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Fermentative capabilities of native yeast strains grown on juices from different *Agave* species used for tequila and mezcal production

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Abstract

The *Asparagaceae* family is endemic from America, being the *Agave* genus the most important. The *Agave* species possess economic relevance and are use as raw material to produce several distilled alcoholic beverages, as bacanora, tequila, and mezcal. The fermentation process has been carry out either spontaneously or by adding a selected yeast strain. The latter is generally responsible for the production of ethanol and volatile compounds. This study comprised five *Agave* species (*A. angustifolia*, *A. cupreata*, *A. durangensis*, *A. salmiana*, and *A. tequilana*) and eight endogenous yeast strains: five of them were non-*Saccharomyces* (*Torulaspora delbrueckii*, *Zygosaccharomyces bisporus*, *Candida ethanolica*, and two *Kluyveromyces marxianus*) and three *Saccharomyces cerevisiae* strains. The results showed that the *S. cerevisiae* strains were not able to grow on *A. durangensis* and *A. salmiana* juices. The *Kluyveromyces marxianus* strains grew and fermented all the agave juices and displayed high ethanol production (48–52 g L^{-1}) and volatile compounds. The ethanol production was higher on *A. angustifolia* juice (1.1–2.8-fold), whereas the volatile compound was dependent on both yeast strain and the *Agave* species. The use of endogenous non-*Saccharomyces* yeast strains is feasible, as they may outperform *S. cerevisiae* regarding the production of fermented beverages from agave plants with a high content of ethanol and aromatic compounds.

Keywords Agave · Yeasts · Ethanol · Fermentation · Volatiles

Introduction

The *Agave* genus comprises several species found in arid and semiarid regions that are spread around Mexico, the Southwestern U.S., Central America, the Caribbean, and Northern South America [1]. Mexico is the biodiversity center of the *Agave* genus, where 272 out of the 310 reported species are found. Agaves are well adapted to the territory's environmental conditions because their morphological and physiological characteristics enable them to withstand harsh environments [2]. The plants allocated in the *Agave* genus have been used for a wide range of applications, such as the production of alcoholic and non-alcoholic beverages, as well as food and fibers products [3]. Recently, they are being used to produce fructo-oligosaccharides intended to be used as prebiotics and dietary fibers [4, 5]. Nowadays, approximately 15 *Agave* species are primarily used to produce alcoholic beverages, e.g., raicilla, bacanora, pulque, tequila, and mezcal [6, 7].

The production of tequila steadily increases each year, reaching export volumes up to 197.0 million liters from an overall production of 273.3 million liters in 2016 [8]. These exports accounted for more than 70% of the total production, which is even higher than its national consumption. In fact, since 1995, tequila exports surpass local consumption, excluding the years 2007–2008. Generally, tequila is a spirit consumed worldwide in more than 50 countries. On the other hand, the production and export of the tequila's smokier "cousin," mezcal, is increasing every year. In 2016, its production volume was 3.9 million liters and, as tequila, more than half of its production volume (2.0 million liters) was exported to 52 countries [9].

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Tequila is produced only from *Agave tequilana* Weber var. Azul, while Mezcal can be produced from several species highlighting the *Agave angustifolia* from the Mexican state of Oaxaca. Other *Agave* species are used for this purpose in different states of Mexico, e.g., *Agave salmiana* widely used in San Luis Potosi and Zacatecas, *Agave cupreata* in Guerrero and *Agave durangensis* is commonly used in Durango [6].

The mezcal production process begins by harvesting agave's mature heads (core), also known as "piñas." Typically, these cores are cooked in order to hydrolyze fructo-oligosaccharides and they are subsequently crushed to extract their juice that is afterwards fermented and finally double distilled [7]. During the artisanal process, fermentation is spontaneously initiated by a wide variety of naturally occurring microorganisms [10]. In this step, microorganisms assimilate sugars (mostly fructose and glucose) and other less available compounds, as substrates for biomass accumulation and to produce ethanol, carbon dioxide, higher alcohols. and esters. All of these contribute to define the chemical composition and the organoleptic properties of the final product. In spite of this, the studies focused on the potential of the *Saccharomyces* and non-*Saccharomyces* species that occur naturally during the fermentation of agave juice are scarce [11–13]. It is well known that the yeast strain is a key factor in the production of ethanol and volatile compounds during the fermentation of alcoholic beverages [14, 15].

Moreover, it has been shown that the yeast strains isolated from wine, display low ethanol and volatile compounds yields when agave juice were used [11]. This may be related to the high saponin content of the agave plant; since it has been demonstrated, it inhibits yeast growth [16]. The isolation and selection of native yeast strains from agave juices is critical in order to increase both ethanol and volatile compounds yields, that commercially available yeasts strains isolated from wine fermentations fail to achieve. It is expected that, agave plants and consequently their juices possess different chemical composition depending on the *Agave* species, the environmental conditions and the harvesting procedures. Hence, it is important to study if diverse yeast strains are able to grow and ferment in different agave juices, in order to determine if strain selection is necessary for each specific juice. Therefore, the fermentation process depends on the *Agave*'s region of origin, production procedures, initial cell concentration, temperature, ethanol concentration [17, 18], and yeast strains.

In the alcoholic beverage industry, yeast fermentative capacity is the most important adaptation feature of a microorganism within the fermentation must [18]. The aim of this work was to evaluate the fermentative capabilities and the generation of volatile compounds by eight endogenous yeast strains from agave juices (five non-*Saccharomyces* strains: *Torulaspora delbrueckii*, *Zygosaccharomyces bisporus*, *Candida ethanolica*, two *Kluyveromyces marxianus*, and three *Saccharomyces cerevisiae*), when grown in juices of five different *Agave* species (*A. angustiforlia*, *A. cupreata*, *A. durangensis*, *A. salmiana*, and *A. tequilana*).

Materials and methods

Raw materials

The cores from different *Agave* species (*Agave angustifolia* Haw, *Agave cupreata*, *Agave durangensis*, *Agave salmiana* ssp. *crassispina*, and *Agave tequilana* Weber var. azul, collected from Oaxaca, Guerrero, Durango, San Luis Potosi, and Jalisco States, respectively) and the juices were obtained as follows: the cores were autoclaved for 18 h at a pressure ranging from 1.0 to 1.1 kg cm^{-2}. The temperature was set at 92 °C and the steam output temperature ranged from 100 to 150 °C. Subsequently, each core was crushed in order to extract the agave juice. All juices were analyzed to quantify saponin levels as well as the carbon/nitrogen ratio (C/N) by using the experimental methods reported by Baccou et al. (1975) [19] and Cheney (1962) [20], respectively. Finally, the agave juice was filtered and stored at − 20 °C until further use.

Nitrogen quantification of the *Agave* juices

The organic and an ammoniacal nitrogen were quantified for the five different types of *Agave* juice used [20]. A calibration curve using L-arginine (30 mM) was used for the organic nitrogen quantification, since it is the predominant amino acid in *A. tequilana* juice [21]. For the ammoniacal nitrogen, ammonium sulfate (7.57 mM) was used to run the calibration curve. This compound is used in the soils where the *Agave* plants are being grown when there is a lack of nitrogen.

For the organic nitrogen, 100 μL of the sample was mixed with 100 μL of a solution of ninhydrin (67 mM) and put in a water bath at 100 °C for 2.5 min. Samples were cooled on ice for 5 min and read in a microplate reader (Bio-Rad 680XR) at 520 nm.

The ammoniacal nitrogen was quantified by adding 1 mL of a solution containing phenol (62.5 g L^{-1}) and nitroferricyanide (2.5 g L^{-1}) in distilled water, to 20 μL of the sample. This solution was mixed in an axial movement and 1 mL of an alkaline hypochlorite solution (31.25 g L^{-1} of sodium hydroxide and 40 mL L^{-1} of 6% chlorine in distilled water) was added and mixed again in an axial manner. The reaction mixture was left for 10 min and then 8 mL of distilled water was added to finally read the absorbance at 630 nm in a microplate reader (Bio-Rad 680XR.

Saponin quantification of the *Agave* juices

Quantification of the saponins present in the *Agave* juices was performed according to the colorimetric method proposed by

Baccou et al. (1977) [19]. This method consisted on the measurement of the sapogenin after an acid hydrolysis. A calibration curve using diosgenin, a predominant steroidal saponin present in the *Agave* plants, was used. The methodology was carried out by mixing 20 μL of the sample in 2 mL of ethyl acetate and adding 1 mL of reagent 1 (5 mL L^{-1} of anisaldehyde in 995 mL L^{-1} of ethyl acetate) and 1 mL of reagent 2 (sulfuric acid 50%) to this solution. The reaction mixture was put in a water bath at 60 °C for 20 min, then mixed and put on ice for 10 min for a final measurement of the absorbance at 430 nm in a spectrophotometer (Thermo Electro Corporation Genesys 10UV).

Strain selection and media

Eight yeast strains isolated from mezcal-producing sites (Table 1) were selected: five non-*Saccharomyces*: *Candida ethanolica* (CeSLPA), *Kluyveromyces marxianus* (KmOFF1 and KmSLP1), *Torulaspora delbrueckii* (TdDI1), *Zygosaccharomyces bisporus* (ZbDGOP), and three *Saccharomyces cerevisiae* strains (ScAR5, ScMC4, and ScZAC1). Which belongs to the strain collection kept by the *Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco* (CIATEJ A.C.) and they were cryogenically preserved (− 80 °C) in a medium consisting of 50% yeast peptone dextrose medium (YPD) and 50% of glycerol. YPD contained 10 g L^{-1} yeast extract (BD, USA), 20 g L^{-1} bacto peptone (BD, USA), and 20 g L^{-1} glucose (Sigma, USA) at pH 4.5.

Yeast growth

The biomass accumulation resulting from the growth of each yeast strain was measured when they were grown on all of the agave juices. Solid medium containing each juice (*A. angustifolia*, *A. cupreata*, *A. durangensis*, *A. salmiana*, and *A. tequilana*) were prepared. These consisted on agave juice (40 g L^{-1}) at pH 4.5 and 2% agar. The incubation temperature was 30 °C and growth was monitored every 24 h for an overall 96-h period. Control medium was YPD agar without agave juice.

Table 1 Mexican States and Agave specie in which the respective yeast strains were isolated

State	*Agave* specie	Substrate	Strain	Code
Oaxaca	*A. angustifolia*	Must	*S. cerevisiae*	ScMC4
			T. delbrueckii	TdDI1
Guerrero	*A. cupreata*	Must	*K. marxianus*	KmOFF1
Durango	*A. durangensis*	Must	*Z. bisporus*	ZbDGOP
San Luis Potosi	*A. salmiana*	Must	*C. ethanolica*	CeSLPA
			K. marxianus	KmSLP1
Zacatecas	*A. tequilana*	Must	*S. cerevisiae*	ScZAC1
Jalisco	*A. tequilana*	Must	*S. cerevisiae*	ScAR5

Fermentative capacity

Batch fermentations of each yeast strain were performed in 250 mL flasks containing 200 mL of the respective agave juice with an initial 140 g L^{-1} sugar concentration at pH 4.5. Only the juice from *A. salmiana* contained an initial 110 g L^{-1}concentration because of its intrinsic levels. All fermentations were initiated by inoculating 10 million cells mL^{-1} and by incubating the culture at 30 °C and 100 rpm. Samples were retrieved every 4 h during a 16-h period. After the initial sampling, samples were taken every 6 h for 24 h, and finally every 8 h for an overall period of 72 h. Batch fermentations were carried out in duplicate.

Biomass measurements

Yeast cells were counted in a hemocytometer (Neubauer chamber) by using aliquots of the ongoing cultures, whereas biomass was quantified by measuring cell dry weight (DW) at the beginning of the fermentation and at the end of the process. The supernatant was stored at 4 °C to further perform the analysis of reducing sugars, ethanol, and volatile compounds.

Analysis of reducing sugars

The quantification of reducing sugars was carried out by using the modified dinitrosalicylic acid (DNS) method [22] as follows: a 100 μL sample was incubated with 100 μL of a DNS solution (10 g L^{-1} sodium hydroxide, 200 g L^{-1} sodium potassium tartrate, 0.5 g L^{-1} sodium metabisulfite, 2 g L^{-1} phenol, and 10 g L^{-1} 3, 5-dinitrosalisylic acid; all reagents were purchased from Sigma ®, USA), in a recirculating water bath at 100 °C for 5 min. After the reaction concluded, the samples were placed on ice for 5 min and 1 mL of distilled water was added to the mixture. The products obtained from the reaction were quantified by spectrophotometry (Bio-Rad 680XR, USA) at 540 nm.

Quantification of ethanol and volatile compounds

The concentration of both ethanol and volatile compounds were assessed by gas chromatography (HP 6890, USA) with a flame ionization detector (FID) at 250 °C, coupled to a headspace system (HP 7694E, USA). The volatile compound injections were performed using the headspace system using a 20 mL vial with 2 mL of the sample. Only the volatile compounds in the headspaces were injected. The temperatures of the vial, loop, and transfer line were 80 °C, 110 °C, and 115 °C, respectively. Additionally, the time to reach the vial

equilibrium was set to 5 min, pressurization time was 0.2 min using high purity helium (99.9999%), the loop fill and equilibrium times were set at 0.2 and 0.5 min, respectively, and injection time was 1 min. The volatile compounds quantified in the sample were acetaldehyde, ethyl acetate, 1-propanol, 2-methyl 1-propanol (isobutanol), 2-methyl 1-butanol, and 3-methyl 1-butanol (amylic alcohol).

Experimental design and statistical analysis

Based on the methodology, the experimental design consisted on a two-way factorial array [23]. The first factor was strain species with eight levels (strains) and the second was agave juice, considered in five levels (species). An overall of 40 runs were performed, in duplicate. To analyze the effect of the individual factors and their interaction, a two-way variance analysis (ANOVA) was performed by taking into account the response of the measured variables by using the SPSS software (v. 24). A variance analysis with sum of squares type IV was applied when there were missing treatments due to a response with a value of zero on the factorial array [24]. In all cases, the Tukey's HSD mean test was performed in order to obtain a detailed comparison between the different treatments [23]. All tests were run with a 95% level of confidence.

Results

Characterization of raw materials

The saponin content was analyzed in all the agave juices, as it has been previously reported that these compounds inhibit yeast growth [16]. Agave contains high levels of these compounds [16, 25]. *A. durangensis* displayed the highest saponin concentration (430.8 ppm) (Table 2) and it was statistically different when compared to the saponin content in *A. salmiana* (357.3 ppm) (Tukey HSD at 95% confidence). The other agave species (*A. angustifolia*, *A. cupreata*, and *A. tequilana*) contain lower saponin levels (307.6 ppm, 315.2 ppm, and 293.1 ppm, respectively) that were statistically different when compared to the first two species, although no significant differences were observed among them (Tukey HSD at 95% confidence). The C/N ratio was also evaluated for all agave juices. The highest ratio (276.9) was observed for *A. tequilana*. This implies that their nitrogen content is low. *A. salmiana* and *A. cupreata* displayed the lowest C/N ratio (94.9 and 68.6) (Table 2).

Yeast growth

The results obtained with all eight yeast strains grown for 24 h in the respective five agave juices are summarized in Table 2. All tested yeast strains successfully grew on *A. angustifolia*, *A. cupreata*, and *A. tequilana* juices. However, only the non-*Saccharomyces* strains (ZbDGOP, KmOFF1, KmSLP1, and CeSLPA) were able to grow on the juices obtained from *A. durangensis* and *A. salmiana*. The *S. cerevisiae* strains (ScAR5, ScMC4, and ScZAC1) were absent on solid media. Additionally, *T. delbrueckii* (TdDI1) did not grow on *A. salmiana* juice, whereas only few *Z. bisporus* colonies (ZbDGOP) were observed on this same juice. As shown in Table 2, *S. cerevisiae* strains grew on those agave juices that contain low saponin levels (*A. tequilana*, *A. cupreata*, and *A. angustifolia*). Thus, this is an important factor that restrict the growth of these strains because of the higher concentration of this compound.

Fermentative capacity

Based on a previously described method, an analysis of variance (ANOVA) was performed in order to evaluate fermentation by all native yeast strains grown on the juices obtained from the agave species used to produce mezcal and tequila. The response variables were cell population, ethanol

Table 2 Yeast growth on solid media supplemented with agave juices

Juice	Strain								Saponin content (ppm)	C/N ratio
	ScAR5	ScMC4	ScZAC1	KmOFF1	KmSLP1	CeSLPA	TdDI1	ZbDGOP		
A. angustifolia	++	++	++	++	++	++	++	++	307.6 ± 2	137.5 ± 1
A. cupreata	++	++	++	++	++	++	++	++	315.2 ± 1	68.6 ± 7
A. durangensis	–	–	–	++	++	++	+	++	430.8 ± 6	237.4 ± 1
A. salmiana	–	–	–	++	++	++	–	+	357.3 ± 4	94.9 ± 5
A. tequilana	++	++	++	++	++	++	++	++	293.1 ± 4	276.9 ± 4

++Normal colony growth

+Low colony growth

-No growth

production, and sugar consumption. All of these were assessed during batch fermentations. The results showed that the *S. cerevisiae* (ScAR5, ScMC4, and ScZAC1) and *T. delbrueckii* (TdDI1) strains do not grow in some of the agave juices, therefore the ANOVA analysis cannot be evaluated under normal circumstances. Because some treatments showed zero values, they may affect the analysis outcome. Because of these constraints, the ANOVA was performed by using type IV sum of squares as recommended in the case of missing treatments within a factorial arrangement [24]. The results showed significant statistical differences in all of the response variables, depending on both *Agave* species and the yeast strain used. The former is the most important factor, followed by yeast strain and by the estimated effect of the agave species-yeast strain interaction (p value < 0.05). Moreover, a Tukey's mean test was performed in order to obtain a detailed comparison between the different treatments [23].

The results obtained from the Tukey post hoc test regarding cell population as a response variable showed that the ZbDGOP and ScZAC1 strains achieved the highest cell populations (1.30×10^8 and 1.32×10^8 cells mL^{-1}) (Fig. 1a). Lower populations were observed for ScAR5 (1.15×10^8 cells mL^{-1}), KmOFF1 (1.13×10^8 cells mL^{-1}), and KmSLP1 (1.11×10^8 cells mL^{-1}). Those strains that displayed the lowest growth were TdDI1 (0.84×10^8 cells mL^{-1}), ScMC4 (0.88×10^8 cells mL^{-1}) and CeSLPA (0.89×10^8 cells mL^{-1}). However, all comparisons were made by considering only those experiments where yeasts exhibited growth. The highest cell population was observed for *A. cupreata* (1.44×10^8 cells mL^{-1}), followed by *A. salmiana* (1.33×10^8 cells mL^{-1}) and *A. angustifolia* (1.03×10^8 cells mL^{-1}). The lowest populations were observed on *A. durangensis* (0.77×10^8 cells mL^{-1}) and *A. tequilana* (0.83×10^8 cells mL^{-1}) (Fig. 1b). These two agave juices were characterized by the highest C/N ratio (237.4 and 276.9 respectively). This indicates a possible nitrogen limitation (Table 2).

Fig. 1c shows the effect of the agave juice-yeast strain interaction. The ZbDGOP and ScZAC1 strains showed the highest mean cell population (1.89×10^8 cells mL^{-1} for both of them) when grown on *A. cupreata*. Conversely, these strains displayed a lower growth on *A. durangensis* juice. The ZbDGOP and TdDI1 strains achieved the lowest cell concentrations (0.38×10^8 cells $mL^{-1}$and 0.36×10^8 cells mL^{-1}, respectively) when grown on the latter juice. Additionally, no significant differences were observed between the KmOFF1 and KmSLP1 yeast strains when growth on all five agave juices (1.13×10^8 cells mL^{-1} and 1.11×10^8 cells mL^{-1}, respectively).

Regarding the concentration of residual reducing sugars (RRS), it was observed that both of the *K. marxianus* strains (KmOFF1 and KmSLP1) consumed almost all the sugars contained on the different agave juices, based on the lowest RRS values displayed by these strains (3.78 g L^{-1} and

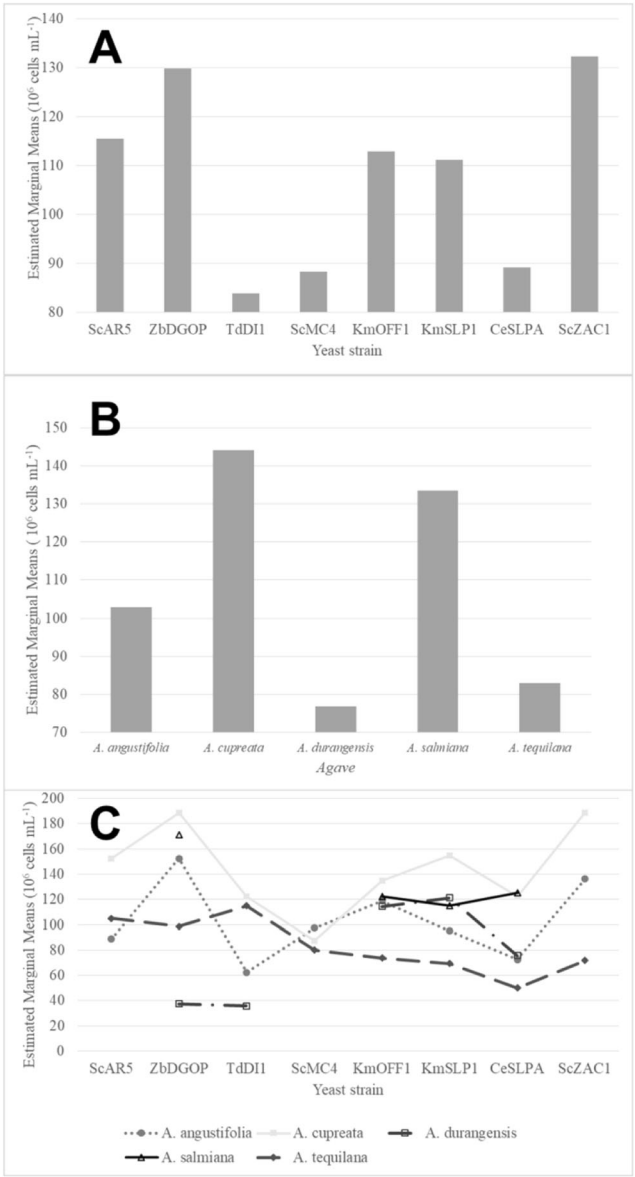

Fig. 1 Estimated marginal means of cell population: **a** yeast strain, **b** Agave species, and **c** strain-agave interaction

5.73 g L^{-1}). In contrast, ScZAC1 and CeSLPA did not consume fermentable sugars (58.11 g L^{-1} and 68.57 g L^{-1}) (Fig. 2a). The lowest RRS concentration (11.23 g L^{-1}) was observed for the *A. cupreata* juice. Therefore, all eight yeast strains were able to consume a high quantity of the sugars contained in this agave. Conversely, the yeast strains consumed a lower amount of the sugars contained on the juice from *A. durangensis* (75.78 g L^{-1}) (Fig. 2b). The effect of the yeast strain-agave juice interaction on RRS showed similar averages for both KmOFF1 and KmSLP1, as lower sugar levels were observed regardless of the agave juice (Fig. 2c). Therefore, both yeast strains are able to adapt in order to grow and to perform fermentation on all five agave juices analyzed in this study. In contrast, the other yeast strains did not adapt to the agave juices and their behavior mainly depended on the

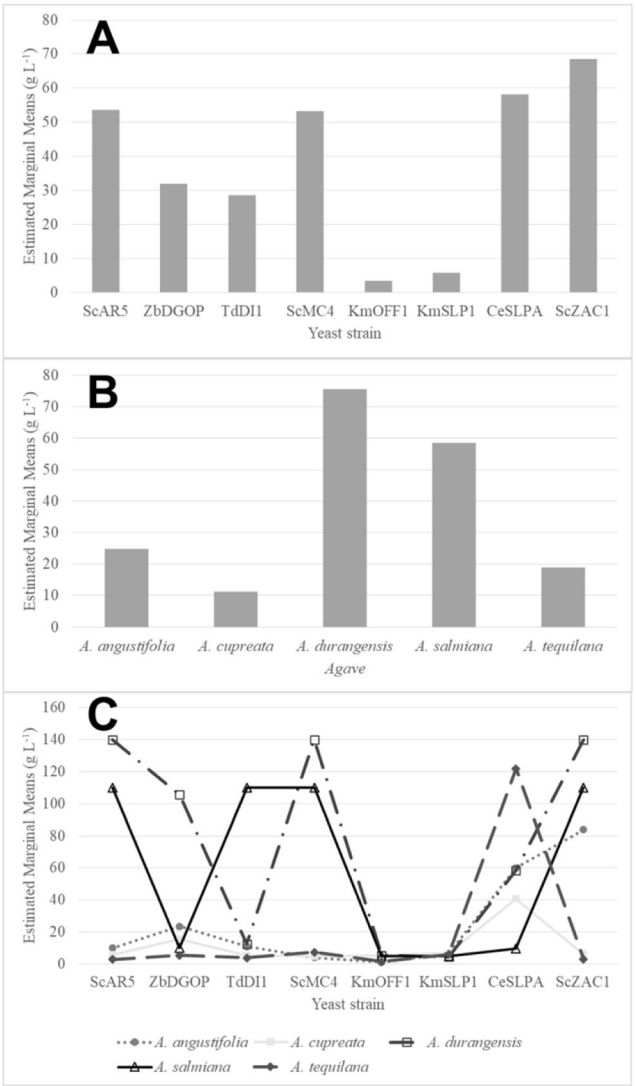

Fig. 2 Estimated marginal means of residual sugars: **a** yeast strain, **b** Agave species, and **c** strain-agave interaction

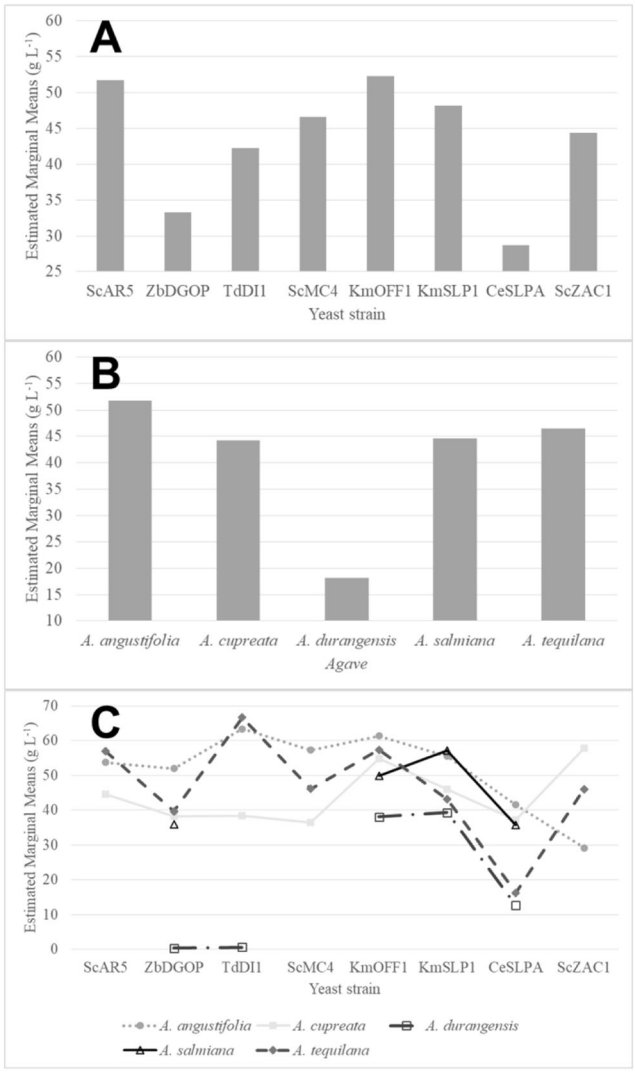

Fig. 3 Estimated marginal means of ethanol concentration: **a** yeast strain, **b** Agave species, and **c** strain-agave interaction

nature of the substrate in which they were initially isolated (Fig. 2c).

Finally, regarding ethanol production, the KmOFF1, ScAR5, KmSLP1, and ScMC4 strains displayed a higher ethanol production when grown on the agave juices (52.27 g L^{-1}, 48.20 g L^{-1}, 51.71 g L^{-1}, and 46.60 g L^{-1}, respectively). As previously mentioned, the agave juices that restricted fermentation were not considered (Fig. 3a). In contrast, the CeSLPA and ZbDGOP strains exhibited low ethanol concentrations (28.66 g L^{-1} and 33.22 g L^{-1}). The agave juice that favored higher ethanol amounts was that from *A. angustifolia* (51.73 g L^{-1}), whereas the lowest levels were observed in the *A. durangensis* juice (18.19 g L^{-1}) (Fig. 3b). Figure 3c shows the influence of agave juice-yeast strain interaction in ethanol production. High ethanol levels were obtained when the juice from *A. angustifolia* was fermented, regardless of the yeast strain used, excepting ScZAC1 (29.24 g L^{-1}). Additionally, ethanol production increased when the juice from *A. tequilana* juice was submitted to fermentation in a strain-independent manner, excepting CeSLPA, that yielded lower ethanol levels (16.3 g L^{-1}). Moreover, the KmSLP1 and KmOFF1 yeast strains were able to produce ethanol in all of the agave juices, although a decreased production was observed on the *A. durangensis* juice (39.3 g L^{-1} and 38.04 g L^{-1}) but it never reached a zero value.

Volatile compounds

Table 3 shows the mean value of volatile compounds productions and the Tukey HSD test at 95% of level of confidence of the volatile compounds produced by all the yeast strains grown on the different agave species as quantified by GC-FID coupled to a headspace. Stars and colored lines represent agave species and the volatile compound produced by the yeast strains, respectively. At the onset of the fermentation

Table 3 Tukey HSD test for the mean production of volatile compounds regarding yeast strain and *Agave* juice

Compounds (mg L^{-1})											
Agave specie	Yeast strain	Acetaldehyde		Ethyl acetate		1-propanol		Isobutanol		Amyl alcohol	
Agave angustifolia	ScAR5	79.07^{ax}	± 8.84	4.40^{av}	± 0.21	19.81^{av}	± 0.87	11.84^{av}	± 1.42	102.77^{av}	± 4.20
	ScMC4	175.30^{ay}	± 0.40	6.47^{av}	± 0.67	14.38 ^{aw}	± 1.73	35.87 ^{av}	± 2.16	128.07 ^{av}	± 3.42
	ScZAZ1	46.15 ^{aw}	± 0.10	449.12^{az}	± 22.57	8.82 ^{aw}	± 0.21	63.86 ^{aw}	± 0.35	4.20 ^{aw}	± 0.88
	KmOFF1	296.03 ^{az}	± 3.32	58.12^{aw}	± 7.83	20.98 ^{av}	± 4.32	316.91 ^{ax}	± 54.32	242.67 ^{ax}	± 13.26
	KmSLP1	74.95 ^{ax}	± 11.14	88.85^{ax}	± 6.81	19.73 ^{av}	± 0.82	186.79 ^{ay}	± 2.06	171.79 ^{ay}	± 3.05
	CeSLPA	42.98 ^{aw}	± 0.67	63.14^{aw}	± 4.85	11.58 ^{aw}	± 0.13	113.46 ^{az}	± 38.85	72.56 ^{az}	± 61.78
	TdDI1	60.72 ^{aw}	± 3.46	9.41^{av}	± 0.11	11.20 ^{aw}	± 0.04	69.72 ^{aw}	± 1.73	23.01 ^{aw}	± 10.61
	ZbDGOP	26.87 ^{av}	± 0.34	8.87^{av}	± 1.13	8.76 ^{aw}	± 0.42	81.32 ^{aw}	± 7.61	176.33 ^{ay}	± 17.35
Agave cupreata	ScAR5	39.59 ^{aw}	± 3.29	6.20^{bv}	± 0.55	27.07^{bx}	± 0.45	15.49^{bv}	± 0.34	98.06 ^{az}	± 6.26
	ScMC4	41.06 ^{aw}	± 8.49	4.31^{bv}	± 1.09	14.44 ^{bv}	± 2.91	20.43 ^{bv}	± 4.01	128.78 ^{av}	± 0.00
	ScZAZ1	78.36 ^{ax}	± 0.90	15.13^{bv}	± 1.12	29.79 ^{bx}	± 0.65	41.71 ^{bv}	± 2.43	173.48 ^{ay}	± 11.16
	KmOFF1	305.26 ^{az}	± 0.50	22.72^{bv}	± 2.76	35.51 ^{by}	± 0.41	116.63 ^{bz}	± 1.90	170.36 ^{ay}	± 2.54
	KmSLP1	82.05 ^{ax}	± 0.04	34.01^{bw}	± 0.00	14.32 ^{bw}	± 2.93	30.89 ^{bv}	± 6.30	88.76 ^{az}	± 0.00
	CeSLPA	30.00 ^{aw}	± 7.09	5.87^{bv}	± 4.71	31.19 ^{bx}	± 2.33	33.01 ^{bv}	± 2.61	86.87 ^{az}	± 5.85
	TdDI1	196.82 ^{ay}	± 0.00	6.73^{bv}	± 2.21	14.32 ^{bw}	± 2.93	30.89 ^{bv}	± 6.30	88.76 ^{az}	± 0.00
	ZbDGOP	44.38 ^{aw}	± 0.01	7.87^{bv}	± 0.30	18.58 ^{b}	± 1.10	41.83 ^{bv}	± 2.43	84.23 ^{az}	± 5.41
Agave durangensis	ScAR5	N.D.		N.D.		N.D.		N.D.		N.D.	
	ScMC4	N.D.		N.D.		N.D.		N.D.		N.D.	
	ScZAZ1	N.D.		N.D.		N.D.		N.D.		N.D.	
	KmOFF1	23.08^{bv}	± 1.71	155.03^{cy}	± 6.02	14.28^{cw}	± 0.26	113.92^{cz}	± 14.86	90.06 ^{cz}	± 0.00
	KmSLP1	30.49^{bv}	± 6.10	205.93^{cy}	± 0.00	16.40^{cv}	± 1.89	70.74^{cz}	± 8.12	101.43 ^{cz}	± 0.00
	CeSLPA	19.28^{bv}	± 7.32	35.56^{cw}	± 0.43	9.74^{cw}	± 3.85	14.98^{cv}	± 2.26	24.64 ^{cw}	± 5.31
	TdDI1	4.18^{bv}	± 1.49	2.57^{cv}	± 0.31	N.D.		N.D.		N.D.	
	ZbDGOP	5.94^{bv}	± 1.21	1.53^{cv}	± 1.17	N.D.		N.D.		N.D.	
Agave salmiana	ScAR5	N.D.		N.D.		N.D.		N.D.		N.D.	
	ScMC4	N.D.		N.D.		N.D.		N.D.		N.D.	
	ScZAZ1	N.D.		N.D.		N.D.		N.D.		N.D.	
	KmOFF1	54.23^{bw}	± 1.54	80.32^{cx}	± 0.43	22.84^{dw}	± 1.65	267.15^{du}	± 12.43	170.22^{by}	± 22.31
	KmSLP1	84.23^{bx}	± 9.21	100.71^{cx}	± 19.68	24.12 ^{dw}	± 4.65	235.84^{du}	± 19.21	287.83 ^{bw}	± 6.73
	CeSLPA	34.09^{bv}	± 0.70	58.38^{cw}	± 0.42	12.28 ^{dv}	± 0.77	81.06^{dz}	± 2.79	88.14 ^{bz}	± 0.95
	TdDI1	N.D.		N.D.		N.D.		N.D.		N.D.	
	ZbDGOP	26.63^{bv}	± 0.37	21.24^{cv}	± 0.94	10.84 ^{dv}	± 0.57	96.14^{dz}	± 1.44	131.22 ^{bv}	± 1.29
Agave tequilana	ScAR5	193.54 ^{ay}	± 3.02	5.43^{bv}	± 0.68	15.69^{ev}	± 2.36	7.25^{bv}	± 1.04	92.90 ^{bz}	± 6.08
	ScMC4	242.70 ^{az}	± 50.48	7.72^{bv}	± 0.70	8.29^{ev}	± 1.45	31.88 ^{bv}	± 7.42	80.87 ^{bz}	± 7.50
	ScZAZ1	65.31 ^{ax}	± 1.39	8.37^{bv}	± 0.66	14.68^{ev}	± 0.86	16.58 ^{bv}	± 1.18	74.17 ^{bz}	± 6.23
	KmOFF1	39.84 ^{aw}	± 0.16	53.65^{bw}	± 1.34	15.89^{ev}	± 0.46	136.24 ^{bz}	± 4.41	157.95 ^{by}	± 2.90
	KmSLP1	33.73 ^{av}	± 1.02	60.82^{bw}	± 5.49	16.51^{ew}	± 0.30	66.93 ^{bz}	± 1.94	109.20 ^{bz}	± 9.79
	CeSLPA	23.24 ^{av}	± 1.39	4.64^{bv}	± 0.52	6.99^{ev}	± 0.04	13.67 ^{bv}	± 0.33	22.92 ^{bw}	± 3.04
	TdDI1	178.77 ^{ay}	± 0.17	6.25^{bv}	± 0.24	9.27^{ev}	± 0.48	53.97 ^{bz}	± 1.16	67.63^{bw}	± 1.54
	ZbDGOP	45.19 ^{aw}	± 1.21	6.92^{bv}	± 0.09	7.86^{ev}	± 0.39	66.84 ^{bz}	± 3.87	116.29^{bz}	± 4.19

Superscript letters ranging from a to e show a significant statistical difference among the Agave species at a 95% confidence level using Tukey HSD test
Superscript letters ranging from u to z show a significant statistical difference among yeast strain at a 95% confidence level using Tukey HSD test

process, the most abundant higher alcohols were amyl alcohol (up to 287.83 mg L^{-1} in *A. salmiana* by KmSLP1), isobutanol (up to 267.15 mg L^{-1} in *A. salmiana* by KmOFF1), and 1-propanol (up to 35.51 mg L^{-1} in *A. cupreata* by KmOFF1). Higher alcohol production was doubled when the *K. marxianus* strain was used instead of the *S. cerevisiae* yeast strains. The KmOFF1 strain also produced amyl alcohol in all the agave juices (ranging from 90.06 to 242.67 mg L^{-1})

(Fig. 3a). The juice from *A angustifolia* stimulated a higher amyl alcohol (up to 242.67 mg L^{-1}) and isobutanol (316.91 mg L^{-1}) production for all the evaluated strains, whereas *A. cupreata* increased 1-propanol production (up to 35.51 mg L^{-1}). The juices from *A. tequilana* and the *A. durangensis* affected the production of higher alcohols. Ethyl acetate was the most abundant ester as it was produced in high amounts by the KmSLP1 yeast strain when compared to the other yeasts grown on *A. durangensis* juice (205 mg L^{-1}). Acetaldehyde (305 mg L^{-1}) was the most abundant aldehyde and its highest concentration was detected when the juice from *A. cupreata* was used.

Discussion

The *S. cerevisiae* strains did not grow on *A. durangensis* and *A. salmiana.* This is consistent with the findings reported by Escalante et al. (2008) [26], as they identified and isolated 11 microorganism species (3 yeasts and 9 bacteria) from the mezcal fermentation process carried out with *A. salmiana* juice. The three isolated yeast strains were non-*Saccharomyces* (*K. marxianus*, *Pichia fermentans*, and *Clavispora lusitaniae*). They were unable to isolate *S. cerevisiae*, probably because of the saponins contained on the juice. Conversely, Verdugo et al. (2011) [27] isolated and identified *S. cerevisiae* strains during the spontaneous fermentation of *A. salmiana* juice, although they did not mention if these *S. cerevisiae* strains are able to ferment when grown on this agave juice.

The results obtained in this work may be explained by the fact that both *Agave* species *(A. salmiana and A. durangensis)* display the highest saponin content (Table 2). It has been shown that these compounds may inhibit yeast growth [28], probably because saponins are known to have hemolytic activity [29]. Some saponins, such as α-tomatinase, have been studied and they were identified as programed cell death inducers in the *Fusarium oxysporum* fungus [30]. Moreover, it has been demonstrated than fungi display two mechanisms to avoid the toxic effects exerted by saponins such as the tomatinase: either by changing their cell membrane composition or by producing specific tomatine-detoxifying enzymes known as tomatinases [31]. Agave saponins also disrupt cell wall integrity, including a decreased 1–3 β-glucans:1–6, β-glucans ratio. Our results showed that non-*Saccharomyces* yeast strains possess the ability to grow on the agave juices that contain higher levels of saponins to consequently perform fermentation. Thus, it is possible that KmSLP1 and KmOFF1 strains have developed their own detoxification metabolism that acts by repairing the cell wall [16]. This may be the consequence of a more efficient adaptation system when compared to that displayed by *S. cerevisiae* yeast strains.

As previously mentioned, both *K. marxianus* strains fermented all the five agave juices. Some studies propose that these yeasts possess an adaptive ability to consume different carbon sources in order to produce biomass [32, 33]. Therefore, regardless the type of juice, both *K. marxianus* strains (KmOFF1 and KmSLP1) assimilated the sugars contained on the culture medium. KmOFF1 and KmSLP1 also displayed higher values of ethanol production and cell population when grown on all five agave juices. These yeast strains are important to produce fermented agave beverages as they have been detected in spontaneous fermentation processes [10, 12, 34].

Regarding ethanol production, the KmOFF1, KmSLP1, ScAR5, and ScMC4 yeast strains displayed a higher ethanol production (Fig. 1c). These results are consistent with those reported by Nonklang et al. (2008) [35], as they observed that *K. marxianus* yield ethanol levels comparable to those produced by *S. cerevisiae* or even higher. In addition, Segura et al. (2015) [13] and Lopez et al. (2012) [12] found that non-*Saccharomyces* strains are characterized by higher ethanol production rates when compared to *S. cerevisiae* strains. This is particularly the case for *K. marxianus* strains isolated from fermentation processes in which *A. tequilana* is used as raw material.

Regarding the generation of volatile compounds, it was observed that both agave species and yeast strain were critical factors. When *A. cupreata* and *A. angustifolia* were used for fermentation, an increased production of higher alcohols was noted. In this context, Pinal et al (1997) [36] highlighted the importance of the C/N ratio regarding the production of higher alcohols. A low C/N ratio enhanced the synthesis of higher alcohols during the production of tequila. In this study, it was observed that the juices from *A. cupreata*, *A. angustifolia*, and *A. salmiana* are characterized by lower C/N ratios when compared to *A. durangensis* and *A. tequilana* (Table 2).

Additionally, the ScZAC1 strain produced a higher level of ethyl acetate by using *A. angustifolia*, juice and, whereas KmSLP1 and the KmOFF1 yeast strains produced a high level of ethyl acetate when grown on *A. durangensis* and a larger quantity of higher alcohols on *A. cupreata* and *A. salmiana.* Both processes, high alcohol and ester production, are linked to nitrogen availability on the media [37]. In this context, the production of ethyl acetate may be directly related to threonine, as it has been reported that high levels of the latter favor a higher concentration of the former during the fermentation process [37]. Therefore, it may be possible that either agave species contain high threonine levels. Alternatively, this amino acid may be produced by the yeasts, as it is essential for growth.

Finally, the KmOFF1 strain generated high acetaldehyde levels when the fermentation is performed on *A. angustifolia* and *A. cupreata.* Thus, *K. marxianus* produces high levels of ethanol, ethyl acetate, higher alcohols, and acetaldehyde,

depending on the type of substrate used during the fermentation process. Some reports mention the potential use of *K. marxianus* strains as an industrial factory of aromatic compounds [38, 39].

As a conclusion, a direct relationship between nutrients and the inhibitors contained on the raw materials was observed based on the adaptation of the yeast strain during the fermentation stage. In this study, three *S. cerevisiae* strains were not able to grow and ferment on *A. durangensis* and *A. salmiana.* It is noteworthy that the KmSLP1 and KmOFF1 strains excelled regarding the production of ethanol and volatile compounds when grown on all agave juices when compared to the other yeast strains used in this work. Therefore, this yeast species may be useful as a starter culture to perform fermentation using different *Agave* species as raw material.

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