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Influence of Freeze-Dried Yeast Starter Cultures on Volatile Compounds of Tchapalo, a Traditional Sorghum Beer from Côte d'Ivoire

Wahauwouélé Hermann Coulibaly^{1,2,*}, Kouadio Florent N'guessan¹, Ibourahema Coulibaly³, Marlène Cot², Peggy Rigou⁴ and Koffi Marcellin Djè¹

¹ Laboratoire de Biotechnologie et Microbiologie des Aliments, Unité de Formation et de Recherche en Sciences et Technologie des Aliments (UFR-STA), Université Nangui Abrogoua, 02 BP 801 Abidjan 02, Ivory Coast; genie_ci@yahoo.fr (K.F.N.); djekoffr@yahoo.fr (K.M.D.)

² Laboratoire d'Ingénierie des Systèmes Biologiques et Procédés, Institut National des Sciences Appliquées (INSA-Toulouse), 135 Avenue de Rangueil, 31077 Toulouse CEDEX 4, France; m_cot@insa-toulouse.fr

³ Laboratoire de Microbiologie, Biologie Industrielle et de Biotechnologie (LMBIB), Unité de Formation et de Recherche en Agroforesterie (UFR-AgroFor), Université Jean Lorougnon Guédé, 12 BP V 25 Daloa 12, Ivory Coast; ibourahema.ujlog@gmail.com

⁴ Plateau d'Analyses des Composés Volatils (PTV), Institut des Hautes Études de la Vigne et du Vin, INRA-SUPAGRO Montpellier, 2 Place Pierre Viala, 34060 Montpellier CEDEX 02, France; rigou@supagro.inra.fr

* Correspondence: wahauwouele@yahoo.fr; Tel.: +225-7-731-604

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Abstract: The production of the Ivorian sorghum beer known as tchapalo remains more or less an empirical process. The use of starter cultures was therefore suggested as the appropriate approach to alleviate the problems of variations in organoleptic quality and microbiological stability. In this study, we evaluated the capacity of *S. cerevisiae* and *C. tropicalis* to produce sorghum beer as freeze-dried starter in mixed or pure cultures. Beers produced with mixed freeze-dried cultures of *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 showed residual sugars and ethanol contents similar to beers obtained with *S. cerevisiae* F12-7 pure culture, but the total sum of organic acids analyzed was the highest with the mixed culture (15.71 g/L). Higher alcohols were quantitatively the largest group of volatile compounds detected in beers. Among these compounds, 2-phenyl ethanol, a higher alcohol that plays an important role in beer flavor, was highly produced with the mixed culture (10,174.8 µg/L) than with the pure culture (8749.9 µg/L).

Keywords: mixed starter; *Saccharomyces cerevisiae*; *Candida tropicalis*; volatile compounds; sorghum beer

1. Introduction

Sorghum beer is a traditional fermented beverage from most of the West African countries where sorghum is produced. It is known as tchapalo in Côte d'Ivoire and by various other names in other African countries. The composition and sensory character of the beer is mainly determined by the metabolism of microorganisms during fermentation. Those microorganisms, by the virtue of their metabolic activities, play an active role in volatile compounds production. Like other beers, sorghum beer is one of the most delicate and labile beverages from a sensory point of view. It has a complex but moderate taste and aroma and shows poor flavor stability. The use of starter culture was thus suggested to alleviate the problems of variations in organoleptic quality and microbiological stability [1,2].

During the brewing of sorghum beer, the alcoholic fermentation stage is performed by several yeast strains, among which *Saccharomyces cerevisiae* was the predominant strain [3–6]. Among the

non-*Saccharomyces* yeasts, the predominant species varied according to geographic origin of the beer. Thus, *Candida tropicalis* was reported as the predominant non-*Saccharomyces* yeast in pito and tchapalo [7,8], while *Clavispora lusitaniae* and *Candida inconspicua* were reported for tchoukoutou and ikigage respectively [9,10]. Previous research aiming to develop starter cultures for sorghum beer production was focused on the use of *S. cerevisiae* as identification studies have revealed the dominance of this species [2,10]. In recent years, growth of non-*Saccharomyces* yeasts has been observed also in fermentation inoculated with *S. cerevisiae*, suggesting a greater contribution of these yeasts to the flavour and quality of alcoholic beverages than previously thought. Thus, the use of *Torulaspota delbrueckii* was evaluated for beer production in both pure and mixed cultures with *Saccharomyces cerevisiae* [11,12]. Other examples of non-*Saccharomyces* yeasts include *Brettanomyces*, which is used in atypical spontaneously fermented Belgian beer or “lambic beer” [13] and *Candida zemplinina* in craft beer fermentation [14].

Various investigations on the use of starter culture for sorghum beer production have been done [15–18]. Beers obtained from inoculation tests with these starters showed organoleptic and sensory characteristics comparable to beers produced in the traditional way. However, these starter cultures were introduced during fermentation in the form of fresh microbial suspensions, which are difficult to maintain for extended periods. To overcome this problem, the use of dried starter cultures, active and stable during storage, is the viable and sustainable solution. Furthermore, such starter preparations require the use of cheap raw materials in order to be economically feasible. An enhanced stability of microorganisms can be achieved by adding many protective compounds, such as disaccharides, polyols, monosaccharides, skim milk, and other organic molecules [19].

Recently, we showed the possibility of producing freeze-dried starter culture for the brewing purpose of tchapalo with sucrose and cassava flour as the best protective agent and support material, respectively [20]. In this study, we aimed to evaluate the capacity of *S. cerevisiae* and *C. tropicalis* to produce sorghum beer as freeze-dried starter culture. Microbiological growth, organic acids, sugars, ethanol and volatile compounds were therefore determined in the produced beers.

2. Materials and Methods

2.1. Yeast Strains

The yeast strains used in this study were *C. tropicalis* C0-7 and *S. cerevisiae* F12-7. They belonged to the culture collection of the Food Technology Department (University of Nangui-Abrogoua, Abidjan, Côte d’Ivoire). They were isolated from traditional sorghum beer from the district of Abidjan (Southern Côte d’Ivoire). They were identified by Polymerase Chain Reaction-Restriction Fragment Length polymorphism (PCR-RFLP) of the Internal Transcribed Spacer (ITS) region and sequencing of D1/D2 domains of the 26S *rRNA* gene [6]. The yeast strains were maintained routinely at $-20\text{ }^{\circ}\text{C}$ in 20% glycerol.

2.2. Production of Freeze-Dried Starter Cultures

The yeast strains of *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 were inoculated into a tube containing 20 mL of yeast extract-peptone-dextrose (YPD) liquid and incubated at $30\text{ }^{\circ}\text{C}$ for 24 h. The cells were harvested by centrifugation at $1300\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. Harvested cells were washed twice in saline solution (0.85% NaCl) and resuspended in the same solution in order to obtain a $20\times$ concentration factor. The initial cell concentration was then calculated by determining the optical density at a wavelength of 650 nm. For the freeze drying, cassava flour and sucrose were used respectively as support material and protective agent. Two grams (2 g) of flour was mixed with 50 mL distilled water and heated to $70\text{--}80\text{ }^{\circ}\text{C}$ for 20–30 min under agitation and then cooled to $30\text{--}40\text{ }^{\circ}\text{C}$. Pure culture of *S. cerevisiae* F12-7 or the mixture of *S. cerevisiae* F12-7/*C. tropicalis* C0-7 (1:2, v/v) was suspended in 25 mL sterile solution of 20 g/L sucrose. Cell suspensions were then blended with cassava solution at 1:1 ratio (v/v). After homogenization, samples were transferred into sterilized flasks, frozen in a

freezer (SANYO, Medical freezer, MDF 235) for 24 h at $-80\text{ }^{\circ}\text{C}$ and subsequently freeze-dried with a freeze drier (Alpha 1–4 Christ, Fischer Scientific, Bioblock, France) at $-60\text{ }^{\circ}\text{C}$ for 32 h under vacuum degree of 6.7–13.3 Pa (50–100 mTorr). Immediately after drying, water content and viability of the freeze-dried biomass were evaluated as previously described by several authors [20].

2.3. Fermentation Conditions

Fermentations were conducted in triplicate under agitation conditions in 1-L sterile Erlenmeyer flasks, closed with dense cotton plugs, containing 500 mL of pasteurized (10 min at $100\text{ }^{\circ}\text{C}$) final sorghum wort obtained from a randomly identified commercial tchapalo brewer at Williamsville-Macaci. The freeze-dried starters were pre-wetted in 100 mL of sterile sorghum wort for 30 min at $30\text{ }^{\circ}\text{C}$ before inoculation. Flasks were simultaneously inoculated with preculture to obtain 10^7 CFU/mL and incubated for 32 h at $28\text{ }^{\circ}\text{C}$ with stirring speed to 1.48 g. Samples of both pure and mixed fermentations were taken from each flask throughout the fermentation process to perform viable cell counts. One hundred μL aliquots of serial dilutions of each sample were plated onto both YPD medium (total yeast population) and Lysine Agar medium (non-*Saccharomyces* yeast population). Lysine Agar medium (Oxoid LTD., Hampshire, England) is a selective medium which does not support the growth of *S. cerevisiae*, and was therefore used for the viable count of the non-*Saccharomyces* yeasts cultured in mixed fermentation. After fermentation, in both pure and mixed cultures, the fermented worts were centrifuged (10 min at $5040\times g$) to remove yeast cells and they were kept at $-20\text{ }^{\circ}\text{C}$ before analysis.

2.4. Analytical Assays

Glucose, maltose, ethanol and organic acids (lactic, malic, oxalic, propionic, tartaric, citric and acetic acids) were separated and quantified by HPLC (Agilent Technologies, 1200 series, UK) using a column Aminex HPX-87H, 300 mm \times 7.8 mm (Biorad) coupled to a refractometer (Agilent Technologies) and a UV diode array detector (Agilent Technologies). The acquisition and processing of data were carried out using the Chromoleon software. The operating conditions for the determination were as follows: oven temperature: $50\text{ }^{\circ}\text{C}$; duration of analysis: 35 min; mobile phase: 5 mM sulfuric acid; flow rate of mobile phase: 0.5 mL/min and injected sample volume: 20 μL . The quantification of the concentration is performed by UV detection at 210 nm. Calibration is carried out from solutions containing the different analytes in the linear ranges of each.

2.5. Volatile Compound Analysis

The analysis of the volatile compounds was carried out as follows [21]. A total of 10 μL of a solution of deuterated standards diluted in ethanol (100 $\mu\text{g}/\text{mL}$) was added to the sample (5 mL). One milliliter of dichloromethane was added and the mixture was gently shaken for 20 min on the shaking table. The samples were centrifuged for 5 min at $412\times g$ at $4\text{ }^{\circ}\text{C}$ to separate the aqueous and organic phases. The organic phase was recovered in a 4-mL vial and a second extraction was performed on the remaining aqueous phase as described above. Both organic phases were mixed and dried with anhydrous Na_2SO_4 to remove all traces of aqueous phase. A total of 1.5 mL of the organic phase was transferred to a vial and evaporated under nitrogen flux to a final volume of 0.5 mL. The sample was then transferred to an insert (Supelco, Bellefonte, PA, USA), returned to the vial and closed.

Samples were analyzed with a Hewlett Packard (Agilent Technologies) 6890 gas chromatograph equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu, Columbia, MD, USA) and coupled to an HP 5973 mass spectrometry detector (HP, now Agilent Technologies). The instrument was controlled and the data analyzed with HP G1701DA ChemStation software (HP, now Agilent Technologies). The gas chromatograph was fitted with a 30 m \times 0.25 mm Phenomenex fused silica capillary column ZB-WAX, 0.25 μm film thickness (Agilent Technologies). The carrier gas was helium, linear velocity 36 cm/s, flow rate 1.0 mL/min in constant flow mode. The initial oven temperature was $40\text{ }^{\circ}\text{C}$ for 3 min. The temperature was increased by $4\text{ }^{\circ}\text{C}/\text{min}$ until it reached $220\text{ }^{\circ}\text{C}$, and was held at this temperature for 20 min. The injector and the transfer line were held at $250\text{ }^{\circ}\text{C}$.

The sample volume injected was 2 μL , and the splitter, at 10:1, was opened after 30 s. The focus liner (Agilent Technologies) was deactivated and tapered with glass wool (2–4 mm). The mass spectrometer quadrupole temperature was set at 150 $^{\circ}\text{C}$ and the source was set at 230 $^{\circ}\text{C}$. For quantification, mass spectra were recorded in Selected Ion Monitoring (SIM) mode with positive ion electron impact at 70 eV.

2.6. Statistical Analysis

The analysis of variance (ANOVA) and Tukey tests and Levene (t -test) were performed with the STATISTICA software, 99th Edition, to compare variables analyzed on the sweet and fermented worts. Statistical differences with $p < 0.05$ were considered significant. The principal component analysis (PCA) was used to compare beer products from the measured variables. PCA allowed grouping the measured variables into new variables called “components” or “factors.” This grouping is based on the correlation of variables. The XLSTAT software (Adinosoft Inc.) was used for the PCA.

3. Results and Discussion

3.1. Evolution of Yeast Biomass during Beer Production

The dry starters used in this study showed residual water contents of 2%–2.5% and viability rates of 9%–11% (not shown).

During sorghum wort fermentation, *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 in both pure and mixed cultures showed three growth phases: 0–4 h was the latency phase; 4–24 h was the exponential phase; and 24–32 h was the stationary phase (Figure 1). The exponential phases of *S. cerevisiae* and *C. tropicalis* strains in mixed culture were higher than that of *S. cerevisiae* strains in pure culture. Thus, values passed from 3.5 to 9 log (CFU/mL) for *S. cerevisiae* F12-7, and from 3.5 to 8.32 log (CFU/mL) for *C. tropicalis* C0-7 in mixed culture. For *S. cerevisiae* in pure culture, the cell population increased from 7 to 10.07 log (CFU/mL) during the same phase. Similar results have been reported by literature [19]. Several studies also reported that the use of mixed cultures resulted in a higher growth rate, better biotransformation and higher yields in products [22]. These findings suggest interaction between the strains involved in the mixed starter. The results also demonstrated that in mixed culture, *C. tropicalis* grew lower than *S. cerevisiae* but it withstood the fermentation. Traditionally, the disappearance of non-*Saccharomyces* species in alcoholic beverage fermentation has been associated with their lower tolerance to ethanol or to toxic compounds [23,24]. However recent studies have shown that the early growth arrest of some non-*Saccharomyces* yeast species in mixed cultures cannot be explained by nutrient depletion or the presence of toxic compounds and, instead, seems to be due to a cell–cell contact mechanism [25].

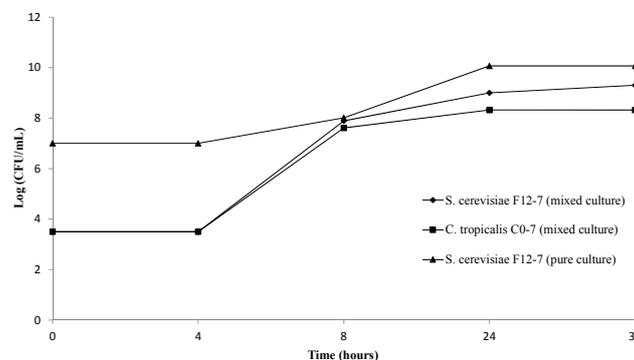


Figure 1. Evolution of yeasts during alcoholic fermentation of sorghum wort with freeze-dried starter cultures.

3.2. Biochemical Characteristics of the Produced Beers

Table 1 shows the biochemical characteristics of beers fermented by the pure and mixed cultures. Except for tartaric and malic acids content, all the others fermentation characteristics of the mixed

culture were similar to those of the *S. cerevisiae* F12-7 pure culture. Tartaric acid was not found in beer obtained with the mixed culture, while it was about 8 mg/L in beer fermented by the pure culture. Contrary to tartaric acid, malic acid was produced during the fermentation and its content was higher in mixed culture (4.7 g/L) than pure culture (2.7 g/L). In the beers produced, the total sum of organic acids analyzed was the highest with the mixed culture (15.71 g/L). These compounds are important in several respects. Firstly, they contribute to lowering the pH during fermentation and, secondly, they influence the sourness attribute, although some acids have their own flavour and aroma characteristics [25]. This is consistent with previous results, which reported that non-*Saccharomyces* yeasts are characterized as secondary compound producers [14,26].

Table 1. Concentration of organic acids, sugars and ethanol in sorghum beers produced with freeze-dried cultures of *S. cerevisiae* F12-7 and *C. tropicalis* C0-7.

	Sorghum Wort	Beer with Mixed Starter	Beer with Pure Starter
Glucose (g/L)	11.1 ± 0 ^a	7.0 ± 0 ^b	6.0 ± 0 ^b
Maltose (g/L)	187.2 ± 0.4 ^a	30.5 ± 2.7 ^b	28.6 ± 1.3 ^b
Ethanol (%)	n.d	4.4 ± 0.9 ^a	4.5 ± 0.8 ^a
Oxalic acid (mg/L)	11.7 ± 0 ^a	11.0 ± 0 ^a	10.0 ± 0 ^a
Citric acid (g/L)	0.1 ± 0 ^a	1.1 ± 0.6 ^b	0.8 ± 0.4 ^b
Tartaric acid (mg/L)	11.0 ± 0.1 ^a	0.0 ^b	8.0 ± 0 ^a
Acetic acid (mg/L)	21.0 ± 0.1 ^a	0.0 ^b	0.0 ^b
Malic acid (g/L)	0.7 ± 0.1 ^a	4.7 ± 0.8 ^b	2.7 ± 0.1 ^c
Lactic acid (g/L)	11.0 ± 0.1 ^a	6.6 ± 0.8 ^b	5.4 ± 0.3 ^b
Propionic acid (g/L)	3.3 ± 0.1 ^a	3.3 ± 0.6 ^a	2.1 ± 0.1 ^a

The values expressed are the means of three measurements. On the same line, the mean values with the same letter are not significantly different ($p > 0.05$).

3.3. Volatile Compounds of the Beers

The use of new fermentation technologies for optimizing beer quality and producing beers with particular flavor profiles is one of the worldwide trends in brewing [11,12,14]. In this context, there is a growing demand for new and improved sorghum beer yeast strains to obtain beers with constant organoleptic characteristics. This demand could be met by non-*Saccharomyces* sorghum beer yeasts, which are described as fermentations improvers and producers of concentrations of fermentation metabolites of brewing importance [4,7,15,16]. A wide range of volatile compounds was detected in the sorghum beer samples, including six esters, four higher alcohols, six acids and one fatty acid (Figure 2). Higher alcohols (propanol, 2-methylpropanol, 3-methylbutanol and 2-phenylethanol) are quantitatively the largest group of volatile compounds (Figure 2A). These compounds are formed during fermentation by two routes, the catabolic Ehrlich pathway and the anabolic Genevois pathway. Both pathways operate in brewer's yeast, but the relative contributions of the two pathways depend greatly on the level of amino acids present in the fermentation medium [25]. Different studies have shown that apiculate yeasts in pure and mixed starters produced lower amounts of higher alcohols when compared to *S. cerevisiae* [11,27–29], which corresponded to our assays. In fact, among the beers, *S. cerevisiae* F12-7 pure culture consistently produced the highest amount of propanol (10,947.8 µg/L) and 2-methylpropanol (50,795 µg/L), while the mixed culture produced the highest amount of 3-methylbutanol (53,054.2 µg/L) and 2-phenylethanol (10,174.8 µg/L). However, these differed from the findings of others authors who observed an increase in alcohol formation in wines produced by mixed cultures, in contrast with those fermented with pure cultures of *S. cerevisiae* [30]. The differences in alcohol production from the various mixed-culture studies could probably be due to the use of different non-*Saccharomyces* yeasts or the degree of yeast succession. In addition, it was reported that the production of higher alcohols depends on the yeast characteristics, medium composition (i.e., wort original extract, amount of zinc, lipids, dissolved oxygen) and fermentation parameters (i.e., temperature, stirring, pressure, fermenter design). So, the pure culture produced the highest

amounts of propanol and 2-methylpropanol, while the mixed culture produced the highest amounts of 3-methylbutanol (isoamyl alcohol) and 2-phenylethanol. Since the alcohols that play the most important role in beer flavour are amyl alcohol, isoamyl alcohol and 2-phenylethanol [25], we may deduce with regard to higher alcohols that beers produced with mixed culture were the best flavored.

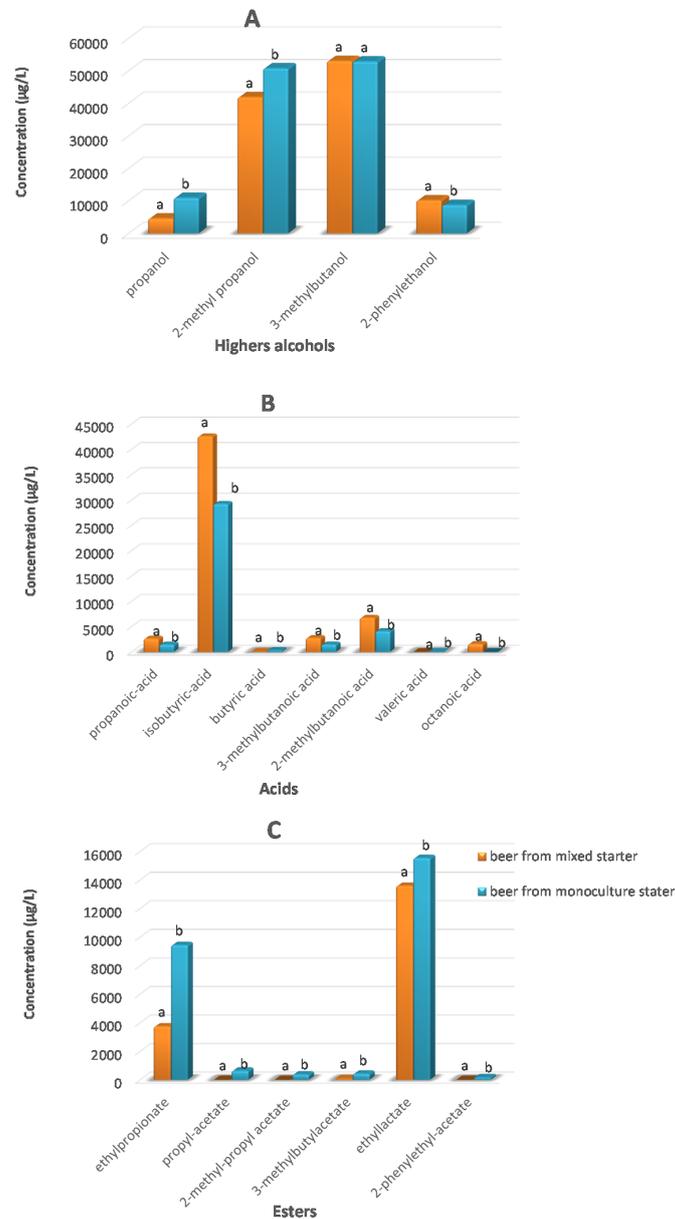


Figure 2. Volatile compounds in sorghum beers produced with mixed and pure freeze-dried starter cultures; (A): Higher alcohols; (B): Acids; (C): Esters. Histograms with the same letters are not statistically different ($p \geq 0.05$).

Acids are quantitatively the second largest group (Figure 2B). Most of the compounds in this group (propanoic, isobutyric, 3-methylbutanoic and 2-methylbutanoic acids) were highly found in beers produced with the mixed culture.

As shown in Figure 2C, esters found in the sorghum beers are acetate and ethyl esters, with ethyl lactate and ethyl propionate forming the bulk of the esters. The highest amount of these compounds was obtained with *S. cerevisiae* F12-7 pure culture. The mixed culture had lower amounts of ester compounds than the pure culture, which is consistent with others studies [31,32]. In contrast, several

authors commented that the presence of apiculate yeasts in the starter did not affect ethyl esters production [29]. These different results may be due to yeast characteristics, medium composition and fermentation parameters, which could affect the production of esters. Ethyl lactate and ethyl propionate were the esters found in the highest quantities in the produced beers. Contrary to our results, ethyl lactate was detected in the traditional sorghum beer produced in Rwanda but not in sorghum beer fermented with a selected strain of *S. cerevisiae* [33]. This is consistent with studies that reported that the synthesis of secondary products by yeasts is an individual and reproducible strain characteristic [28]. Ethyl lactate, which has a mild, buttery and creamy odor with hints of fruit and coconut, can have a significant influence on the taste and character of sorghum beers. However, like other esters, it can to some extent be the basis of organoleptic defects.

Each group of volatile compounds (higher alcohols, acids and esters) was reduced to two main components (F1 and F2) by the PCA. These main components explained the total variation in the data, with the first component (F1) accounting for more than 97% of the variation. Among higher alcohols, the compounds that mainly contributed positively (F loading > 0.8) to F1 were 2-methylpropanol and 3-methylbutanol (Figure 3A). Ethyl lactate and isobutyric acid were the compounds that mainly contributed positively to F1 for esters and acids, respectively. As shown in Figure 3, the beer produced with the mixed culture was widely separated from the beer obtained with *S. cerevisiae* pure culture.

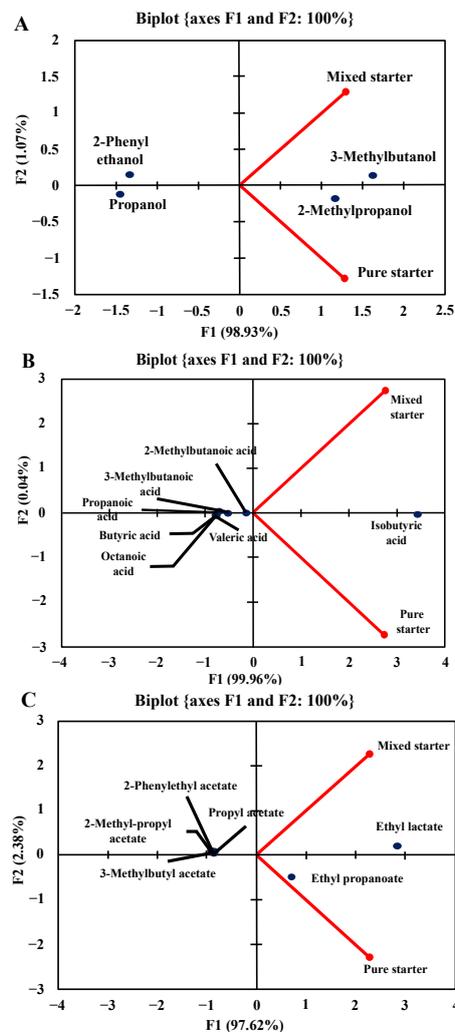


Figure 3. Principal component analysis loadings for (A) Higher alcohols, (B) Acids and (C) Esters of sorghum beers produced with freeze-dried cultures of *S. cerevisiae* F12-7 and *C. tropicalis* C0-7.

4. Conclusions

The sorghum beers produced from freeze-dried mixed and pure cultures of *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 showed ethanol concentrations within the range of traditional beers. But the mixed culture produced the highest content of organic acids, especially malic acid. Analysis of volatile compounds demonstrated that produced beers differed on their higher alcohol, acid and ester contents. The beers produced from pure culture of *S. cerevisiae* F12-7 were characterized by a relatively high concentration of esters, while those from the combination of *S. cerevisiae* and *C. tropicalis* were instead characterized by a high concentration of 2-phenylethanol, a higher alcohol that plays an important role in beer flavour. The mixed freeze-dried culture of *S. cerevisiae* F12-7 and *C. tropicalis* C0-7 is thus proposed as a promising starter for the production of sorghum beers and more studies should be conducted, especially examining the sensory qualities of the product and general acceptability by consumers compared to traditional beer.

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Conflicts of Interest: The authors declare no conflict of interest.

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