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The effects of co-culturing non-*Saccharomyces* yeasts with *S. cerevisiae* on the sugar cane spirit (cachaça) fermentation process

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Abstract Twenty non-*Saccharomyces* strains were previously tested in pure culture for their ability to grow in 12 % ethanol, their β-glucosidase activity, flocculation, glycerol, ethanol and acetic acid production, fermentation kinetics and their production of volatile compounds. Of these 20 strains, three strains, namely, *Pichia anomala* UFLA CAF70, *P. anomala* UFLA CAF119 and *Pichia caribbica* UFLA CAF733, were evaluated in co-culture with *Saccharomyces cerevisiae* UFLA CA11. Of the mixed inocula, the mixture of *P. caribbica* UFLA CAF733 and *S. cerevisiae* UFLA CA11 gave the highest ethanol concentration (75.37 g/L), the lowest levels of residual glucose (1.14 g/L) and fructose (19.92 g/L), and the highest volumetric productivity (Q_p) of ethanol. Twenty-three minor volatile compounds were identified in the fermented sugar cane juice. The mixed culture of *P. caribbica* UFLA CAF733 and *S. cerevisiae* UFLA CA11 gave the highest concentration of volatile compounds with good sensory descriptors; these compounds included ethyl esters (290.13 μg/L), acetates (715.21 μg/L) and monoterpenic alcohols (195.56 μg/L). This mixed culture also gave the lowest concentration of volatile acids (1774.46 μg/L) and aldehydes (121.10 μg/L). In principal component analysis, the mixed inoculum of UFLA CAF733 and UFLA CA11 was positively characterized by ethyl hexanoate, 2-phenylethanol, linalool, nonanoic acid, ethyl butyrate, phenylethyl acetate, diethylsuccinate, hexanoic acid, and geraniol. In conclusion, we found that clear improvements could be achieved in the fermentation process with mixed, rather than pure, *S. cerevisiae* culture. The use of the non-*Saccharomyces* strain *P. caribbica* UFLA CAF733 in co-culture with *S. cerevisiae* UFLA CA11 may therefore be an interesting means by which to improve the quality of cachaça.

Keywords Cachaça · Fermentation · Non-*Saccharomyces* · Mixed inoculum

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Introduction

Cachaça is a unique sugar cane spirit produced exclusively in Brazil with an alcohol content of between 38 and 48 % v/v at 20 °C. This spirit is obtained by the distillation of fermented sugar cane juice (Brazil 2005) and is the third most-consumed distilled beverage worldwide and the second most-consumed beverage in Brazil. The annual cachaça production is approximately 1.3 billion liters, with an average of 11 L being consumed per individual per year (Campos et al. 2010).

The microbiota of traditional fermentation processes is complex and consists of both yeasts, such as *Kluyveromyces marxianus*, *Pichia heimii*, *Hanseniaspora*

uvarum, *Pichia subpelliculosa*, *Debaryomyces hansenii*, *Pichia methanolica*, *Saccharomyces cerevisiae*, and bacteria, such as certain lactic and acetic acid bacteria and several bacteria belonging to enterobacteriaceae family (Schwan et al. 2001). Artisanal cachaça spirits normally have unique smell and taste due to the metabolites and volatile substances that are produced during the fermentation process of this complex mixture of yeast and bacteria species.

The use of selected strains of *S. cerevisiae* in cachaça production allows for a faster process start-up, a lower risk of contamination from spontaneous fermentation, a more rapid and uniform rate of fermentation, a lower competition for essential nutrients, a higher yield, a lower level of residual sugars and the maintenance of beverage flavor properties (Campos et al. 2010). Many previous studies have investigated the influence of non-*Saccharomyces* yeasts on the final quality of alcoholic beverages, including wine (Viana et al. 2008; Zott et al. 2008) and tequila (Arellano et al. 2008; Arrizon et al. 2006). The effects of *S. cerevisiae* and non-*Saccharomyces* species interactions on beverage quality have also been studied in recent years; for example, Alvarez et al. (2012) compared *K. marxianus* and *S. cerevisiae* in the agave fermentation process of tequila production. These researchers demonstrated that *K. marxianus* increased the concentrations of major and minor volatile compounds associated with the organoleptic quality of tequila, making it a more suitable biocatalyzer for the industrial production of tequila than the commonly used *S. cerevisiae*.

Many precursors of the aromatic components of juices, musts and wines, such as the monoterpenes (e.g., limonene, linalool oxide, linalool, geraniol, nerol, citronellol and a-terpineol) are initially present in a di-glycosidically bound and non-volatile form (Swangkeaw et al. 2011). It is known that non-*Saccharomyces* species can release enzymes (e.g., β-glucosidase) with the capacity to transform inactive compounds into their active aromatic forms, thereby resulting in improvements in the sensory qualities of wines (Maturano et al. 2012). To date, however, aroma release has mainly been enhanced using commercial enzyme preparations of fungal origin, mainly from *Aspergillus* spp. The composition of these preparations varies, but they typically contain a mixture of non-specific glucanases (Villena et al. 2007). Enzymatic hydrolysis with β-glucosidase is an alternative method that may modify the natural aroma distribution pattern, depending on the substrate specificity of the β-glucosidase activity (Swangkeaw et al. 2011). Although there is information about the influence of non-*Saccharomyces* yeasts on the quality of fermented beverages, there are no reports in the literature specifically addressing the influence of non-*Saccharomyces* strains grown in mixed culture on cachaça quality. The aim of this study was therefore to select non-*Saccharomyces* yeast strains for use in co-culture with *S. cerevisiae* and to evaluate the use of mixed *S. cerevisiae* and non-*Saccharomyces* cultures in the fermentation of sugar cane juice.

Materials and methods

Yeast strains

Twenty non-*Saccharomyces* strains isolated from fruit wine fermentation, coffee fermentation and sugar cane silage were evaluated (Table 1). A *S. cerevisiae* strain (UFLA CA11) that was commercialized in Brazil as starter culture for cachaça production was used in the mixed inocula. All strains used in the present study were from the microbial collection at the Microbial Physiology Laboratory/Department of Biology from the Federal University of Lavras (UFLA) in Brazil.

Inocula preparation

Yeast strains maintained in 20 % glycerol at −80 °C were re-activated and multiplied using YPD (1 % yeast extract, 2 % peptone and 2 % glucose) as described below. Using a platinum loop, yeast strains were inoculated into tubes containing 1 mL YPD and were incubated at 28 °C. After 24 h, the contents of these tubes were transferred to new tubes containing 9 mL YPD and incubated for 24 h at 28 °C. The resulting yeast cultures (10 mL) were transferred to Erlenmeyer flasks containing 90 mL YPD, which were subsequently incubated for 24 h at 28 °C and 200 rpm. Yeast cells were later separated from the medium by centrifugation ($RCF = 4053$, 5 min, 25 °C) and washed twice with sterile distilled water (Duarte et al. 2010). The non-*Saccharomyces* strains and *S. cerevisiae* strain were used in all steps of this work in populations of 10^7 and 10^8 cells/mL, respectively.

Table 1 Non-*Saccharomyces* yeasts, their sources and biotechnological parameters evaluated for the selection of non-*Saccharomyces* strains

Yeasts			Parameters		
Code	Species	Source	Growth in ethanol 12 %	% Floc (10 min)	% Floc (30 min)
UFLA CE1	*Torulaspora delbrueckii*	Sugar cane silage fermentation	+	100.00	94.56
UFLA CAF58	*Torulaspora delbrueckii*	Coffee fermentation	+	96.98	90.94
UFLA CAF725	*Pichia guilliermondii*	Coffee fermentation	+	97.21	80.49
UFLA CA32	*Torulaspora delbrueckii*	Coffee fermentation	+	97.25	92.78
UFLA CE10	*Candida glabrata*	Sugar cane silage fermentation	+	95.61	93.24
UFLA CAF16	*Torulaspora delbrueckii*	Coffee fermentation	+	96.50	91.25
UFLA FT1	*Pichia ofuraensis*	Tropical fruit fermentation	+	96.81	81.67
UFLA FT32	*Arxula adeninivorans* (Syn. *Blastobotrys adeninivorans*)	Tropical fruit fermentation	+	98.15	85.93
UFLA CAF712	*Pichia gulliermondii*	Coffee fermentation	+	96.68	80.73
UFLA CAF719	*Pichia caribbica*	Coffee fermentation	+	97.51	90.65
UFLA CAF76	*Hanseniaspora uvarum*	Coffee fermentation	+	95.88	76.29
UFLA CE19	*Candida glabrata*	Sugar cane silage fermentation	+	94.02	92.69
UFLA CAF70	*Pichia anomala*	Coffee fermentation	+	97.51	90.65
UFLA CAF119	*Pichia anomala*	Coffee fermentation	+	69.81	64.53
UFLA CAF61	*Rhodotorula mucilaginosa*	Coffee fermentation	+	96.98	92.75
UFLA CE20	*Candida glabrata*	Sugar cane silage fermentation	+	95.61	77.03
UFLA CE5	*Torulaspora delbrueckii*	Sugar cane silage fermentation	+	95.33	92.21
UFLA CE6	*Candida glabrata*	Sugar cane silage fermentation	+	96.98	90.94
UFLA CAF733	*Pichia caribbica*	Coffee fermentation	+	94.26	74.66
UFLA CAF731	*Pichia guilliermondii*	Coffee fermentation	−	np	np

Floc flocculation, *np* not performed

Screening of non-*Saccharomyces* yeast strains for co-culture with *S. cerevisiae*

Twenty non-*Saccharomyces* yeasts strains were screened based on (1) their capacity to grow in 12 % ethanol and (2) their flocculation capacity. After these preliminary tests, a number of strains were evaluated (in pure culture) for their fermentation performance (sugar consumption, ethanol production, glycerol and acetic acid production and fermentation kinetics). The fermentation experiments were performed in flasks containing 180 mL of sterile sugar cane juice at 16 °Brix (adjusted with distilled water) maintained at 28 °C without agitation.

After the evaluation of fermentation performance, strains with lower acetic acid production, higher ethanol yields and lower residual sugar levels, were evaluated for their quantitative β-glucosidase activity. The strains with the highest β-glucosidase activity were used in a new fermentation assay assessing their production of desirable volatile compounds (e.g., ethyl esters, acetates and monoterpenic alcohols). All experiments were carried out in triplicate.

Growth in 12 % ethanol

The capacity to grow in 12 % ethanol was assessed by plating on "ESA" medium (0.5 % yeast extract, 0.5 % peptone, 2 % glucose, 12 % (v/v) ethanol, 0.015 % sodium metabisulphite, 1.5 % agar). Yeast growth was observed after 48 h of incubation at 30 °C (Valles et al. 2008).

Flocculation

The yeast strains were inoculated in 10 mL of sterile YPD medium and incubated at 28 °C for 24 h. After the incubation, the tubes were stirred (10 s), and the

flocculation capacity was observed visually. The strains were classified as flocculent yeasts (FY) when they formed cellular aggregations in static culture and formed clumps again after being dispersed by shaking. Alternatively, the strains were classified as non-flocculent yeasts (NF) when the cells did not form clumps after dispersal by shaking (Valles et al. 2008). For the spectrophotometric determination, the obtained cell suspensions were centrifuged, and the cells were resuspended in 5 mL of Helm's buffer (3 mM calcium chloride, 50 mM acetate-acetic buffer, pH = 4.5). The degree of flocculation of the different strains was recorded as the ratio between the optical density measured at 600 nm of culture suspension and the optical density measured after 30 min at rest ($OD_{30} \times 100/OD_0$). The following flocculation scale was established: a ratio >90 %, 0 (no flocculation), a ratio between 70 and 90 %, 1 (low flocculation), a ratio between 30 and 70 %, 2 (medium flocculation), a ratio <30 %, 3 (high flocculation) (Valles et al. 2008).

β-Glucosidase activity assay

The quantitative assay for β-glucosidase activity was performed by measuring the amount of *p*-nitrophenol (*p*NP) (Sigma) released from an artificial substrate, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) (Sigma) as described previously (Swangkeaw et al. 2011). The cell-free culture medium corresponding to the enzyme solution (0.1 mL) was mixed with 0.2 mL of a 0.002 M solution of *p*NPG in 0.1 M citrate phosphate buffer at pH 5.0. The reaction mixture was subsequently incubated at 30 °C for 30 min. The enzymatic reaction was stopped by adding 2.0 mL of 0.25 M Na_2CO_3 (Merck). The released *p*NP was assessed spectrophotometrically at 405 nm (Swangkeaw et al. 2011), and the measured enzymatic activity was expressed as nanomoles of *p*NP per milliliter per hour.

HPLC analysis

Ethanol, glycerol, acetic acid, and carbohydrates (glucose, sucrose and fructose) were identified and quantified using high-performance liquid chromatography (HPLC). All analyses were performed using a Shimadzu chromatograph (LC-10A*i*, Shimadzu Corp., Japan) that was equipped with a dual detection system consisting of a UV detector (SPD-10A*i*) and a refractive index detector (RID-10A). A Shimadzu ion exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm) was operated at a temperature of 30 °C using 100 mM perchloric acid as the eluent at a flow rate of 0.6 mL/min. Acids were detected using UV absorbance (210 nm), while the sugars, glycerol and ethanol were detected using RID. The compounds were identified by comparing their retention times to the retention times of certified known standards. The quantification was performed using an external calibration methodology. All samples were examined in duplicate (Duarte et al. 2011).

GC-FID analysis

Major volatile compounds were analyzed directly after the filtration of samples (0.22-μm pores) without any other prior treatments. The minor volatile compounds were determined after extraction with dichloromethane as previously described in Duarte et al. (2010). This analysis was performed using a gas chromatography (GC) Shimadzu model 17A, equipped with an flame ionization detector (FID) and using a capillary column of silica DB Wax (30 m × 0.25 mm i.d. × 0.25 μm) (J&W Scientific) operated under conditions described by (Duarte et al. 2011). The volatile compounds were analyzed by the injection of 1 μL of each sample in the split mode (1:10), and the subsequent compound identification was accomplished by comparing the retention times of the sample peaks with those of known standards that were injected in the same conditions. The resulting measurements were expressed in 4-nonanol (internal standard) equivalents. For the major volatile compounds, 4-nonanol was used at a concentration of 123.76 mg/L; for the minor volatile compounds, 4-nonanol was used at a concentration of 312 μg/L (Duarte et al. 2010, 2011).

Evaluation of fermentation performance

To evaluate fermentation performance, the conversion factors of the substrates (sucrose, glucose and fructose) into ethanol ($Y_{p/s}$), glycerol ($Y_{g/s}$) and acetic acid ($Y_{ac/s}$) was calculated, along with the volumetric productivity of ethanol (Q_p) and its conversion efficiency (E_f) (Duarte et al. 2011; Oliveira et al. 2004). The equations used in this work are presented below.

$$\left[Y_{p/s} = \left(P_f - P_i\right)/\left(S_i - S_f\right)\right];$$
$$\left[Y_{g/s} = \left(g_f - g_i\right)/\left(S_i - S_f\right)\right];$$
$$; \left[Y_{ac/s} = \left(Ac_f - Ac_i\right)/\left(S_i - S_f\right)\right];$$
$$\left[Q_p = \left(P_f - P_i\right)/t_f\right]$$

In this equation, P_i is the initial concentration of ethanol, P_f is the ethanol concentration at the end of fermentation, S_i is initial substrate concentration, S_f is substrate concentration at the end of fermentation, g_i is initial glycerol concentration, g_f is glycerol concentration at the end of fermentation, Ac_i is the initial acetic acid concentration, Ac_f is the concentration of acetic acid at the end of fermentation and t_f is the total fermentation time. To calculate the substrate concentration, the content of sucrose was mathematically converted into the corresponding amounts of fructose and glucose.

Co-fermentation with *S. cerevisiae* UFLA CA11

The three pre-selected non-*Saccharomyces* strains were used in co-culture with *S. cerevisiae* UFLA CA11. The fermentation experiments were carried out in flasks containing 250 mL of sugar cane juice 16 °Brix at 28 °C without agitation. In addition to its use in the three mixed inocula, *S. cerevisiae* UFLA CA11 was also used in pure culture as a fermentation control. During fermentation, samples were collected to evaluate fermentation performance and to determine the yeast population profile as described below. The fermented sugar cane juice was submitted for GC-FID analysis and HPLC analysis.

Analysis of growth and survival

Yeast growth was determined by plate counting. Samples were collected throughout the fermentation period and diluted appropriately using sterile 0.1 % peptone water. In the mixed cultures, the counting of the pre-selected non-*Saccharomyces* cells were performed using medium lysine agar (LA) (containing 66 g/L lysine medium (Himedia), 10 mL/L 50 % potassium lactate (Himedia), pH 4.8); the number of *S. cerevisiae* cells was given as the difference between the total plate count using YPD and the plate count using LA (Nissen et al. 2003). YPD and LA plates were incubated at 28 °C and counted after 48 h of incubation.

Statistical analysis

Principal Component Analyses were performed using XLstat 7.5.2 software (Addinsoft's, New York, NY, USA). The software SISVAR 5.1 (Lavras, MG, Brazil) was used for the Scott-Knott test.

Results and discussion

Screening of non-*Saccharomyces* strains

Preliminary tests

The strain *P. guilliermondii* UFLA CAF731 was unable to grow in the 12 % ethanol. The ability to grow in 12 % ethanol is a very important selection factor for any strains to be used in cachaça production because ethanol concentrations reach values of approximately 8.8 % during the fermentation process (Campos et al. 2010).

The sedimentation capacity was checked visually and all nineteen yeasts were able to sediment such that this parameter could not be used to exclude any strains (data not shown). In the spectrophotometric determination after 10 min, only the *Pichia anomala* UFLA CAF119 strain had flocculation, at a level of 69.81 % (Table 1), which was categorized as a medium flocculation level (2). After 30 min, *P. anomala* UFLA CAF119, *P. caribbica* UFLA CAF733, *H. uvarum* UFLA CAF76 and *Candida glabrata* UFLA CE 20 had flocculation values of 64.53 % (2—medium flocculation), 74.66 % (1—low flocculation), 76.29 % (1—low flocculation) and 77.03 % (1—low flocculation), respectively (Table 1). Flocculation analysis represents an easy and low-cost method for the separation of cells from fermented sugar cane during cachaça production (Soares 2010). Although strains UFLA CAF119, UFLA CAF733, UFLA CAF76 and UFLA CE20 had the highest flocculation values, all nineteen strains were used in subsequent steps of sugar cane juice fermentation.

Fermentation kinetics and the alcohol, sugar and acetic acid profiles

The strains *P. ofuraensis* UFLA FT1, *Arxula adeninivorans* (syn. *Blastobotrys adeninivorans*) UFLA FT32, *C. glabrata* UFLA CE10, *C. glabrata* UFLA

CE19, *C. glabrata* UFLA CE20 (75 % of the *C. glabrata* tested strains) and *H. uvarum* UFLA CAF76, were not able to ferment sugar cane juice and therefore left high levels of residual sucrose in the culture medium (Table 2). Consequently, these yeasts produced notably low levels of ethanol, ranging from 1.64 g/L (UFLA FT1) to 13.3 g/L (UFLA CAF76). Two others strains, UFLA CAF725 and UFLA CE5, had high levels of residual fructose and glucose and also produced low levels of ethanol (Table 2). In addition to its low concentration of produced ethanol, strain UFLA CAF725 also produced only 0.39 g/L of glycerol (Table 2).

The strains *Torulaspora delbrueckii* UFLA CE1, *T. delbrueckii* UFLA CAF58, *T. delbrueckii* UFLA CAF16, *P. guilliermondii* UFLA CAF712 and *C. glabrata* UFLA CE6 had the highest concentrations of both glycerol (ranging from 5.96 g/L to 6.61 g/L) and ethanol (ranging from 48.67 to 59.58 g/L). The highest glycerol value was measured with UFLA CE1 fermentation. A high level of glycerol production from sugar cane juice fermentation by non-*Saccharomyces* strains was reported by Nova et al. (2009); these data combined with the volatile compound data led the authors to conclude that yeast dynamics during the fermentation process can influence the final quality of the resulting beverage. The strains with the highest concentration of glycerol and ethanol also had high values for conversion factors $Y_{g/s}$ and $Y_{p/s}$ (Table 2). As proposed by Oliveira et al. (2004), strains with values of $Y_{p/s}$ between 0.42 and 0.45 g/g are grouped in the medium level, while strains with $Y_{p/s}$ values ranging from 0.451 to 0.49 g/g are grouped in the high level. The latter is the case for strain *T. delbrueckii* UFLA CAF58, which has a $Y_{p/s}$ value of 0.49 g/g (Table 2). Based on the theoretical maximum value of $Y_{p/s}$ (0.51 g/g), the strain UFLA CAF58 was the yeast that converted sugar to ethanol with the highest efficiency. Indeed, it had an E_f value of 95.47 %, placing it in the highly efficient category (Duarte et al. 2010; Oliveira et al. 2004). Although UFLA CAF58 also had a high E_f value, the percentage of sugars consumed (*Conv*) was only 64.97 %, which is an intermediate value for the 19 non-*Saccharomyces* strains evaluated in this work. Besides the aforementioned strains (UFLA CE1, UFLA CAF58, UFLA CAF16, UFLA CAF712 and UFLA CE6), the strains UFLA CAF733, UFLA CAF119, UFLA CAF70, UFLA CAF719, UFLA CAF32 and UFLA CAF61, showed high values of E_f, ranging from 72 to 93.07 % (listed in decreasing order, Table 2).

The strain UFLA CE6 was the most efficient producer of ethanol per unit time with a Q_p of 1.24 g/L h, indicating that this strain produced ethanol more rapidly than the other yeast strains under study. The strains UFLA CAF58, UFLA CAF16 and UFLA CAF712 showed the second largest Q_p value (1.10 g/L h). In addition to their high Q_p values, strains UFLA CE6 and UFLA CAF16 were also the most efficient in terms of glucose, fructose and sucrose conversion with values of 76.35 and 72.74 %, respectively, for the parameter *Conv*. The combination of high values for $Y_{p/s}$, E_f, Q_p and *Conv* indicates that such yeasts are able to grow in sugar cane juice and efficiently convert sugars into ethanol (Duarte et al. 2010), which is a major compound in cachaça (Silva et al. 2009). Indeed, the two main alcohols in the cachaça beverage are glycerol and ethanol. For this reason, strains with high values of substrate conversion into ethanol ($Y_{p/s}$) and glycerol ($Y_{g/s}$), high volumetric productivity of ethanol (Q_p) and high conversion efficiency (E_f) were selected for subsequent steps of this work.

The sugar cane juice fermented by *P. anomala* UFLA CAF119 and *P. caribbica* UFLA CAF733 contained 1.18 and 1.12 g/L of acetic acid and had $Y_{ac/s}$ values of 0.0141 and 0.0109 g/g, respectively (Table 2). Given that Brazilian law (Brazil 2005) allows acetic acid in cachaça production (the volatile acidity of acetic acid is 150 mg/100 mL of anhydrous alcohol), the strains UFLA CAF119 and UFLA CAF733 were also selected for use in further screening steps.

Quantitative test of β-glucosidase activity

The eleven strains selected based on their fermentative performance and profile of sugars (glucose, fructose and sucrose), ethanol, glycerol and acetic acid were next evaluated for level of β-glucosidase activity. The results in Table 3 show that higher β-glucosidase activity levels were found in *P. anomala* UFLA CAF119, *P. caribbica* UFLA CAF733 and *P. anomala* UFLA CAF70 (letters *a*, *b* and *c* in the Scott-Knott test). The other yeasts studied had lower values of β-glucosidase activity (letter *d* in the Scott-Knott test). The highest value of β-glucosidase activity, 0.495 nmol *p*NP/mL h, was found for the strain UFLA CAF119 followed by values of 0.312 and

Table 2 Concentrations (g/L) of sugars, alcohols and acetic acid by HPLC in fermented sugar cane juice and kinetics parameters for non-*Saccharomyces* strains

Yeast	Compounds (g/L)						Parameters					
	Sucrose	Glucose	Fructose	Glycerol	Ethanol	Acetic acid	$Y_{p/s}$ (g/g)	E_f (%)	*Conv.* (%)	Q_p (g/L h)	$Y_{g/s}$ (g/g)	$Y_{ac/s}$ (g/g)
UFLA CE1	0.70 ± 0.25	8.64 ± 0.42	47.60 ± 4.49	6.61 ± 0.09	48.67 ± 1.18	0.04 ± 0.02	0.43	84.20	66.55	1.01	0.0583	0.0003
UFLA CAF58	0.32 ± 0.04	13.71 ± 1.57	44.51 ± 3.13	6.41 ± 0.16	52.90 ± 0.21	0.08 ± 0.04	0.49	95.47	64.97	1.10	0.0590	0.0007
UFLA CAF725	58.20 ± 1.30	39.23 ± 5.61	48.48 ± 5.07	0.39 ± 0.04	5.06 ± 0.15	0.32 ± 0.00	0.19	37.83	14.98	0.11	0.0149	0.0122
UFLA CAF32	0.33 ± 0.07	20.14 ± 2.23	51.28 ± 4.12	5.85 ± 0.14	42.95 ± 0.48	0.00 ± 0.00	0.46	89.35	56.77	0.89	0.0620	0.0000
UFLA CE10	148.34 ± 2.48	6.67 ± 0.42	2.67 ± 0.31	4.12 ± 0.50	3.02 ± 0.25	0.70 ± 0.04	0.25	49.90	6.70	0.06	0.3470	0.0591
UFLA CAF16	0.48 ± 0.08	12.20 ± 0.75	32.85 ± 0.34	6.35 ± 0.59	52.62 ± 2.44	0.03 ± 0.01	0.43	84.89	72.74	1.10	0.0523	0.0002
UFLA FT1	104.86 ± 45.53	4.35 ± 0.45	2.57 ± 1.29	0.63 ± 0.14	1.64 ± 0.03	0.30 ± 0.13	0.03	5.25	34.31	0.03	0.0102	0.0048
UFLA FT32	120.77 ± 6.33	7.90 ± 0.09	6.94 ± 0.43	4.06 ± 0.29	7.76 ± 0.10	0.58 ± 0.04	0.21	41.88	20.38	0.16	0.1117	0.0160
UFLA CAF712	0.65 ± 0.08	13.73 ± 1.56	44.29 ± 3.30	6.44 ± 0.17	52.86 ± 0.20	0.13 ± 0.01	0.45	88.32	66.66	1.10	0.0549	0.0011
UFLA CAF719	0.59 ± 0.09	22.09 ± 2.03	54.55 ± 2.71	6.73 ± 0.22	42.30 ± 0.53	0.01 ± 0.00	0.45	88.14	54.92	0.88	0.0715	0.0001
UFLA CAF76	129.88 ± 18.56	6.01 ± 1.72	1.98 ± 0.11	6.03 ± 1.15	13.30 ± 0.73	0.55 ± 0.18	0.40	78.14	18.74	0.28	0.1805	0.0163
UFLA CE19	148.24 ± 4.99	12.64 ± 0.16	2.71 ± 1.51	3.77 ± 0.06	2.84 ± 0.31	0.67 ± 0.06	0.18	35.20	8.96	0.06	0.2381	0.0426
UFLA CAF70	4.28 ± 0.09	21.80 ± 2.66	59.18 ± 4.64	2.14 ± 0.09	34.44 ± 0.29	0.31 ± 0.01	0.39	76.03	50.95	0.72	0.0241	0.0035
UFLA CAF119	0.32 ± 0.09	22.26 ± 3.01	59.54 ± 6.37	4.12 ± 0.02	31.95 ± 0.73	1.18 ± 0.05	0.38	74.85	50.47	0.67	0.0492	0.0141
UFLA CAF61	0.29 ± 0.03	15.63 ± 1.42	46.80 ± 2.51	6.03 ± 0.33	49.97 ± 0.94	0.08 ± 0.01	0.47	93.07	62.66	1.04	0.0573	0.0008
UFLA CE20	157.87 ± 10.36	8.66 ± 0.97	4.02 ± 0.46	0.70 ± 0.02	3.24 ± 0.28	0.51 ± 0.02	–	–	–	0.07	–	–
UFLA CE5	14.38 ± 1.09	39.28 ± 4.91	60.97 ± 7.32	11.98 ± 0.06	19.40 ± 0.13	0.55 ± 0.06	0.34	66.80	33.05	0.40	0.2104	0.0096
UFLA CE6	12.23 ± 0.12	6.17 ± 2.37	23.10 ± 3.20	5.96 ± 0.08	59.58 ± 1.24	0.14 ± 0.08	0.44	85.87	76.35	1.24	0.0438	0.0011
UFLA CAF733	0.43 ± 0.03	16.15 ± 0.03	45.86 ± 0.09	3.61 ± 0.10	37.79 ± 0.33	1.12 ± 0.06	0.37	72.00	62.23	0.79	0.0351	0.0109

Table 3 β-Glucosidase activity (nmol *p*NP/mL h) for different non-*Saccharomyces* yeasts strain

Yeast	β-Glucosidase
UFLA CAF119	$0.495^{a} \pm 0.360$
UFLA CAF733	$0.312^{b} \pm 0.0256$
UFLA CAF70	$0.054^{c} \pm 0.0034$
UFLA CAF712	$0.027^{d} \pm 0.0026$
UFLA CAF16	$0.012^{d} \pm 0.0023$
UFLA CE1	$0.012^{d} \pm 0.0076$
UFLA CE6	$0.007^{d} \pm 0.0014$
UFLA CAF61	$0.007^{d} \pm 0.0014$
UFLA CAF719	$0.006^{d} \pm 0.0003$
UFLA CAF58	$0.006^{d} \pm 0.0009$
UFLA CAF32	$0.006^{d} \pm 0.0008$

Values identified by the same letters are not significantly different at the 0.05 level (Scott-Knott test)

0.054 nmol *p*NP/mL h for the strains UFLA CAF733 and UFLA CAF70, respectively (Table 3). These three strains were therefore selected along with UFLA CAF712, UFLA CAF16 and UFLA CE1 for steps of this work. Although the last three strains showed no significant differences ($p < 0.05$) in their β-glucosidase activity, they were selected instead based on their fermentation performance and their alcohol, sugar and acetic acid profiles (Table 2). The current literature (Comitini et al. 2011; Rodrígues et al. 2010; Villena et al. 2007) contains considerable discussion on the β-glucosidase activity of wine yeast. To the best of our knowledge, no published reports have assessed the role of β-glucosidase activity in non-*Saccharomyces* yeast acting in the transformation of aroma precursors from sugar cane juice. Of the strains evaluated in this work, those with highest β-glucosidase activity produced a fermented sugar cane juice with the highest volatile compound levels, as shown below.

Metabolite profile of pre-selected non-*Saccharomyces* strains

Thirty-one volatile compounds (VOCs) were identified in the sugar cane juice fermented by the six strains with the highest β-glucosidase activity (Table 4). Based on their VOC profiles, certain strains with the highest levels of ethyl esters, acetates and monoterpenic alcohols were considered more suitable for sugar cane juice fermentation.

The highest concentrations of higher alcohols were measured in the sugar cane juice fermented by strains UFLA CAF16 (59019.54 μg/L) and UFLA CE1 (57684.65 μg/L) (Table 4). However, these two yeasts also had the lowest concentration of ethyl esters. Additionally, strain UFLA CE1 also had the lowest concentration of acetates (427.95 μg/L) (Table 4). Conversely, the strains UFLA CAF119 and UFLA CAF70 had the highest concentrations of ethyl esters, of 344.16 and 205.55 μg/L, respectively (Table 4). Ethyl esters are important in beverage quality as these compounds lead to "fruity" aromatic descriptors, such as "apple," "papaya" (Meilgaard 1975) and "green apple" (Meilgaard 1975; Siebert et al. 2005).

In addition to having a high concentration of ethyl esters, UFLA CAF119 had the highest concentration of acetates (2957.39 μg/L) followed by UFLA CAF733 (2289.37 μg/L). These two strains also had the highest concentration of monoterpenic alcohols with values of 284.74 μg/L (UFLA CAF119) and 144.48 μg/L (UFLA CAF733). Acetate and the monoterpenic alcohols are also associated with positive aromatic descriptors, such as "sweet" (propyl acetate), "perfumed" (ethyl butyrate), "roses" (phenylethyl acetate) (Meilgaard 1975) and "citrus-like" (linalool) (Czerny et al. 2008).

Interestingly, the three strains with the highest β-glucosidase activity produced a fermented sugar cane juice with high amounts of ethyl esters and monoterpenic alcohols, suggesting that these yeasts were more efficient in fermenting sugar cane juice and producing the VOCs that positively influence the resulting aromatic characteristics of the fermented sugar cane juice. Although the expected increase in the levels of some volatile compounds was not correlated with β-glucosidase activity, this increase could be explained by the general metabolism of the yeast strains (Rodríguez et al. 2010).

Most of the volatile compounds identified in fermented sugar cane juice have been identified in sugar cane spirits (cachaça) by several authors (Campos et al. 2010; Cardeal et al. 2008; de Souza et al. 2009; Duarte et al. 2011; Nonato et al. 2001; Silva et al. 2009), indicating that with the results from fermented sugar cane is possible to infer that the use of different yeast strains can affect the sensory qualities of the final beverage product.

To help with the interpretation of the results from Table 4, principal component analysis (PCA) was

Table 4 Concentrations (μg/L) of volatile compounds in sugar cane juice fermented by different non-*Saccharomyces* yeasts

No.	Compounds	Yeast						Descriptors
		UFLA CAF119	UFLA CAF733	UFLA CAF712	UFLA CE1	UFLA CAF16	UFLA CAF70	
	Higher alcohols (6)							
1	1-Propanol	261.48 ± 28.31	239.10 ± 4.49	114.98 ± 20.04	179.34 ± 38.32	159.36 ± 16.97	228.01 ± 25.50	Alcohol (C)
2	2-Methyl-1-butanol 3-Methyl-1-butanol	12561.97 ± 71.27	12153.16 ± 376.42	14958.26 ± 1092.34	19726.37 ± 123.32	21768.37 ± 1845.23	9956.95 ± 1717.34	Alcohol, banana, medicinal, solvent (C)
3	1-Pentanol	nd	nd	nd	nd	nd	16.38 ± 5.72	Alcohol, banana, sweetish, aromatic (C)
4	1,2-Propanediol	nd	nd	nd	nd	nd	17.82 ± 3.70	–
5	Furfuryl alcohol	nd	nd	nd	11.50 ± 3.39	nd	18.03 ± 7.11	Sugar cane, Woody (C)
6	2-Phenylethanol	11010.66 ± 53.59	10720.19 ± 421.96	33443.59 ± 287.75	37767.44 ± 1025.07	37091.81 ± 2394.62	5867.62 ± 750.34	Roses, sweetish, perfumed (C)
	Total higher alcohols	23834.11	23112.43	48516.83	57684.65	59019.54	16104.81	
	Ethyl esters (3)							
7	Ethyl octanoate	nd	nd	nd	nd	nd	14.82 ± 3.81	Apple, sweetish, fruity (C); sweet (B)
8	Diethylsuccinate	243.96 ± 2.11	144.70 ± 4.27	34.57 ± 0.10	39.10 ± 3.38	27.11 ± 0.32	144.67 ± 9.21	–
9	Diethyl malate	100.20 ± 4.95	34.00 ± 2.00	17.81 ± 1.65	9.17 ± 0.09	0.00	46.06 ± 8.74	–
	Total esters	344.16	178.70	52.38	48.27	27.11	205.55	
	Acetates (6)							
10	Ethyl acetate	nd	nd	2.81 ± 0.60	nd	nd	nd	Solvent, fruity, sweetish (C)
11	Propyl acetate	1180.39 ± 27.47	937.95 ± 15.98	452.45 ± 9.56	331.31 ± 47.77	592.60 ± 25.07	197.76 ± 47.22	

Table 4 continued

No.	Compounds	Yeast						Descriptors
		UFLA CAF119	UFLA CAF733	UFLA CAF712	UFLA CE1	UFLA CAF16	UFLA CAF70	
12	Isobutyl acetate	17.35 ± 0.81	14.89 ± 0.28	16.33 ± 6.18	9.23 ± 0.40	35.52 ± 10.18	23.49 ± 5.17	Banana, sweet, fruity (C)
13	Ethyl butyrate	3.54 ± 0.17	11.34 ± .357	32.50 ± 11.98	12.84 ± 2.29	22.62 ± 2.29	4.10 ± 0.81	Papaya, butter, sweetish, Apple, perfumed (C)
14	Isoamyl acetate	1242.83 ± 23.26	947.01 ± 22.75	5.78 ± 0.37	8.01 ± 0.38	nd	164.65 ± 11.92	Banana, Apple, solvent (C)
15	Phenylethyl acetate	513.28 ± 2.14	378.18 ± 1.80	95.67 ± 8.45	66.56 ± 13.12	80.37 ± 11.84	53.52 ± 3.46	Roses, honey, Apple, sweetish (C); flowery (B)
	Total acetates	2957.39	2289.37	605.54	427.95	731.11	443.52	
	Monoterpenic alcohols (2)							
16	Linalool	182.18 ± 82.67	116.01 ± 11.61	71.53 ± 46.73	125.44 ± 12.43	163.69 ± 45.47	30.52 ± 3.90	Aniseed, terpenoid (C); citrus like, bergamot (A)
17	Guaiacol	102.56 ± 5.06	28.47 ± 2.86	nd	7.46 ± 0.89	nd	nd	–
	Total monoterpenic alcohols	284.74	144.48	71.53	132.90	163.69	30.52	
	Aldehydes (3)							
18	Acetaldehyde	382.87 ± 20.98	330.72 ± 59.34	82.28 ± 13.23	99.49 ± 27.26	148.78 ± 3.09	244.76 ± 15.84	Green leaves, fruity (C)

Table 4 continued

No.	Compounds	Yeast						Descriptors
		UFLA CAF119	UFLA CAF733	UFLA CAF712	UFLA CE1	UFLA CAF16	UFLA CAF70	
19	Octanal	239.02 ± 36.36	282.71 ± 14.86	39.79 ± 10.81	30.27 ± 0.95	78.64 ± 10.64	29.20 ± 5.77	Orange peel, bitter, aldehyde, vinous (C); citrus-like, Green (A)
20	Furfural	nd	nd	nd	12.51 ± 3.97	29.48 ± 5.37	21.82 ± 1.55	Paper, husk (C)
	Total aldehydes	621.89	613.43	122.07	142.27	256.90	295.78	
21	Propionic acid	nd	nd	nd	9.81 ± 6.07	18.74 ± 7.45	nd	Acetic acid, Milk (C); vinegar (B)
22	Isobutyric acid	208.71 ± 4.89	152.01 ± 1.11	265.30 ± 33.97	289.76 ± 7.43	267.30 ± 27.07	124.22 ± 5.53	Sweaty, bitter (C); cheese, rancid (B)
23	Butyric acid	nd	nd	nd	10.40 ± 5.02	nd	25.17 ± 5.68	Buttery, cheesy, sweaty (C)
24	Hexanoic acid	52.20 ± 4.24	11.63 ± 3.60	68.25 ± 8.68	52.27 ± 7.59	91.18 ± 6.08	20.35 ± 1.13	Goaty, fatty acid, vegetable oil, sweaty (C)
25	Heptanoic acid	29.08 ± 8.35	16.68 ± 1.68	nd	7.88 ± 1.50	nd	nd	–
26	2-Ethyl caproic acid	nd	nd	nd	nd	15.17 ± 1,28	nd	–
27	Octanoic acid	121.42 ± 5.99	96.29 ± 14.53	335.95 ± 18.27	188.79 ± 17.74	408.03 ± 30.60	63.72 ± 3.17	Fatty acid, vegetable oil (C); rancid, harsh (B)
28	Nonanoic acid	nd	56.90 ± 4.91	7.73 ± 0.25	nd	nd	nd	–
29	Decanoic acid	744.17 ± 62.96	2673.99 ± 236.99	360.43 ± 83.06	50.51 ± 3.65	472.39 ± 83.39	44.78 ± 8.49	Waxy, tallow, caprylic, rancid, soapy (C); fatty (B)

Table 4 continued

No.	Compounds	Yeast						Descriptors
		UFLA CAF119	UFLA CAF733	UFLA CAF712	UFLA CE1	UFLA CAF16	UFLA CAF70	
30	Benzoic acid	nd	nd	nd	18.98 ± 0.61	32.16 ± 1.02	nd	–
	Total volatile acids	1155.58	4163.08	1037.66	628.40	1304.97	278.24	
	Others (1)							
31	1,1-Dietoxyethane	17.72 ± 3,50	17.98 ± 2.01	11.74 ± 3.22	9.36 ± 2.21	15.09 ± 0.05	13.65 ± 2.16	–

Nd not detected. (A) Czerny et al. (2008); (B) Siebert et al. (2005); (C) Meilgaard (1975)

applied to the VOCs data. The first (PC1) and second (PC2) principal component accounted for 43.46 and 27.74 % of the total variance, respectively (Fig. 1). The PC1 was characterized by high positive values for acetaldehyde, 1-propanol, diethylsuccinate and diethyl malate, while the PC2 was characterized by positive values for butyric acid, 1-pentanol, ethyl octanoate and 1,2-propanediol, and negative values of isoamyl acetate, phenylethyl acetate and propyl acetate (Fig. 1b). The strains UFLA CAF712 and UFLA CAF16 were grouped at the negative part of the PC1 and PC2 (Fig. 1a) and were correlated with ethyl butyrate, 2-phenylethanol, 2-methyl-1-butanol + 3-methyl-1-butanol, octanoic acid, hexanoic acid and isobutyric acid. Alternatively, the strains UFLA CAF119 and UFLA CAF733 had positive values for PC1 and negative values for PC2 and were characterized by diethylsuccinate, acetaldehyde, isoamyl acetate, phenylethyl acetate, octanal and propyl acetate (Fig. 1a). The strain UFLA CAF70 had positive values for PC1 and PC2 and was correlated with butyric acid, 1-pentanol, ethyl octanoate and 1,2-propanediol. Finally, the strain UFLA CE1 had positive values for PC2 and negative values for PC1.

Based on the results of Table 4 and the PCA (Fig. 1a, b), the strains UFLA CAF119, UFLA CAF733 and UFLA CAF70 were determined to be the most efficient in terms of producing VOCs with good descriptors (Table 4) that can positively influence the final beverage quality.

Evaluation of three pre-selected non-*Saccharomyces* strains in co-culture with *S. cerevisiae* UFLA CA11

Growth and survival analysis of yeasts in co-culture

Three non-*Saccharomyces* strains, *P. anomala* UFLA CAF119, *P. caribbica* UFLA CAF733 and *P. anomala* UFLA CAF70, behaved similarly during the fermentation process, with only slight population increases (data not shown). At the end of fermentation, the counts for UFLA CAF119, UFLA CAF733 and UFLA CAF70 cultured in LA were 7.69, 7.83 and 7.74 log CFU/mL, respectively. The small differences between the populations of the three pre-selected strains indicated that all three strains were able to survive in fermenting medium with high levels of ethanol (Table 5), which is an essential characteristic

for a starter culture to be used in cachaça production (Campos et al. 2010).

In co-culture, the population of UFLA CA11 decreased slightly at the end of the fermentation process (data not shown). After 24 h of fermentation, the population of UFLA CA11 was approximately 8.5 log CFU/mL in co-culture, while it was 8.81 log CFU/mL in pure culture. This reduction in the *S. cerevisiae* UFLA CA11 population was not observed in previous research on the co-inoculation of non-*Saccharomyces* and *S. cerevisiae* (Bely et al. 2008; Viana et al. 2009, 2011), indicating that the behavior of non-*Saccharomyces* and *S. cerevisiae* strains are directly influenced by the substrate (e.g., sugar cane juice or grape juice), the inoculum ratios, the temperature, the specific yeast species and the unique strain–strain interactions.

Fermentation kinetics and alcohol, sugar and acetic acid profiles

The residual content of sucrose was similar for the three mixed inocula and for the pure culture of UFLA CA11 (Scott-Knott test, $p < 0.05$) (Table 5). Interestingly, the residual content of glucose and fructose for the mixed inocula followed the same pattern as when the non-*Saccharomyces* strains UFLA CAF733, UFLA CAF70 and UFLA CAF119 were used in pure culture (listed from most to least efficient) (Table 2).

The mixed inoculum of UFLA CAF733 and UFLA CA11 was the most efficient (letter *b* in the Scott-Knott test) in terms of sugar consumption, leaving a residual sugars content of only 1.14 g/L (glucose) and 19.92 g/L (fructose). This result suggests that there is a synergistic interaction between these strains with respect to sugar consumption. The pure culture of UFLA CA11 and mixed inoculum of UFLA CAF119 and UFLA CA11 left a similar (letter *a* in the Scott-Knott test) residual content of glucose and fructose and were only half as efficient as the other two inocula studied inocula. The fact that residual sugars are found in the fermentation of sugar cane juice in cachaça production has been previously reported (Duarte et al. 2011; Nova et al. 2009; Vicente et al. 2006). However, no data have yet addressed the incremental sugar consumption of mixed non-*Saccharomcyes* and *S. cerevisiae* cultures. The lowest residual sugar

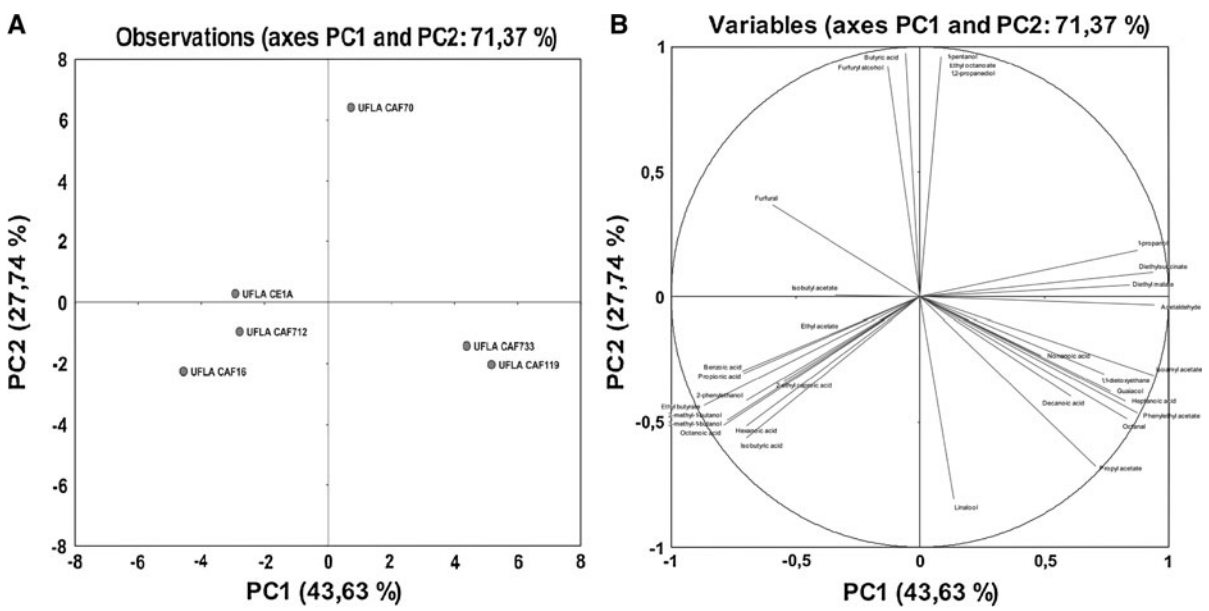

Fig. 1 Principal component analysis of volatile compounds (**b**) in sugar cane juice fermented by different non-*Saccharomyces* strain (**a**)

Table 5 Concentrations (g/L) of sugars, alcohols and acetic acid by HPLC in fermented sugar cane juice and kinetics parameters for different mixed inocula and for *S. cerevisiae* UFLA CA11

Inoculum	Compounds						Kinetics parameters					
	Sucrose	Glucose	Fructose	Glycerol	Ethanol	Acetic acid	$Y_{p/s}$ (g/g)	Efic (%)	Conv. (%)	Q_p (g/L h)	$Y_{g/s}$ (g/g)	$Y_{ac/s}$ (g/g)
UFLA CAF70 + UFLA CA11	$0.57^{a} \pm 0.01$	$1.64^{b} \pm 1.08$	$21.98^{b} \pm 5.00$	$7.63^{a} \pm 0.30$	$74.10^{a} \pm 4.37$	$0.34^{a} \pm 0.01$	$0.48^{a} \pm 0.02$	$94.98^{a} \pm 4.02$	$86.33^{a} \pm 3.94$	$1.95^{a} \pm 0.10$	$0.0499^{b} \pm 0.002$	$0.0022^{a} \pm 0.0$
UFLA CAF733 + UFLA CA11	$1.04^{a} \pm 0.12$	$1.14^{b} \pm 0.03$	$19.92^{b} \pm 0.48$	$7.92^{a} \pm 0.16$	$75.37^{a} \pm 0.80$	$0.16^{b} \pm 0.00$	$0.48^{a} \pm 0.01$	$94.33^{a} \pm 1.17$	$87.61^{a} \pm 0.30$	$1.98^{a} \pm 0.02$	$0.0505^{b} \pm 0.001$	$0.0010^{b} \pm 0.0$
UFLA CAF119 + UFLA CA11	$0.90^{a} \pm 0.12$	$8.16^{a} \pm 0.75$	$41.35^{a} \pm 3.02$	$7.67^{a} \pm 0.45$	$60.48^{b} \pm 1.45$	$0.14^{c} \pm 0.00$	$0.49^{a} \pm 0.03$	$96.02^{a} \pm 4.94$	$70.99^{b} \pm 2.27$	$1.59^{b} \pm 0.02$	$0.0621^{a} \pm 0.006$	$0.0011^{b} \pm 0.0$
UFLA CA11	$0.91^{a} \pm 0.12$	$8.92^{a} \pm 0.02$	$41.94^{a} \pm 0.05$	$7.84^{a} \pm 0.28$	$59.28^{b} \pm 0.14$	$0.04^{d} \pm 0.00$	$0.47^{a} \pm 0.01$	$92.19^{a} \pm 2.20$	$70.87^{b} \pm 0.53$	$1.56^{b} \pm 0.03$	$0.0622^{a} \pm 0.003$	$0.0004^{c} \pm 0.0$

Values identified by the same letters are not significantly different at the 0.05 level (Scott-Knott test)

levels, especially for fructose, can reduce the risk of stuck fermentation; indeed, high fructose to glucose ratios are the main cause of stuck fermentation (Berthels et al. 2004).

As expected given the low residual sugar levels, the ethanol content was higher (letter *a* in the Scott-Knott test) for the mixed inocula of UFLA CAF733 and UFLA CA11 and of UFLA CAF70 and UFLA CA11, with concentrations of 75.37 and 74.10 g/L, respectively (Table 5). Although these two mixed inocula produced the highest ethanol concentrations, no significant differences were found in their $Y_{p/s}$ and E_f parameters. However, for the parameter *Conv*, the UFLA CAF733 and UFLA CA11 and the UFLA CAF70 and UFLA CA11 inocula were the most efficient with values of 87.61 and 86.33 %, respectively (Table 5). Additionally, these mixed inocula showed the highest volumetric productivity values for ethanol (Q_p), of 1.98 and 1.95 g/L h, respectively (Table 5). Not only did these mixed inocula have the lowest concentrations of glucose and fructose, they also had the highest concentrations of ethanol and high values of Q_p, indicating that they could produce a high yield of cachaça, as proposed by Campos et al. (2010) for pure *S. cerevisiae* culture.

The pure culture of *S. cerevisiae* UFLA CA11 produced the lowest acetic acid levels (Table 5) followed by mixed inocula UFLA CAF119 + UFLA CA11 (0.14 g/L), UFLA CAF733 + UFLA CA11 (0.16 g/L) and UFLA CAF70 + UFLA CA11 (0.34 g/L). Comparing the acetic acid concentrations from the pure non-*Saccharomyces* cultures (Table 2) to those of the mixed inocula suggests that interactions with UFLA CA11 diminished the non-*Saccharomyces* acid production. In the other words, the strains that produced the highest acetic acid levels in pure culture also had the lowest acetic acid levels in co-culture with *S. cerevisiae* (Tables 2, 5). The $Y_{ac/s}$ followed the same pattern, with the lowest value of 0.0004 g/g being observed for the pure culture of UFLA CA11 (Table 5). Although high concentrations of acetic acid can negatively influence beverage quality (Duarte et al. 2010; Oliveira et al. 2004), the values found in this work were lower than those resulting in diminished quality and high acidity, as defined by Brazilian law (Brazil 2005).

The glycerol content was similar for all mixed inocula (Table 5) with no statistically significant differences observed between them. However, the

mixed inoculum of UFLA CAF119 and UFLA CA11 had a value of 0.0621 g/g for $Y_{g/s}$, corresponding to the highest $Y_{g/s}$ value (Table 5).

Metabolite profile of volatile compounds in pre-selected non-*Saccharomyces* strains

Table 6 shows the major volatile compounds found in sugar cane juice fermented with three different mixed inocula and with a pure culture of UFLA CA11. All mixed inocula resulted in an increase in the concentration of 2-methyl-1-butanol and 3-methyl-1-butanol, which are the two major higher alcohols of cachaça (Duarte et al. 2011). With the exception of the methanol and furfuryl alcohol levels, the mixed inoculum UFLA CAF733 + UFLA CA11 had the highest levels of the other seven higher alcohols (Table 6). The most abundant alcohol was 3-methyl-1-butanol, which had a concentration of 100.90 mg/L in the juice fermented using the mixed inoculum UFLA CAF733 and UFLA CA11.

Ethyl acetate was the only acetate identified among the major volatile compounds, and it was found to be at its highest concentration (106.94 mg/L) when UFLA CAF119 was co-cultured with UFLA CA11 (Table 6). Ethyl acetate is the main acetate of cachaça (Duarte et al. 2011; Nonato et al. 2001) and it is also the most important acetate in terms of its sensory qualities (de Sousa et al. 2012); however, at concentrations above 150 mg/L, ethyl acetate can negatively affect beverage quality (Mallouchos et al. 2003). In terms of the volatile acids, the lowest level of propionic acid was found in the pure culture of UFLA CA11 (1.39 mg/L), and the lowest level of butyric acid was found for mixed inoculum of UFLA CAF733 + UFLA CA11 (0.79 mg/L). Fermentation with a pure culture of UFLA CA11 resulted in the lowest concentrations of acetaldehyde (8.27 g/L) and acetoin (1.25 mg/L) (Table 6).

Twenty-three minor volatile compounds were also identified in fermented sugar cane juice (Table 7). Of the five identified ethyl esters, ethyl butyrate (27.84 μg/L), ethyl hexanoate (99.24 μg/L), diethyl-succinate (120.11 μg/L) and diethyl malate (15.47 μg/L) were found in high concentrations when sugar cane juice was fermented with a mixed inoculum of UFLA CAF733 + UFLA CA11 (Table 7). This mixed inoculum also produced a fermented sugar cane juice with the highest total concentration of ethyl esters (290.13 μg/L). These high ester concentrations suggest that the mixed inoculum of CAF733 + UFLA CA11 has great potential as a starter culture for cachaça production because esters are key aromatic compounds associated with favorable aromatic descriptors as "fruity" (Czerny et al. 2008; Siebert et al. 2005), "apple-perfumed" (Meilgaard 1975), "green apple" (Meilgaard 1975; Siebert et al. 2005) and "sweet" (Siebert et al. 2005). The highest concentrations of the four identified acetate compounds (isoamyl acetate, isobutyl acetate, propyl acetate, phenylethyl acetate) and the highest total acetate concentration (715.21 μg/L) were found in the co-culture of UFLA CAF733 and UFLA CA11. As expected, isoamyl acetate and phenylethyl acetate were the most abundant acetates. Previous works (Moreira et al. 2008; Rojas et al. 2003; Viana et al. 2009) demonstrated that these two acetates have their concentrations increased by non-*Sacchomyces* yeasts. In addition to the highest acetate concentrations, the mixed inoculum of UFLA CAF733 and UFLA CA11 also had the highest total concentration of monoterpenic alcohols (195.56 μg/L) (Table 7), including linalool (151.31 μg/) and geraniol (29.50 μg/L) (Table 7). These results can be correlated with the β-glucosidase activity, as previously described (Swangkeaw et al. 2011). The concentrations of all volatile acids were increased by the use of three non-*Saccharomcyes* strains in mixed inocula with *S. cerevisiae* UFLA CA11 (Table 7). The mixed inoculum of UFLA CAF119 and UFLA CA11 produced the highest concentrations of isobutyric acid (61.32 μg/L), octanoic acid (1076.37 μg/L), decanoic acid (377.75 μg/L) and total volatile acids (3486.81 μg/L). The lowest total aldehyde content (121.10 μg/L) was found in sugar cane juice fermented by the mixed inoculum of UFLA CAF733 and UFLA CA11, while the highest content (223.31 μg/L) was found in juice fermented by the mixed inoculum of UFLA CAF119 and UFLA CA11 (Table 7). Low aldehyde and volatile acid concentrations are desirable for beverage quality, as their aromatic descriptors include such terms as "bitter" and "wax" (Meilgaard 1975), and "rancid" and "sweaty" (Siebert et al. 2005). Although no studies to date have addressed yeast selection specifically for the fermentation process of cachaça production, several groups have reported using the kinetic parameters (Arellano et al. 2008) and volatile compounds levels (Arellano et al. 2008; Arrizon et al. 2006; Pinal et al.

Table 6 Concentrations (mg/L) of major volatile compounds in sugar cane juice fermented by different mixed inocula

No.	Compounds	Yeast				Descriptors
		UFLA CAF733 + UFLA CA11	UFLA CAF119 + UFLA CA11	UFLA CAF70 + UFLA CA11	UFLA CA11	
	Alcohols (9)					
1	1-Propanol	8.53 ± 0.83	5.93 ± 1.13	5.28 ± 0.41	6.24 ± 0.00	Alcohol (C)
2	2-Methyl-1-propanol	22.76 ± 2.47	21.25 ± 0.50	15.02 ± 1.47	15.06 ± 1.28	–
3	2-Methyl-1-butanol	17.98 ± 0.90	15.00 ± 6.17	17.53 ± 0.84	11.27 ± 0.15	Alcohol, banana, medicinal, solvent (C)
4	3-Methyl-1-butanol	100.90 ± 1.17	55.84 ± 2.91	85.44 ± 2.05	44.57 ± 0.17	Alcohol, banana, sweetish, aromatic (C)
5	2-Heptanol	nd	nd	nd	0.73 ± 0.07	Coconut (C)
6	1,2-Propanediol	5.73 ± 0.05	4.30 ± 0.25	5.70 ± 0.69	2.24 ± 0.23	–
7	2-Phenylethanol	24.49 ± 1.36	17.56 ± 0.74	19.64 ± 3.66	17.91 ± 1.27	Roses, sweetish perfumed (C)
8	Methanol	nd	nd	nd	0.17 ± 0.08	Alcohol, solvent (C)
9	Furfuryl alcohol	35.14 ± 3.21	72.77 ± 8.68	36.19 ± 12.73	26.32 ± 1.72	Sugar cane, Woody (C)
	Total alcohols	2151.53	192.65	184.80	124.51	
	Acetates (1)					
10	Ethyl acetate	6.00 ± 0.71	106.94 ± 10.67	92.42 ± 4.26	2.36 ± 0.02	Solvent, fruity, sweetish (C)
	Total acetates	6.00	106.94	92.42	2.36	
	Volatile acids (2)					
11	Propionic acid	3.67 ± 0.53	5.40 ± 2.26	3.52 ± 2.46	1.39 ± 0.06	Acetic acid, Milk (E); vinegar (B)
12	Butyric acid	0.79 ± 0.51	2.96 ± 0.83	2.14 ± 1.22	nd	Sweaty (A); cheese, rancid (B)
	Total volatile acids	4.46	8.36	5.66	1.39	
	Aldehydes (1)					
13	Acetaldehyde	9.83 ± 0.44	10.01 ± 0.51	10.62 ± 3.38	8.27 ± 0.84	Green leaves, fruity (C)
	Total aldehydes	9.83	10.01	10.62	8.27	
	Others (1)					
14	Acetoin	4.39 ± 0.46	5.68 ± 0.56	6.48 ± 0.07	1.25 ± 0.07	Fruity, moldy, Woody (C)

nd not detected. (A) Czerny et al. (2008); (B) Siebert et al. (2005); (C) Meilgaard (1975)

2009) obtained from the fermented agave used for tequila production to suggest that yeast selection can play an important role in the resulting distilled beverage flavor and aroma.

PCA was applied to the data from Tables 6 and 7. The PC1 and PC2 accounted for 53.55 and 31.02 % of the total variance, respectively. The PC1 enabled the differentiation between the mixed inocula and the pure culture of *S. cerevisiae* UFLA CA11 (Fig. 2a). In the positive side of PC1 and the negative side of PC2, the mixed inocula UFLA CAF70 + UFLA CA11 and UFLA CAF119 + UFLA CA11 were characterized more by ethyl acetate, butyric acid, decanoic acid, propionic acid, acetoin, acetaldehyde, furfural and isoamyl acetate (Fig. 2a, b). The mixed inoculum of UFLA CAF733 and UFLA CA11 was positively characterized in PC1 and PC2 by ethyl hexanoate, 2-phenylethanol, linalool, nonanoic acid, ethyl butyrate, phenylethyl acetate, diethylsuccinate, hexanoic acid, and geraniol (Fig. 2a, b). The pure culture of *S. cerevisiae* UFLA CA11 was characterized by methanol and 2-heptanol (Fig. 2a, b).

Table 7 Concentrations (μg/L) of minor volatile compounds in sugar cane juice fermented by different mixed inocula

No.	Compounds	Yeast				Descriptors
		UFLA CAF733 + UFLA CA11	UFLA CAF119 + UFLA CA11	UFLA CAF70 + UFLA CA11	UFLA CA11	
	Ethyl Esters (5)					
1	Ethyl butyrate	27.84 ± 1.65	15.00 ± 3.05	21.21 ± 3.99	12.59 ± 4.40	Fruity (A, B); papaya, butter, sweetish, Apple perfumed (C)
2	Ethyl hexanoate	99.24 ± 14.59	67.70 ± 9.47	69.53 ± 13.69	88.95 ± 11.93	Fruity, green Apple (B, C)
3	Ethyl octanoate	27.47 ± 8.30	34.70 ± 3.53	33.84 ± 8.80	43.72 ± 8.24	Apple, fruity (C); sweet (B)
4	Diethylsuccinate	120.11 ± 12.43	106.68 ± 19.96	100.71 ± 19.24	86.05 ± 25.45	–
5	Diethyl malate	15.47 ± 2.51	nd	14.53 ± 3.61	nd	–
	Total ethyl esters	290.13	224.08	239.82	231.31	
	Acetates (4)					
6	Isoamyl acetate	376.22 ± 14.01	340.89 ± 15.61	328.61 ± 45.44	215.58 ± 39.29	Banana, Apple, solvent (C)
7	Isobutyl acetate	35.49 ± 6.34	29.36 ± 3.81	34.12 ± 3.95	27.53 ± 2.04	Banana, sweet, fruity (C)
8	Propyl acetate	56.64 ± 12.15	52.09 ± 1.78	39.85 ± 12.21	51.84 ± 6.00	Solvent, sweet, fragrant (C)
9	Phenylethyl acetate	246.86 ± 16.09	183.89 ± 21.92	189.97 ± 39.30	130.26 ± 15.25	Apple, honey, roses, sweet (C); flowery (B)
	Total acetates	715.21	603.23	592.55	425.21	
	Monoterpenic alcohols (4)					
10	Linalool	151.31 ± 57.18	105.57 ± 17.36	103.72 ± 38.45	94.77 ± 11.93	Citrus-like, bergamot (A); aniseed, terpenoid (C)
11	*b*-Citronellol	13.80 ± 6.76	19.75 ± 5.33	nd	13.97 ± 3.32	Citronella (D)
12	Geraniol	29.50 ± 5.54	26.83 ± 2.21	27.83 ± 4.87	23.52 ± 1.84	Rose-like, citrus-like (A
13	Guaiacol	0.95 ± 0.13	2.37 ± 0.09	1.33 ± 0.41	0.46 ± 0.05	–
	Total monoterpenic alcohols	195.56	154.52	132.88	132.72	
	Volatile Acids (6)					
14	Isobutyric acid	43.94 ± 1.96	61.32 ± 15.05	60.89 ± 13.30	48.82 ± 8.79	Sweat, bitter (C); cheese, rancid (B)
15	Hexanoic acid	181.96 ± 34.16	158.88 ± 17.11	151.02 ± 5.74	119.40 ± 8.91	Fatty acids, vegetable oil (C), cheese, sweaty (B)
16	Heptanoic acid	37.92 ± 12.49	nd	26.98 ± 5.89	23.14 ± 6.78	–
17	Octanoic acid	1072.58 ± 121.12	1076.37 ± 40.45	942.75 ± 171.62	863.17 ± 62.23	Fatty acids, vegetable oil (C); rancid, harsh (B)
18	Nonanoic acid	78.82 ± 47.62	38.03 ± 11.46	46.81 ± 7.31	32.17 ± 9.54	–
19	Decanoic acid	359.24 ± 63.15	377.75 ± 21.05	360.88 ± 83.59	339.56 ± 126.23	Wax, tallow, rancid, soap (C); fatty (B)
	Total volatile acids	1774.46	3486.81	1589.33	1426.26	
	Aldehydes (2)					
20	Octanal	99.28 ± 12.96	202.49 ± 13.71	197.17 ± 23.46	129.24 ± 53.77	Orange peel, bitter, aldehyde, vinous (C); citrus-like, Green (A)
21	Furfural	21.82 ± 4.41	20.82 ± 7.39	17.97 ± 5.97	11.51 ± 2.37	Paper, husk (C)
	Total aldehydes	212.10	223.31	215.14	140.75	
	Others (2)					

Table 7 continued

No.	Compounds	Yeast				Descriptors
		UFLA CAF733 + UFLA CA11	UFLA CAF119 + UFLA CA11	UFLA CAF70 + UFLA CA11	UFLA CA11	
22	1,1-Dietoxyethane	20.39 ± 3.77	22.31 ± 4.60	20.37 ± 3.58	19.92 ± 1.85	–
23	2,3-Butanedione	nd	37.51 ± 11.11	50.03 ± 7.35	nd	Buttery (A)
	Total others	20.39	59.82	70.40	19.92	

nd not detected. (A) Czerny et al. (2008); (B) Siebert et al. (2005); (C) Meilgaard (1975); (D) Ribéreau-Gayon et al. (2006)

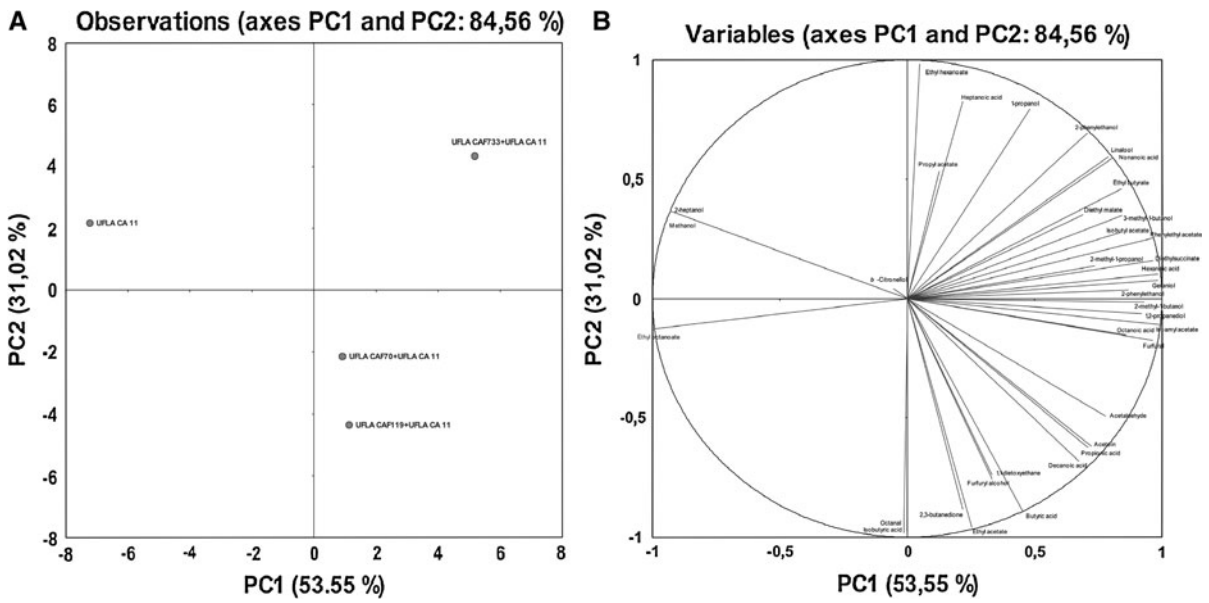

Fig. 2 Principal component analysis of volatile compounds (**b**) in sugar cane juice fermented by different mixed inocula and pure culture of *S. cerevisiae* UFLA CA11 (**a**)

Conclusions

Based on the results of this work, the co-inoculation of *S. cerevisiae* UFLA CA11 with three different non-*Saccharomyces* strains, namely *P. anomala* UFLA CAF70, *P. caribbica* UFLA CAF733 and *P. anomala* UFLA CAF119 improved the fermentation of sugar cane juice. The use *P. caribbica* UFLA CAF733 with *S. cerevisiae* UFLA CA11 left low concentrations of residual sugars (glucose, fructose and sucrose), had correspondingly high levels of sugar conversion (*Conv*), produced high concentrations of ethanol and had high volumetric productivity of ethanol (Q_p). Additionally, the mixed inoculum of UFLA CAF733 and UFLA CA11 produced increased concentrations of desirable volatile compounds, such as ethyl hexanoate, 2-phenylethanol, linalool, ethyl butyrate, phenylethyl acetate, diethylsuccinate and geraniol. Such increases in the levels of desirable volatile compounds combined with the high ethanol yield suggest that mixed inocula can be used to produce cachaça. The use of the non-*Saccharomyces* strain *P. caribbica* UFLA CAF733 in mixed inoculum with *S. cerevisiae* UFLA CA11 may be an interesting alternative to improve the quality of cachaça, supporting the idea that mixed inocula can be used to produce a beverage with a unique and favorable aromatic profile.

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References

Alvarez AL, Pérez ALD, Aguirre CS, Rodríguez LM, García JC (2012) Ethanol yield and volatile compound content in fermentation of agave must by *Kluyveromyces marxianus* UMPe-1 comparing with *Saccharomyces cerevisiae* baker's yeast used in tequila production. J Biosci Bioeng 113:614–618. doi:10.1016/j.jbiosc.2011.12.015

Arellano M, Pelayo C, Ramírez J, Rodriguez I (2008) Characterization of kinetic parameters and the formation of volatile compounds during the tequila fermentation by wilds yeasts isolated from agave juice. J Ind Microbiol Biotechnol 35:835–841. doi:10.1007/s10295-008-0355-4

Arrizon J, Fiore C, Acosta G, Romano P, Gschaedler A (2006) Fermentation behaviour and volatile compounds production by agave and grape must yeasts in high sugar *Agave tequiliana* and grape must fermentations. Antonie Van Leeuwenhoek 89:181–189. doi:10.1007/s10482-005-9022-1

Bely M, Stoeckle P, Masneuf-Pomarède I, Dubourdieu D (2008) Impact of *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. Int J Food Microbiol 122:312–320. doi:10.1016/j.ijfoodmicro.2007.12.023

Berthels NJ, Otero RRC, Bauer FF, Thevelein JM, Pretorius IS (2004) Discrepancy in glucose and fructose utilization during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res 4:683–689. doi:10.1016/j.femsyr.2004.02.005

Brazil (2005) Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n°. 13, de 29 de junho de 2005. Aprova o regulamento técnico para fixação dos padrões de identidade e qualidade para aguardente de cana e para cachaça. Brasília: Diário Oficial da União, seção 1, pp 3–4, de 30 de junho de 2005

Campos CR, Silva CF, Dias DR, Basso LC, Amorin HV, Schwan RF (2010) Features of *Saccharomyces cerevisiae* as a culture starter for the production of the distilled sugar cane beverage cachaça in Brazil. J Appl Microbiol 108:1871–1879. doi:10.1111/j.1365-2672.2009.04587.x

Cardeal ZL, de Souza PP, da Silva MDRG, Marriot PJ (2008) Comprehensive two-dimensional gas chromatography for fingerprint pattern recognition in *cachaça* production. Talanta 74:793–799. doi:10.1016/j.talanta.2007.07.021

Comitini F, Gobbi M, Domizio P, Romani C, Lencioni L, Mannazzu I, Ciani M (2011) Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiol 28:873–882. doi:10.1016/j.fm.2010.12.001

Czerny M, Christlbauer M, Christlbauer M, Fischer A, Granvogl M, Hammer M et al (2008) Re-investigation on odour thresholds of key food aroma compounds and development of an aroma language based on odour qualities of defined aqueous odorant solutions. Eur Food Res Technol 228:265–273. doi:10.1007/s00217-008-0931-x

de Souza PP, Cardeal ZL, Augusti R, Morrison P, Marriott PJ (2009) Determination of volatile compounds in Brazilian distilled cachaça by using comprehensive two-dimensional gas chromatography and effects of production pathways. J Chromatogr A 1216:2881–2890. doi:10.1016/j.chroma.2008.10.061

de Souza APG, Vicente MA, Klein RC, Fietto LG, Coutrim MX, Afonso RJCF, Araújo LA, da Silva PHA, Bouillet LEM, Castro IM, Brandão RL (2012) Strategies to select yeast starter culture for production of flavor compounds in *cachaça* fermentations. Antonie Van Leeuwenhoek 101:379–392. doi:10.1007/s10482-011-9643-5

Duarte WF, Dragone G, Dias DR, Oliveira JM, Teixeira JA, Silva JBA, Schwan RF (2010) Fermentative behavior of *Saccharomyces* strains during microvinification of raspberry juice (*Rubus idaeus* L.). Int J Food Microbiol 143:173–182. doi:10.1016/j.ijfoodmicro.2010.08.014

Duarte WF, de Sousa MVF, Dias DR, Schwan RF (2011) Effect of co-inoculation of *Saccharomyces cerevisiae* and *Lactobacillus fermentum* on the quality of the distilled sugar cane beverage cachaça. J Food Sci 76:C1307–C1318. doi:10.1111/j.1750-3841.2011.02412.x

Mallouchos A, Komaitis M, Koutinas A, Kanellaki M (2003) Evolution of volatile byproducts during wine fermentations using immobilized cells on grape skins. J Agric Food Chem 51:2402–2408. doi:10.1021/jf026086s

Maturano YP, Assaf LAR, Toro ME, Nally MC, Vallejo M, de Figueroa LIC, Combina M, Vazquez F (2012) Multi-enzyme production by pure and mixed culture of *Saccharomyces* and non-*Saccharomyces* yeast during wine fermentation. Int J Food Microbiol 155:43–50. doi:10.1016/j.ijfoodmicro.2012.01.015

Meilgaard MC (1975) Flavor chemistry of beer: Part II: Flavor and threshold of 239 aroma volatiles. MBAA Tech Q 12:151–168

Moreira N, Mendes F, de Pinho PG, Hogg T, Vasconcelos I (2008) Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed culture in grape must. Int J Food Microbiol 124:231–238. doi:10.1016/j.ijfoodmicro.2008.03.025

Nissen P, Nielsen D, Arneborg N (2003) Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* in mixed cultures bye a cell–cell contact-mediated mechanism. Yeast 20:331–341. doi:10.1002/yea.965

Nonato AE, Carazza F, Silva FC, Carvalho CR, Cardeal ZL (2001) A headspace solid-phase microextraction method for the determination of some secondary compounds of Brazilian sugar cane spirits by gas chromatography. J Agric Food Chem 49:3533–3539. doi:10.1021/jf000896r

Nova MXV, Schuler ARP, Brasileiro BTRV, Morais MA Jr (2009) Yeast species involved in artisanal cachaça fermentation in the three stills with different technological levels in Pernambuco, Brazil. Food Microbiol 26:460–466. doi:10.1016/j.fm.2009.02.005

Oliveira ES, Rosa CA, Morgano MA, Serra GE (2004) Fermentation characteristics as criteria for selection of

cachaça yeast. World J Microbiol Biotechnol 20:19–24. doi:10.1023/B:WIBI.0000013286.30695.4e

Pinal L, Cornejo E, Arellano M, Herrera E, Nuñez L, Arrizon J, Gschaedler A (2009) Effect of *Agave tequiliana* age, cultivation field location and yeast strain on tequila fermentation process. J Ind Microbiol Biotechnol 36:655–661. doi:10.1007/s10295-009-0534-y

Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D (2006) Varietal aroma. In: Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D (eds) Handbook of enology. The chemistry of wine and stabilization and treatments, vol 2. Wiley, England, pp 187–206

Rodrígues ME, Lopes CA, Barbagelata RJ, Barda NB, Caballero AC (2010) Influence of *Candida pulcherrima* Patagonian strain on alcoholic fermentation behaviour and wine aroma. Int J Food Microbiol 138:19–25. doi:10.1016/j.ijfoodmicro.2009.12.025

Rojas V, Gil JV, Piñaga F, Manzanares P (2003) Acetate ester formation in wine by mixed cultures in laboratory fermentations. Int J Food Microbiol 86:181–188. doi:10.1016/S0168-1605(03)00255-1

Schwan RF, Mendonça AT, Silva JJ, Silva JR, Rodrigues V, Wheals AE (2001) Microbiology and physiology of cachaça (aguardente) fermentations. Antonie Van Leeuwenhoek 79:89–96. doi:10.1023/A:1010225117654

Siebert TE, Smyth HE, Capone DL, Neuwöhoner C, Pardon KH, Skouroumounis GK et al (2005) Stable isotope dilution analysis of wine fermentation products by HS-SPME-GC-MS. Anal Bioanal Chem 381:937–947. doi:10.1007/s00216-004-2992-4

Silva CLC, Vianna CR, Cadete RM, Santos RO, Gomes FCO, Oliveira ES, Rosa CA (2009) Selection, growth, and chemo-sensory evaluation of flocculent starter culture strains of *Saccharomyces cerevisiae* in the large-scale production of traditional Brazilian cachaça. Int J Food Microbiol 131:203–210. doi:10.1016/j.ijfoodmicro.2009.02.027

Soares EV (2010) Flocculation in *Saccharomyces cerevisiae*: a review. J Appl Microbiol 110:1–18. doi:10.1111/j.1365-2672.2010.04897.x

Swangkeaw J, Sukanda V, Butzuke CE, Vichitphan K (2011) Characterization of β-glucosidase from *Hanseniaspora* sp. and *Pichia anomala* with potentially aroma-enhancing capability in juice and wine. World J Microbiol Biotechnol 27:423–430. doi:10.1007/s11274-010-0474-8

Valles BS, Bedriñana RP, Queipo AL, Alonso JJM (2008) Screening of cider yeasts for sparkling cider production (Champenoise method). Food Microbiol 25:690–697. doi:10.1016/j.fm.2008.03.004

Viana F, Gil JV, Genovés S, Vallés S, Manzanares P (2008) Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiol 25:778–785. doi:10.1016/j.fm.2008.04.015

Viana F, Gil JV, Vallés S, Manzanares P (2009) Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Haseniaspora osmophila* and *Saccharomyces cerevisiae*. Int J Food Microbiol 135:68–74. doi:10.1016/j.ijfoodmicro.2009.07.025

Viana F, Belloch C, Vallés S, Manzanares P (2011) Monitoring a mixed starter of *Hanseniaspora vineae–Saccharomyces cerevisiae* in natural must: impact on 2-phenylethyl acetate production. Int J Food Microbiol 151:235–240. doi:10.1016/j.ijfoodmicro.2011.09.005

Vicente MA, Fietto LG, Castro IM, dos Santos ANG, Coutrim MX, Brandão RL (2006) Isolation of *Saccharomcyes cerevisiae* strains producing higher levels of flavoring compounds for production of "cachaça" the Brazilian sugarcane spirit. Int J Food Microbiol 108:51–59. doi:10.1016/j.ijfoodmicro.2005.10.018

Villena MA, Iranzo JFU, Pérez AIB (2007) β-Glucosidase activity in wine yeasts: application in enology. Enzyme Microb Technol 40:420–425. doi:10.1016/j.enzmictec.2006.07.013

Zott K, Miot-Sertier C, Claisse O, Lonvaud-Funel A, Masneuf-Pomarede I (2008) Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. Int J Food Microbiol 125:197–203. doi:10.1016/j.ijfoodmicro.2008.04.001