.63 ± 2,12	0.67 ± 0.07	3	9.22 ± 0.46	9.25 ± 0.92	0.63 ± 0.11	4
9	24	20	1	11.03 ± 0.42	8.76 ± 1,26	0.67 ± 0.22	2	10.08 ± 0.04	9.85 ± 1.63	0.6 ± 0.10	6
10	24	20	5	11.83 ± 0.81	9.13 ± 1,66	0.77 ± 0.23	2	10.74 ± 0.25	10.25 ± 0.07	0.7 ± 0.04	5
11	72	20	5	10.37 ± 1.79	8.3 ± 0,53	0.66 ± 029	5	10.84 ± 0.48	9.6 ± 1.41	0.685 ± 0.05	6
12	48	15	5	10 ± 0.36	7.76 ± 1,33	0.65 ± 0.27	3	10.29 ± 0.04	9.3 ± 0.28	0.66 ± 0.03	5
13	48	25	5	10.33 ± 0.70	8.26 ± 0,55	0.69 ± 0.26	4	9.27 ± 0.69	8 ± 0.57	0.63 ± 0.03	5
14	72	20	1	11.27 ± 0.32	9.13 ± 0,25	0.72 ± 0.36	5	12.56 ± 0.23	8.4 ± 1.70	0.58 ± 0.13	5
15	48	20	3	10.77 ± 0.93	8.9 ± 0,17	0.57 ± 0.33	4	9.87 ± 0.01	9.8 ± 0.14	0.66 ± 0.03	5

Control cultures pure	Ethanol (% v/v)	Glycerol (g/L)	Acetic acid (g/L)	P NS (days)
H. uvarum BHu9	5.73 ± 0.03	5 ± 0.35	0.55 ± 0.04	22
C. membranaefaciens BCm71	1.6 ± 0.02	1.1 ± 0.05	0.2 ± 0.014	22
S. cerevisiae BSc114	12.51 ± 0.56	8.4 ± 0.70	0.52 ± 0.03	

X_1: time of permanence of non-*Saccharomyces*; X_2: temperature; X_3: inoculum size of non-*Saccharomyces*; P: permanence time of non-*Saccharomyces*.
Values are means ± standard deviation of three independent experiments.

indicate how a variation of factors affects ethanol levels. According to Fig. 2, the minimum production of ethanol by *H. uvarum* BHu9/*S. cerevisiae* BSc114 was reached under the following conditions: permanence of *H. uvarum* BHu9 prior to *S. cerevisiae* inoculation of 48 h and 37 min, a fermentation temperature of 25 °C and an initial inoculum size of 5×10^6 cells/mL. Optimum fermentation conditions for

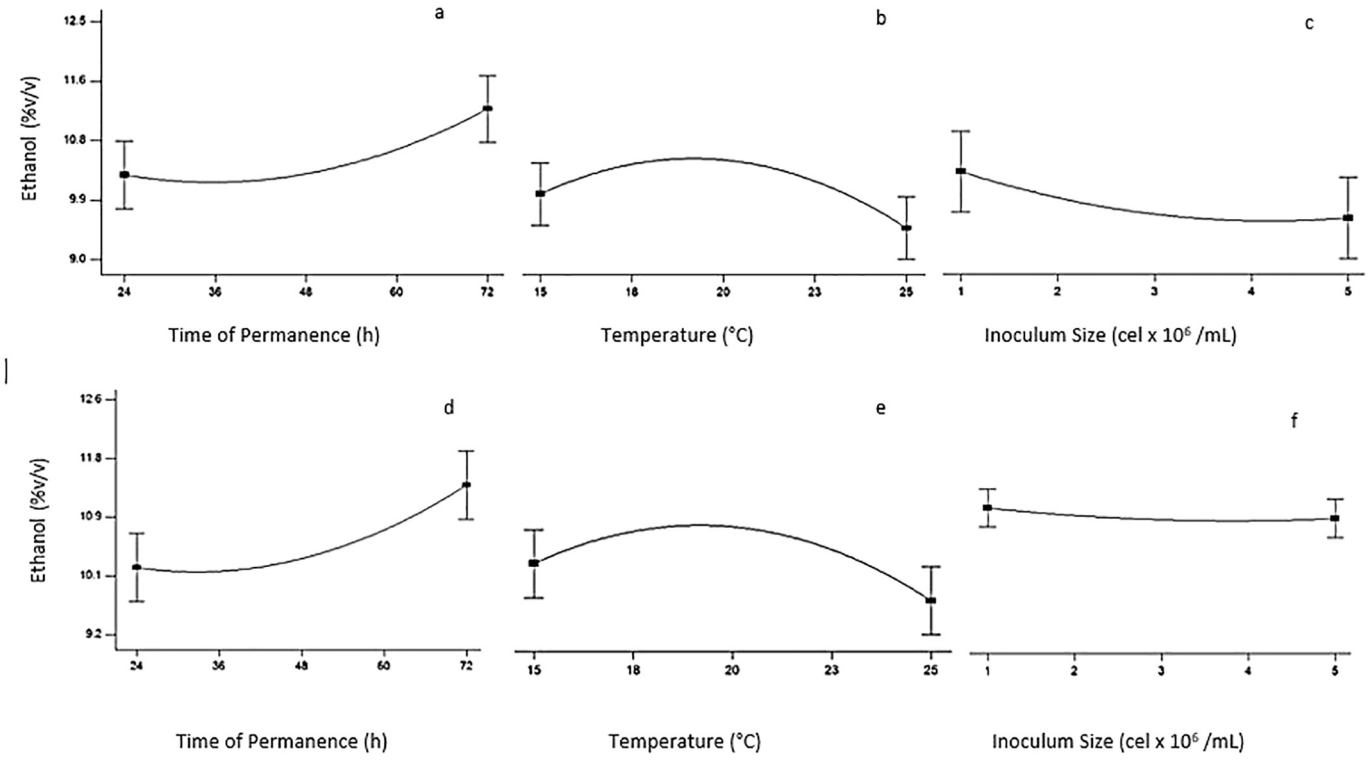

Fig. 1. Ethanol production obtained with a polynomial equation of *Hanseniaspora uvarum* BHu9/*Saccharomyces cerevisiae* BSC114 and *Candida membranaefaciens* Cm71/*Saccharomyces cerevisiae* BSC114 co-cultures: a) time of permanence of *Hanseniaspora uvarum* BHu9, b) temperature of the fermentation during *Hanseniaspora uvarum* BHu9 as a pure culture, c) inoculum size of *Hanseniaspora uvarum* BHu9 at the beginning of the fermentation process, d) time of permanence of *Candida membranaefaciens* BCm71, e) temperature of the fermentation during *Candida membranaefaciens* BCm71 as a pure culture and f) inoculation size of *Candida membranaefaciens* BCm71at the beginning of the fermentation. In each case, the other two factors remained constant at the central point (3×10^6 cells/mL for inoculation size, 48 h for time of persistence and 20 °C for temperature).

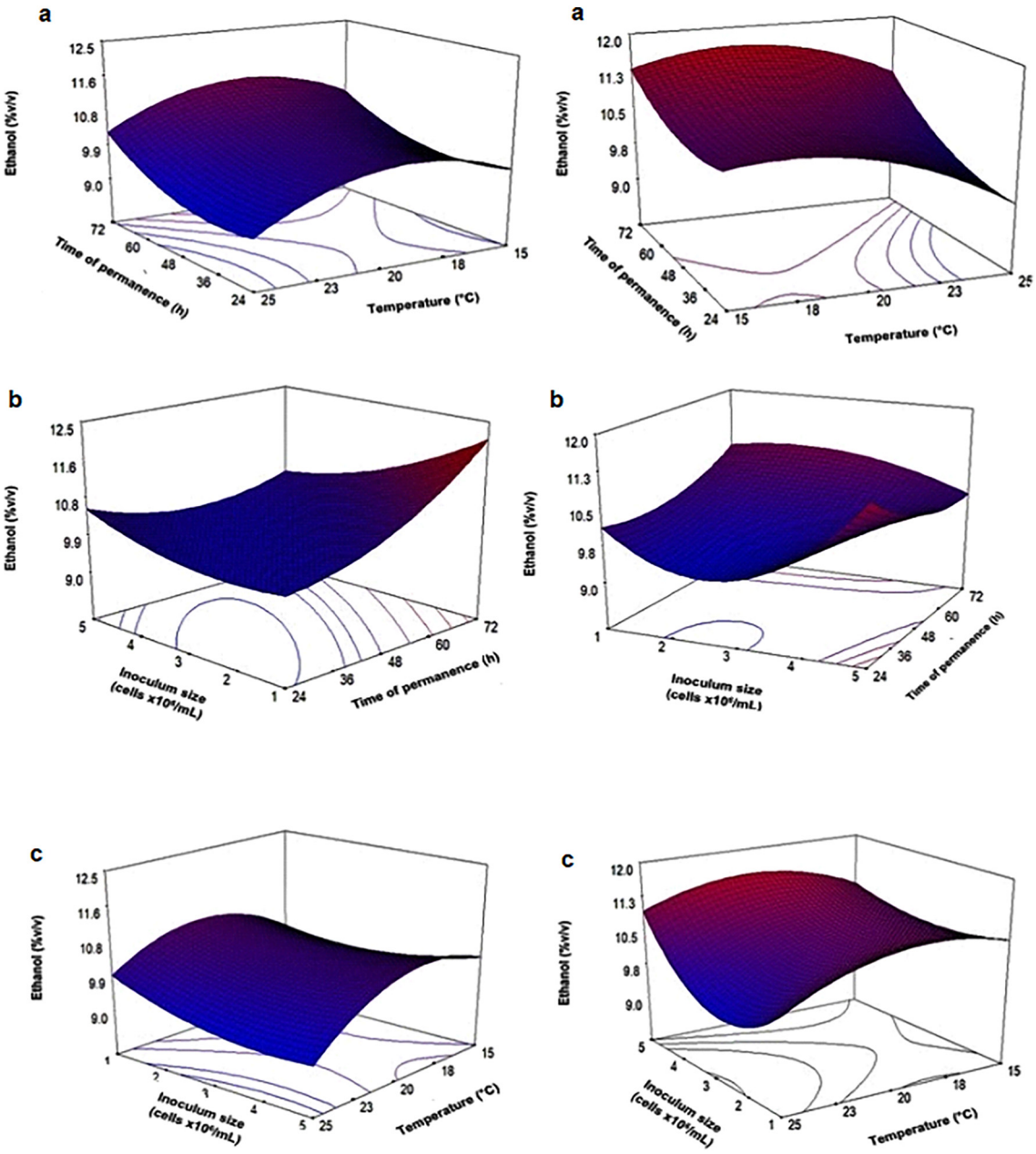

Fig. 2. Response surface of resulting ethanol levels after fermentation of two co-cultures. The left panel corresponds to parameters assayed for BHu9 and the right panel to BCm71 parameters. The "Y" axis registers the final ethanol yield. Ethanol levels are represented as a function of a) temperature and inoculum size interactions b) time of permanence of BHu9 or BCm71 and initial BHu9 or BCm71 inoculum size and c) time of permanence of BHu9 or BCm71 and fermentation temperature.

wine with reduced ethanol level with *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 were: permanence of *C. membranaefaciens* BCm71 prior to *S. cerevisiae* inoculation of 24 h 15 min, a fermentation temperature of 24.94 °C and an initial inoculum size of 2.72×10^6 cells/mL.

Growth of the different yeast populations was monitored periodically. In the case of *H. uvarum/S. cerevisiae*, the two yeasts coexisted for 3 days in 66.6% of the experimental runs. In the case of BCm71/BSc114, *C. membranaefaciens* coexisted for 2 days with *S. cerevisiae* in 60% of the experimental runs (Table 2). Coexistence was independent of the three factors assayed (X_1, X_2 and X_3) (Figs. S2 and S3, Supplementary data). In Fig. S4 are shown population dynamics of *H. uvarum* BHu9, *C. membranaefaciens* BCm71 and *S. cerevisiae* BSc114 under pure conditions. It is important highlight that both non-*Saccharomyces* population remained throughout fermentative process (22 days). Furthermore, all yeasts assayed reached higher populations than the co-inocula conditions (Figs. S2, S3 and S4, Supplementary data).

Final glycerol and acetic acid production by *H. uvarum/S. cerevisiae* ranged between 8.0 ± 0.57 and 11.0 ± 0.07 g/L and 0.56 ± 0.06 and 0.79 ± 0.0, respectively. In the case of *C. membranaefaciens/S. cerevisiae*, glycerol levels ranged from 7.7 ± 0.87 to 9.7 ± 0.66 g/L and acetic acid values ranged from 0.53 ± 0.12 to 0.77 ± 0.23 (Table 2). Both compounds are considered quality parameters and presented acceptable values for wine.

3.2. Validation analysis

Two treatments and one control were carried out to confirm optimized conditions by RSM analysis (Table 3).

Ethanol production and sugar consumption in T1, after that 48 h

Table 3
Model validations with optimal parameters for fermentation of sterile grape must of *Vitis vinifera* L. cv Malbec.

Treatment	Strains inoculated at *t*0	Time of persistence of NS yeast (h:min)	Temperature (°C)	Inoculation level ($\times 10^6$ cells/mL)
T1	*H. uvarum* BHu9	48:37	25	5
T2	*C. membranaefaciens* BCm71	24:15	24.94	2.72
T3 (control)	*S. cerevisiae* BSc114	–	25	2

*t*0: yeast inoculation in grape must at time zero.

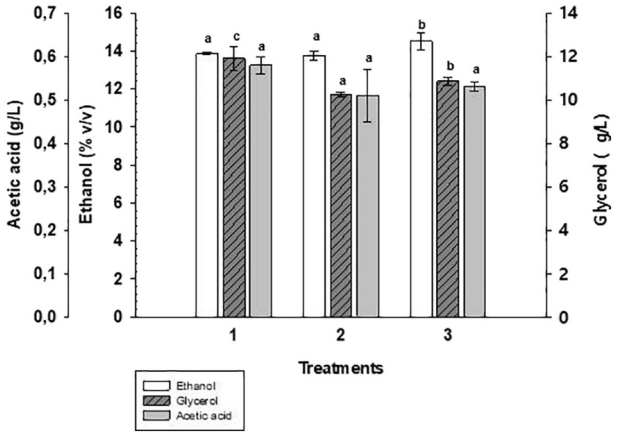

Fig. 3. Ethanol (% v/v). Glycerol (g/L) and acetic acid (g/L) productions in T1. T2 and T3. Different letters indicate significant differences.
T1: *Hanseniaspora uvarum* BHu9/*Saccharomyces cerevisiae* BSc114 co-culture.
T2: *Candida membranaefaciens* BCm71/*Saccharomyces cerevisiae* BSc114 co-culture.
T3: *Saccharomyces cerevisiae* BSc114 pure culture (control).

37 min (prior to BSc114 inoculation), *Hanseniaspora uvarum* BHu9 consumed 72.53 g/L of sugars (30.8%) and produced 1.89% (v/v) of ethanol. With respect to T2, after 24 h 5 min (prior to BSc114 inoculation), *Candida membranaefaciens* BCm69 consumed 37.08 g/L of sugars (15.57%) and produced 0.5% (v/v) of ethanol.

Fig. 3 shows the final chemical parameters of the wines obtained. As could be expected, ethanol levels in wines fermented with *H. uvarum* BHu9/*S. cerevisiae* BSc114 (T1) and *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 (T2) co-cultures were significantly lower than with a pure *S. cerevisiae* BSc114 (T3) culture ($p < 0.05$). The ethanol values attained for *H. uvarum* BHu9/*S. cerevisiae* BSc114 and *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 were 13.86% (v/v) ± 0.06 and 13.76% (v/v) ± 0.25, respectively, while the control treatment reached values of 14.5% (v/v) ± 0.46. The rate of sugars converted into ethanol (sugar consumed (g/L)/ethanol produced (% v/v)) for T1, T2 and T3 was 17.18, 17.31 and 16.30 g/L, respectively.

Glycerol production was significantly higher for *H. uvarum* BHu9/*S. cerevisiae* BSc114 (T1) than for *S. cerevisiae* BSc114 (control): 11.9 ± 0.57 g/L and 11 ± 0.2 g/L, respectively. In contrast, *S. cerevisiae* BSc114 (control) registered a higher glycerol concentration than *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 (T2) co-culture (10.25 ± 0.1 g/L). With regard to acetic acid concentrations, there was no significant difference between treatments (Fig. 3). The final pH values reached with T1, T2 and T3 were 3.49 ± 0.08, 3.71 ± 0.01 and 3.63 ± 0.06, respectively.

Regarding fermentation kinetics, *H. uvarum* BHu9/*S. cerevisiae* BSc114 (T1) and *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 (T2) both finished alcoholic fermentation after 9 days, while *S. cerevisiae* BSc114 (control) finished fermentation after 8 days (Fig. 4a). *H. uvarum* BHu9/*S. cerevisiae* BSc114 showed the lowest fermentation kinetics, while *S. cerevisiae* BSc114 registered highest values of CO_2 production. As can be observed in Fig. 4a, CO_2 release in T2 (BCm71/BSc114) exhibited an intermediate behavior between T1 and T3.

The population dynamics of each yeast species inoculated in the

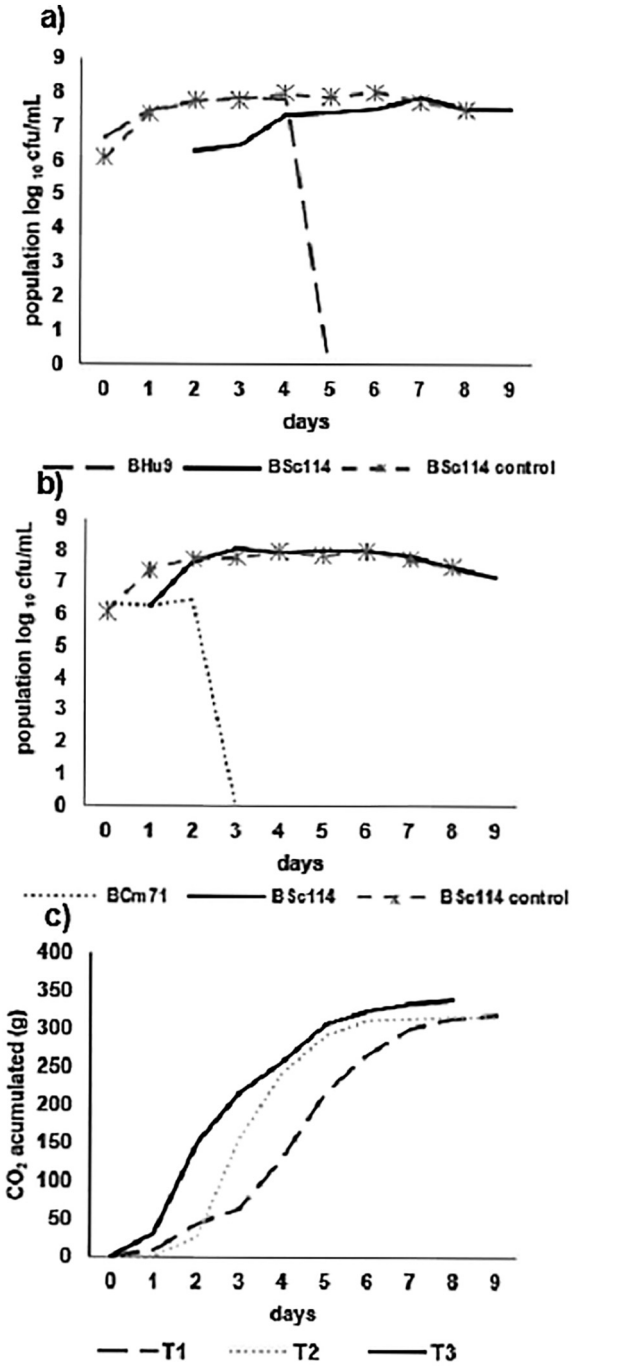

Fig. 4. Development of validation fermentations: a) Fermentation kinetics (measured as CO_2 release) of T1, T2 and T3. Arrows indicate inoculation of *Saccharomyces cerevisiae* BSc114 b) population dynamics of *Hanseniaspora uvarum* BHu9/*Saccharomyces cerevisiae* BSc114 co-culture (T1) compared with BSc114 control (T3) and c) population dynamics of *Candida membranaefaciens* BCm71/*Saccharomyces cerevisiae* BSc114 co-culture (T2) compared with *Saccharomyces cerevisiae* BSc114 control (T3).

different treatments are displayed in Fig. 4b and c. In T1, the *H. uvarum* BHu9 population increased from its initial concentration of 5×10^6 cells/mL to a concentration of about 7×10^7 cells/mL on Day 4 (Fig. 4b). On the contrary, *C. membranaefaciens* BCm71 did not show any increase and from the second day, BCm71 became undetectable (Fig. 4c). In T1, *H. uvarum* BHu9 maintained high populations up to day 4, after which concentrations were below the detection limit of the technique applied. Meanwhile, in the same treatment the *S. cerevisiae* BSc114 population increased from their initial concentration of 2×10^6 cells/mL to 7.8×10^7 cells/mL, reaching its maximum population on day 7 (Fig. 4b). In the *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 (T2) co-culture, *C. membranaefaciens* BCm71 kept its population until day 2 and it became undetectable from day 3 onward. *S. cerevisiae* BSc114 reached populations of about 10^8 cells/mL, similar to the control treatment (Fig. 4c).

It is important to highlight that the interaction period of *Saccharomyces* and non-*Saccharomyces* yeasts in experimental runs of the BB design and the validations test was different. In the latter case, the yeasts of both co-cultures assayed, T1 and T2, coexisted for 2 days and 1 day, respectively.

3.3. Sensorial analysis

Principal component analysis (PCA) was performed in order to characterize the sensorial attributes of the wines. The wine tasters analyzed 19 sensorial attributes (Fig. 5). PC1 explains 66.1% of the total variability, while PC2 explains 33.9%. The attributes that best described the first component (with their respective eigenvector values indicated in parenthesis) were red (−0.46) and brown color (0.57). These attributes allowed a clear separation of T3 and T1 from T2.

PC2 was descripted by attributes such as vanilla (0.48), bitterness (0.49) and blackberry (−0.29). This component shows a separation between T1 and T3. Wines fermented with *H. uvarum* BHu9/*S. cerevisiae* BSc114 (T1) appeared to be defined by varietal typicity, plum, strawberry, red color and acidity. Wines produced with *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 co-culture (T2) were related to attributes like brown color, blackberry, cherry and mouthfeel. Vanilla, astringency, chili peppers, hotness, bitterness and sweetness were related to wine fermented by *S. cerevisiae* BSc114 (control) (Fig. 5).

4. Discussion

It is commonly known that non-*Saccharomyces* yeasts can strongly affect the chemical composition and flavor of wine (Rossouw and Bauer, 2016). In addition, non-*Saccharomyces* yeasts are generally recognized as non-ethanologenic and/or present a low efficiency of sugar/ethanol conversion compared to *S. cerevisiae* (Contreras et al., 2014). However, the influence of these yeasts on the final wine composition could be affected by fermentation factors such as temperature, pH, sugar and nitrogen availability, culture type (single, mixed or sequential), and starter inoculum size (Ribéreau-Gayon, 2000; Torija et al., 2003).

In the present work, time of permanence of NS yeasts significantly affected ethanol production by both co-cultures assayed, even though each yeast strain behaved differently. Several research groups have studied the effect of different co-inoculation periods of yeasts in wines for diverse purposes, but in general, no correlations were found between co-inoculation times and ethanol values (Ciani et al., 2006; Clemente-Jimenez et al., 2005; Gobbi et al., 2013). In the present study, permanence of *H. uvarum* BHu9 of 48 h prior to addition of *S. cerevisiae* BSc114 registered the lowest ethanol level in the wines assayed. This can be related to high sugar consumption through oxidative metabolism by *H. uvarum* BHu9 at the beginning of the winemaking, which would have diminished the sugars in the grape juice available for fermentation by *S. cerevisiae*. However, when permanence of *H. uvarum* BHu9 was extended to 72 h results were not as expected, because wines showed higher ethanol levels. A possible explanation is that after depletion of oxygen in the grape juice after 48 h, *H. uvarum* population, fully adapted to grape must fermentation, started to ferment sugars into ethanol.

An unexpected result was registered for *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114, because the co-culture produced less ethanol when both strains remained together for a longer period of time (when *S. cerevisiae* was inoculated to 24 h). Recent studies have elucidated that certain *S. cerevisiae* strains produce antimicrobial molecules like small peptides or amino acids, short and medium chain fatty acids and *Killer*-like toxins (Branco et al., 2015; Ciani et al., 2016; Wang et al., 2016). Some of these molecules, e.g. antimicrobial peptides, are derived from glycolytic enzyme, and they are probably linked to sugar metabolism of *S. cerevisiae* as has been reported by Wang et al. (2016). More knowledge is required to find out whether NS strains produce

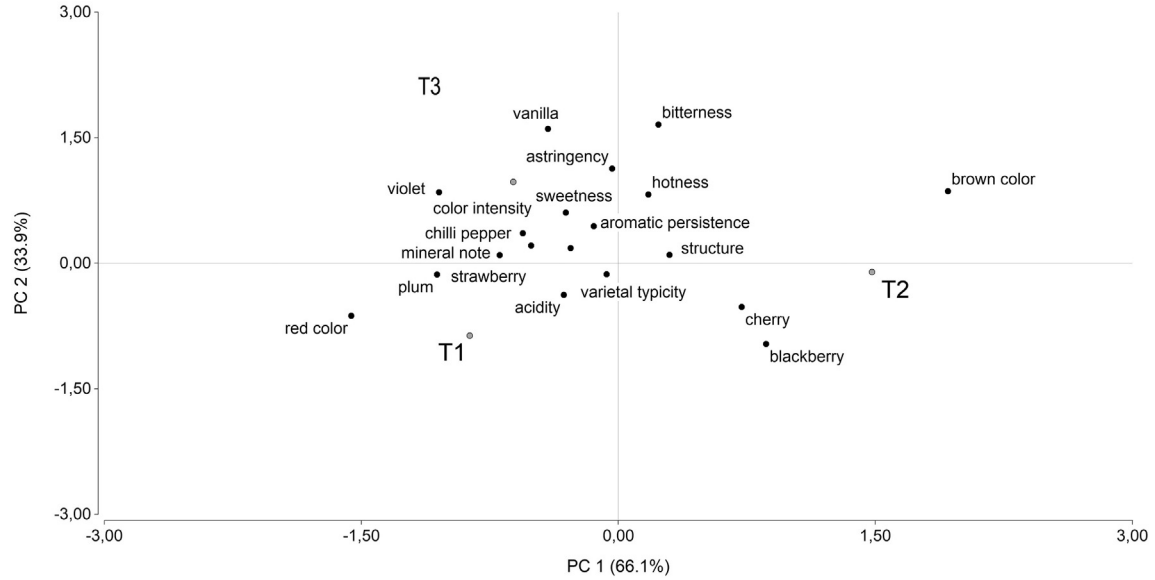

Fig. 5. Principal component analysis (PCA) of sensorial attributes evaluated by wines tasters in T1: *Hanseniaspora uvarum* BHu9/*Saccharomyces cerevisiae* BSc114 co-culture, T2: *Candida membranaefaciens* BCm71/*Saccharomyces cerevisiae* BSc114 co-culture and T3: *Saccharomyces cerevisiae* BSc114 pure culture (control).

antimicrobial compounds that affect the sugars metabolism of *S. cerevisiae* strains.

In this context, it is important to emphasize the role of environmental conditions in fermentations when analyzing the interaction between different yeasts (Wang et al., 2016). High sugar concentrations, low pH values, availability of nitrogen and other essential compounds, decreasing oxygen and increasing ethanol levels are some of the main factors that could affect the strain or specific interactions between *Saccharomyces* and non-*Saccharomyces* yeasts such as synergism, mutualism, competition or amensalism during the fermentation process.

According to the results of the population dynamics of the present study, we can infer that t exist strong interaction between non-*Saccharomyces* and *S. cerevisiae* yeasts assayed because of non-*Saccharomyces* yeasts were detected only the first stage. Another possible explanation is that certain conditions (such as: nitrogen limitation, low oxygen availability, increasing ethanol, SO_2 presence and low temperature) induce a viable but non-culturable (VBNC) cellular state of the NS yeasts (Maturano et al., 2016; Wang et al., 2016).

It is well known that growth and permanence of yeast populations is strongly influenced by the fermentation temperature (Fleet and Heard, 1993; Torija et al., 2003). Several authors have suggested that certain NS species have a better chance to grow and persist at low temperatures than *Saccharomyces* species (Heard and Fleet, 1988; Sharf and Margalith, 1983). Low temperature can increase tolerance of some yeast species to ethanol and high sugar concentrations of the initial grape must (Gao and Fleet, 1988; Torija et al., 2003). Accordingly, *H. uvarum* BHu9 and *C. membranaefaciens* BCm71 were selected for their ability to grow at low temperature (15 °C) (Mestre et al., 2017). However, in the present study the two NS strains showed lowest fermentation activity (based on fermentation kinetics) at the lowest temperature assayed (15 °C), which means that sugar consumption was very little (data not shown). Consequently, high sugar concentrations remained available, which were subsequently fermented by *S. cerevisiae* BSc114. Under the present experimental conditions, ethanol concentration decreased with a higher initial temperature of the process. An explanation would be that at low temperature the rigidity of the lipid bilayer membrane increases (or the fluidity decreases). This can affect activity of transmembrane proteins, like those involved in nutrient uptake (García-Ríos et al., 2016; Redón et al., 2011). Hence, activity of membrane-associated enzymes is reduced, greatly decreasing membrane transport (Aguilera et al., 2007; Murata et al., 2006; Vigh et al., 1998). Although sugar transport is mediated by facilitated diffusion, reduced enzymatic activity in the membrane would hamper sugar transport to the inside of the cell and therefore its uptake (Ribéreau-Gayon et al., 2006). Under the present assay conditions, the experimental runs at high initial temperature (25 °C) registered lowest ethanol production as has been mentioned above. This could partially be explained by the fact that NS yeasts kept their membrane in optimal conditions under the high assay temperature, which facilitated diffusion of hexoses into the cytoplasm and subsequently their rapid metabolism. Based on the results of a previous study (Mestre et al., 2017), *H. uvarum* BHu9 and *C. membranaefaciens* BCm71 require high amounts of sugar to produce ethanol. This enological trait of NS yeasts enhances a reduction in available sugar which is subsequently fermented by *S. cerevisiae*. In this context, our results coincide with those reported by Torija et al. (2003), who observed that the ethanol yield of *S. cerevisiae* was lower at higher fermentation temperature. Similarly, Ribéreau-Gayon et al. (2006) reported that elevated temperature generally yielded a lower alcohol concentration.

The inoculum size is another key parameter in microbial fermentation (Medina et al., 1997; Papagianni and Moo-Young, 2002; Rao et al., 2004). We have not found information about the inoculum size of NS yeasts and its effect on the ethanol concentration in wines. According to our results, different inoculum sizes of the NS yeasts assayed did not significantly affect the final ethanol production ($p > 0.05$).

With regard to sensory analysis, wines fermented by non-*Saccharomyces*/*Saccharomyces* co-cultures under optimized conditions were associated with higher aromatic complexity characterized by the presence of red fruit aromas intimately linked to the presence of esters in wines. According to reported results by Erten and Campbell (2001), the ability of non-*Saccharomyces* yeasts to utilize sugars through oxidative metabolism could favor the production of desirable esters and other wine flavors.

High ethanol concentrations can negatively affect the sensory attributes of wine (Guth and Sies, 2002). For example, high alcohol content masks certain flavor-related volatile compounds and it also increases the perception of bitterness, hotness and sweetness (Williams and Rosser, 1981; Tilloy et al., 2014). Moreover, elevated ethanol levels decreases perception of fruit aromas in wines (Escudero et al., 2007). Our results have demonstrated that wines fermented with *S. cerevisiae* BSc114 (T3) were associated with the parameters previously mentioned.

With respect to the color parameters evaluated, a brown color was strongly related to fermentation with *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114. This color is a negative parameter according to wine consumers. *Candida* spp. has been reported as ascorbic acid producer (Abbas, 2006), and this compound it combines with SO_2. As consequence, a depletion of SO_2 in the medium occurs. Hence the antioxidant activity decrease and production of yellow pigments (browning) increase (Li et al., 2008). The authors also reported that ascorbic acid in the presence of O_2 (due to periodical remounting) can be oxidized to dehydroascorbic acid and H_2O_2. This latter reagent is a highly oxidative compound of anthocyanin. On the other hand, *C. membranaefaciens* BCm71 presents β-glucosidase activity (Mestre et al., 2017). These enzymes can act on monoglucoside-anthocyanins and, consequently release the anthocyanidin, which spontaneously converts into brown or colorless compounds (Manzanares et al., 2000).

It has been reported that *H. uvarum* is able to produce and release higher amounts of organic acids than other NS and *S. cerevisiae* yeasts (Hong and Park, 2013). Under the current assay conditions, this treatment registered the lowest pH values. It is well known that phenolic compounds (especially anthocyanins) are more stable in acid medium presenting a red tonality (Ribéreau-Gayon et al., 2006). In contrast, low pH values promote a higher percentage of free SO_2 in the medium, being more effective as antioxidant. This fact could be the reason why T1 is strongly related to the red tonality.

In conclusion, under the current assay conditions both co-inoculations produced the desired response regarding our main goal to produce wines with reduced ethanol. However, the quality of the wines differed.

Further research should be carried out to assess both strategies under different conditions. Vinifications should be performed with fresh grape must without sterilization and the fermentation scale should be increased. It would also be interesting to study the volatile compositions of the wines using GC–MS in order to acquire more comprehensive and detailed knowledge. This is necessary to transfer a fully reliable strategy to the industry.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.08.016.

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