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Applicability of *Saccharomyces cerevisiae* Strains for the Production of Fruit Wines Using Cocoa Honey Complemented with Cocoa Pulp

Running title: Fruit wine made with cocoa honey and cocoa pulp

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SUMMARY

Research background. Cocoa honey (CH) and cocoa pulp (CP) are both fruit pulps highly appreciated but, until now, CH is less processed than CP. In this work, it was investigated the applicability of strains of *S. cerevisiae* to ferment CH complemented with CP, to obtain fruit wines and improve CH commercialization.

Experimental approach. The selection of a strain, previously isolated from *cachaçaria* distilleries in Brazil, took place based on its fermentation performance. The conditions for fermentation with *S. cerevisiae* L63 were then studied in relation to: volumetric proportion (φ_{CH}) of CH (complemented with CP), sucrose addition (γ_{suc}), temperature (T) and inoculum size (N_0). The best conditions were applied in order to obtain fermentation profiles.

Results and conclusions. S. cerevisiae L63 (N_0=10^7–10^8 cell/mL) is capable to ferment φ_{CH} of 90 and 80 % (*V/V*) for 24 or 48 h with γ_{suc} of 50 and 100 g/L at T=28–30 °C resulting in

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wines with ethanol contents from 8 to 14 % (*V/V*). Additionally, the φ_{CH}=90 % (*V/V*) wine resulted in the lowest residual sugar concentration (<35 g/L) than the φ_{CH}=80 % (*V/V*) wine (~79 g/L) which could be classified as a sweet wine. In general, *S. cerevisiae* L63 resulted in a similar fermentation performance than a commercial strain tested, indicating its potential for fruit pulp fermentation.

Novelty and scientific contribution. Therefore, *S. cerevisiae* L63 is capable to ferment CH complemented with CP to produce fruit wines with good commercial potentials that may also benefit small cocoa producers by presenting a product with greater added value.

Key words: alcoholic beverage, clarification, ethanol content, fruit pulp, Placket-Burman

INTRODUCTION

The information obtained through the recent investigation in relation to fermentation of different fruit pulps (*1-3*) is valuable since it allows a better understanding of the processes which is crucial to obtain products of higher quality and competitiveness. Furthermore, it can contribute to the reduction of post-harvest losses, which are common in tropical countries. A few examples of tropical fruits used to obtain fruit wines are: yellow mombin (*4*), pineapple (*5*), cashew (*6*), mango (*7*), guava (*8*) and carambola (*9*). Fermentation can be performed by different microorganisms; however, *Saccharomyces cerevisiae* has been the yeast most traditionally applied in fruit wine production due to its versatility (*10-12*).

Cocoa (*Theobroma cacao* L.) is known worldwide for its beans which are used in the production of chocolate and cocoa butter (*13*). Cocoa pulp (CP) is one of the by-products resulting from the processing of its fruit and is highly consumed in the preparation of juice, but it has also been proposed for the production of fruit wine (*14-16*). Another by-product of cocoa processing is a mucilagenous pulp called cocoa honey (CH), this pulp is obtained (in much smaller volume) after the initial pulping and it drips from the cocoa beans just before they start to ferment. Both CP and CH are rich in nutrients such as sugars, fibers and bioactive compounds (*17,18*). The pectin content in CH is higher than in CP, reaching ~2.5 % (*m/V*) (*19*). It is due to its pectin content, and also to its high perishability, that CH has more often been used for the production of artisanal jelly (*19,20*) and for fresh consumption. However, it is known that CH also has the potential to produce fruit wine, as observed previously by Leite *et al.* (*17*) and Magalhães-Guedes *et al.* (*21*). Consequently, the combined application of CH and CP can result in differentiated fruit wines.

Thus, in view of the importance of cacao cultivation for the southern region of Bahia (Brazil) and, in order to stimulate the use of CH to obtain products of greater added value, the present work proposed to select a strain of *S. cerevisiae* (previously isolated from different *cachaça* distilleries) and define the best conditions for the selected strain, *S. cerevisiae* L63, to ferment CH complemented with CP in order to obtain fruit wines.

MATERIALS AND METHODS

Materials

The cocoa honey (CH) used was donated by the company Du Kakau (Una, Bahia, Brazil) and, according to the manufacturer, it was obtained by cold pressing and filtration, with no added sugars and/or preservatives. The cocoa pulp (CP), produced by the company Sabiá (Ilhéus, Bahia, Brazil), was acquired at local business (Ilhéus, Bahia, Brazil). Both substrates were kept frozen until they were used. The citrus pectin was donated by the company Herbstreith & Fox (Germany). All other chemicals were purchased locally from reliable suppliers.

Microorganisms: maintenance, cultivation and inoculum preparation

The strains of *Saccharomyces cerevisiae* investigated in this research (L13, L37, L63 and L67), were previously isolated and identified by Silva *et al.* (*18*) and characterized according to pectinase activity by Carvalho *et al*. (*22*). They belong to the culture stock of the Applied Microbiology Laboratory (LABMA/UESC, Ilhéus, Bahia, Brazil) and they are preserved in a 15 % (*m/V*) glycerol solution at -80 °C. A lyophilized commercial yeast was also used for performance comparison: *S. cerevisiae* CA-11 (recommended for the production of *cachaça*, a Brazilian distilled spirit made from sugarcane) which was donated by the company LNF (Bento Gonçalves, Rio Grande do Sul, Brazil).

The concentrated cellular solutions were obtained by cultivating each strain in 25 mL of Sabouraud broth at 28 °C for 48 h (Solab, SL-200; Piracicaba, São Paulo, Brazil); after cultivation, 100 µL aliquots were spread over the surface of the same solid medium that was incubated once again at 28 °C for 24 h. Then, the cell growth from the surface of the Petri dishes was scraped with 2 mL aliquots of sterile distilled water. The collected volume had its total cell count determined in a Newbauer chamber; these values were used to obtain the initial concentration of cells (N_o, cell/mL), named “inoculum 1”, used for the preliminary tests and the study of fermentation conditions. For the activation and inoculum preparation of the commercial strain, 3 g of the lyophilized cells were used following the same procedure.

To promote adaptation to the fermentation must, when obtaining the fermentation profiles, “inoculum 2” was prepared with about 30 mL of “inoculum 1”, 50 mL of CH and 2.5 g of sucrose, with sterile distilled water added until reaching a volume of 100 mL. Cultivation occurred at 30 °C for 12 h (SPLabor, SP-222; Presidente Prudente, São Paulo, Brazil), without stirring, and then the cell mass was separated by centrifugation (15,000*g*/5 min) (Thermo scientific, 16R; Jundiaí, São Paulo, Brazil) and the supernatant was discarded. The cells were resuspended in 15 mL of sterile distilled water and the total cell count was determined as described above. The same procedure was followed for the strain CA-11.

Selection of the strain

The cultivation of the strains (L13, L37, L63 and L67) was carried out in 250 mL Erlenmeyers flasks containing 150 mL of culture medium. The performance of each strain during incubation was observed for 4–5 days in relation to cell count (*N*, cell/mL), soluble solids content (*SS*, °Brix), pH and flocculation (visual analysis); the alcohol content (φ_{etol}, % *V/V*) was only analyzed at the end of fermentation. Initially, the strains were cultivated in solutions of commercial pectin (20 g/L) and sucrose (100 g/L) in a phosphate buffer (0.5 M/pH 4.0) with N_o around $1{\cdot}10^7$–$2{\cdot}10^7$ cell/mL. Incubation was carried out at 30 °C and agitation of 150 rpm (SPLabor, SP-222; Presidente Prudente, São Paulo, Brazil). Then, the cultivation of strains L13 and L63 was evaluated in pure CH (φ_{CH}=100 % *V/V*) with initial conditions of pH 3.43 and 16.8 °Brix, under the same temperature and N_o, however, without sucrose and agitation.

Experimental design for fermentation conditions

The best conditions for fermentation of CH and CP by the yeast *S. cerevisiae* L63 were investigated applying the statistical tool of experimental design (*23*) with a Placket Burman matrix (PB8). Considering the volume of 200 mL of medium, the independent variables (factors) and their lowest (-1) and highest (+1) levels were: volumetric proportion of CH (added or not with CP) (φ_{CH}) from 80 to 100 % (*V/V*), sucrose concentration (γ_{suc}) from 0 to 100 g/L, temperature (*T*) from 28 to 32 °C and initial cell count (N_o) from $1{\cdot}10^6$ to $1{\cdot}10^8$ cell/mL. The dependent variables (responses) investigated over 72 h of fermentation were: ethanol content (φ_{etol}, % *V/V*), cell count (*N*, cell/mL), pH (*pH*), soluble solids content (*SS*, °Brix) and absorbance read at 600 nm (*A*). In order to use the same CH batch for all of the PB8 experiments it was not possible to perform the central condition (level zero) in triplicate but only in one replicate.

Statistical analysis

The responses obtained with the experimental design were applied to the Effects Analysis (*23*) performed at 85 % of confidence using the statistical software Protimiza Experimental Design (*24*). In this analysis, the individual effects of each factor were considered statistically significant with $p<0.15$ which indicates that, by increasing the factor from its lowest level to its highest level it is possible to obtain a statistically significant alteration (positive or negative) in the average response.

Fermentative profiles

The profiles for concentration of sugar, organic acid and alcohol (γ) in addition to *N*, *SS*, pH and *A* were obtained for fermentations (200 mL) performed at 28 °C and $N_o=6.5\cdot10^7$ cell/mL, with samples being collected between 3 and 48 h. For both values of $\varphi_{CH}=90$ and 80 % (*V/V)* fermentations were carried out with $\gamma_{suc}=50$ g/L for 24 h and with $\gamma_{suc}=100$ g/L for 48 h. For comparison, the commercial yeast strain was used under the same conditions. CH, CP and the volumetric proportions of both (without the addition of sucrose) were analyzed by chromatography for sugar, organic acid and alcohol concentrations. Due to batch limitation in CH and CP it was only possible to perform one replicate in the analysis.

Physicochemical analysis

The soluble solids were determined with a digital refractometer (Akso, RHB90 / São Leopoldo, Rio Grande do Sul, Brazil) on a °Brix scale (*SS*, % *m/V*) and pH was determined with a pH meter (Bel, PHS3BW / Rio de Janeiro, Rio de Janeiro, Brazil). The ethanol content (φ_{etol}, % *V/V*) was determined with a refractometer (TEKCOPLUS, RETK-75; Hong Kong, China). To assess the clarification of the fermented must, the absorbance (*A*) was read at 600 nm in a spectrophotometer (Bel, SP 2000 UV; Rio de Janeiro, Rio de Janeiro, Brazil).

Concentrations (γ, g/L) of sucrose, glucose, fructose, ethanol, methanol, glycerol and citric, lactic and acetic acids, were determined in a system for high-performance liquid chromatography (HPLC, Hitachi Primaide; Tokyo, Japan) with an ion exchange column (Bio-Rad, Aminex HPX-87H, 300 *x* 7.8 mm; Hercules, California, USA) and an infrared detector (Knauer 98.00; Berlin, Germany). The samples were diluted 20 times in ultrapure water and filtered in a PVDF membrane filter (0.45 µm) with an injection volume of 20 µL. For the analytical run, an isocratic system was used with the mobile phase of H_2SO_4 (0.005 N) and a flow rate of 0.6 mL/min at room temperature (~26 °C) and a 20 min running time. The acquisition and integration of the peaks was performed using the Star Chromatography

Workstation v. 6.0 (Varian, California, USA) software based on standard curves previously prepared with chromatographic standards (Sigma-Aldrich, USA).

RESULTS AND DISCUSSION

Strain selection

The fermentative performance of the four strains of *S. cerevisiae* (L13, L37, L63 and L67), in a medium containing pectin and sucrose (to mimic concentration in cocoa honey *in natura*) was evaluated over 98 h and, in general, the pH remained stable, varying between 3.88–3.97 at *t*=0 h and between 3.68–3.82 at *t*=98 h. These results are in line with what has been reported by other researchers, for example, with fermentation of pear and kiwi pulps by *S. cerevisiae* WLS2 (*25*). The strains L13 and L63 showed the greatest *SS* reductions throughout cultivation and L67 resulted in the lowest reduction. The cell count (*N*) remained in the range of 10^7 cell/mL, and a stronger flocculation with the strains L37 and L67 was observed, indicating that flocculation was induced by agitation for these two strains. Agitation is one of the factors that favors cell-to-cell adhesion and promotes flocculation (*26*). Although flocculation is appreciated in the industrial production of *cachaça* (since it facilitates the separation of cells at the end of the process, without the need for centrifugation or filtration), in this preliminary tests, flocculation was observed to be detrimental to the performance of fermentations (especially for φ_{etol}). Strains L13 and L63 showed more stable profiles for *N* and *SS* (probably due to their reduced flocculation) and, for this reason, these two strains were selected to continue the study. In sequence, static fermentations were carried out for 93 h with CH and the ethanol contents (φ_{etol}) obtained at the end of fermentation were similar: L13=8.78 % (*V/V*) and L63=8.67 % (*V/V*). Under these conditions it was observed (for both strains) that the maximum *N* were around $1 \cdot 10^8$ cell/mL (between 69 and 72 h), in addition, pH showed little variation around 3.5 and the greatest reductions (~65 %) in *SS* values were observed around 42 h.

Based on the previous study by Carvalho *et al.* (*22*) in which the highest production of pectinases by the strain L63 strain was reported (up to 3.5 times more pectinases than the strain L13, for example), the strain L63 was chosen for CH fermentation in order to favor clarification due to the hydrolysis of pectin. For the simultaneous fermentation and clarification of cocoa pulp (CP), for example, commercial pectinases and cellulases were added together with the strain CA-11 in the work of Duarte *et al.* (*16*). Dias *et al.* (*14*) also used pectinases as a pretreatment of CP for fermentation. To exemplify the clarification of the medium, Fig. S1 shows a comparison of CH before (Fig. S1a) and after fermentation (Fig. S1b) by the strain

L63 (Fig. S1c). The obtained results are promising, however, it is necessary to emphasize that the strains L13, L37 and L67 also have good potential for fermentation of fruit pulps and should be better investigated in subsequent studies.

Study of the best conditions for fermentation

Fermentations were carried out and the obtained responses: φ_{etol} and *N*, were considered the main responses to be analyzed and *pH*, *SS* and *A* were analyzed as complementary responses at *t*=0 h and *t*=72 h. From the results presented in Table 1 it is possible to observe that the highest φ_{etol} obtained corresponded to run 2 (72 h) and run 6 (48 and 72 h), however, it is also important to observe the φ_{etol} value of run 9 which was obtained in a shorter time (24 h) and, consequently, represents a higher productivity. These alcohol contents are in accordance with the range stipulated for fruit wines in Brazil: 4–14 % (*V/V*) (*27*). For the response *N*, it is possible to observe that, regardless of N_o, its value did not exceed the factor of 10^8 (Table 1) which was also confirmed by Dias *et al.* (*14*). Thus, a range of N_o=10^7–10^8 cell/mL is suggested considering possible difficulties in larger scales in respect to preparation of the inoculum solution and cell count. A similar inoculum ($1{\cdot}10^8$ cell/mL) was used for fermentation of CP by Duarte *et al.* (*16*). In relation to the response *pH*, small variations were once again observed during fermentation (Table 1). The clarification of the musts can also be proven out due to the reduction of the responses *SS* and *A* throughout fermentation time; the greatest reductions (> 70 %) of *SS* were obtained in runs 5 and 9 and, for more than half of the runs performed, *A* was reduced in more than 95 % (Table 1). The clarification associated with fermentation can be understood, for example, when related to the consumption of sugar, due to cell growth, and/or the enzymatic hydrolysis of pectin.

Insert Table 1

Considering the Analysis of Effects (Table 2) for the response *N*, the only factor with a positive and statistically significant effect (p<0.15) at all times was N_o and, only at 24 h, it was φ_{CH}. For φ_{etol}, N_o was also statistically significant and positive for the first part of fermentation (24 and 48 h), with its greatest effect in the first 24 h. The concentration of sucrose (γ_{suc}) also presented positive and statistically significant effects for the second half of fermentation (48 and 72 h). In general, this analysis suggest that *N* can be increased in the first 24-48 h hours of fermentation with the increase of γ_{suc}, N_o and φ_{CH}, as demonstrated in the obtained responses in Table 1. Additionally, in order to increase φ_{etol}, fermentation can be extended up to 48 h with a higher γ_{suc} value. Obtaining a fermented product with higher ethanol content is

interesting as it can be used, for example, to obtain a distilled spirit, denominated in Brazil as *aguardente de fruta* (*27*).

Insert Table 2

Considering the best φ_{etol} values (Table 1), run 9 also presented (within 24 h) *pH*, *SS* and *A* values of: 3.46, 5.4 °Brix and 0.078, respectively, and run 6 (within 48 h): 3.62, 7.0 °Brix and 0.088, respectively. Between these two fermentations, run 9 was carried out with a higher φ_{CH} and lower γ_{suc} than run 6. The results also indicate that increasing the fermentation time up to 72 h was not advantageous for φ_{etol} since the observed increments did not justify extending the fermentation time to more than 48 h. Additionally, in account to runs 6 and 9, both were performed with initial *SS* values around 19 °Brix which is close to the condition performed by Leite *et al.* (*17*) with CH. For fermentation of other fruit pulps with *S. cerevisiae,* values from 16 to 20 °Brix have been reported (*1,28*).

In general, the results obtained are in accordance with the CH and CP fermentations carried out by other researchers. The fermentation of CH by a commercial strain (*S. cerevisiae* AWRI726), for example, was also evaluated by experimental design by Magalhães-Guedes *et al.* (*21*), considering the factors: temperature, soluble solids and fermentation time. In this example, the authors obtained the highest alcohol content (15 % *V/V*) under the conditions of 25 °C, 13 °Brix (with sucrose addition) and a longer fermentation time (96 h), furthermore, an increase in temperature to 28 °C and in time to 112 h indicated to be favorable for fermentation. In a complementary study, this same research group obtained an alcohol content of 16 % (*V/V*) when fermenting CH with the same strain but at 20 °C and 144 h (*17*).

In another example, fermentation of CP by *S. cerevisiae* UFLA CA 1162 resulted in an alcohol content of about 8 % (*V/V*) and fermentation was carried out for 30 days at 22 °C followed by a period of wine maturation (*16*). Also, CP fermentation by *S. cerevisiae* CA1183 resulted in about 11 % (*V/V*) of ethanol and pH=3.7, after 64 h at 25 °C with an additional period of maturation (*14*). An alcohol content of approximately 10 % (*V/V*) and pH=3.6 was obtained when using 80 % (*V/V*) of CP (previously clarified with pectinases) complemented with *Hibiscus sabdariffa* L. extract fermented at 30 °C for 168 h by *S. cerevisiae* var. *bayanus* (*15*).

As temperature (*T*) was not statistically significant ($p>0.15$) in the range analyzed for the two responses (Table 2), the lowest value (28 °C) was chosen, however, based on the preliminary tests and other studies of literature with *S. cerevisiae* (*6,7,15,28*), the temperature range of 28–30 °C can be suggested as well. As the increase in φ_{CH} up to 100 % (*V/V*) indicated

to slow down *N*, but had no effect over φ_{etol}, both φ_{CH} (90 and 80 % *V/V*) were chosen due to the possibility of achieving a differentiate sensorial profile for fruit wines. Additionally, considering the time of 24 h as eligible for obtaining a fruit wine, γ_{suc}=50 g/L was selected; considering 48 h, in order to obtain higher alcohol contents (for distillation, for example), γ_{suc}=100 g/L was selected.

Fermentation profiles and composition of fruit wines

Fig. 1 presents the concentrations of sugar and citric acid in CH and CP and, according to the results, CH presented a higher concentration of sucrose (27.75 g/L), and CP had higher concentrations of citric acid (γ_{cit}=8.17 g/L), fructose (γ_{fru}=64.12 g/L) and glucose (γ_{glu}=64.28 g/L). Thus, the two selected φ_{CH} for the fermentation musts can be easily understood as a reflection of their individual compositions (Fig. 1).

Insert Fig. 1

Fermentations conducted for 24 h (Fig. 2) and 48 h (Fig. 3) with both φ_{CH} were investigated in relation to the concentration of sugars, alcohols and acids (Fig. 2a, 2c, 3a and 3c) and in relation to *N*, *SS*, *pH* and *A* (Fig. 2b, 2d, 3b and 3d). For these fermentations, it was not possible to obtain N_o = $1 \cdot 10^8$ cell/mL as desired, however, the actual value employed ($6.5 \cdot 10^7$ cell/mL) was within the suggested range for inoculum concentration. In general, the fermentative profiles obtained indicated an increase in φ_{etol} and *N* associated with the consumption of sugars and simultaneous clarification, which is in agreement with other studies (*16*,*17*). Table 3 also shows the compilation of the final values presented by the L63 fermented fruit wines in comparison with the commercial strain for both φ_{CH} (90 and 80 % *V/V*) and times (24 and 48 h) evaluated.

In regard to the fermentations performed for 24 h with γ_{suc}=50 g/L, it was observed (Fig. 2a and 2c) that for both φ_{CH} there was a marked decrease in γ_{suc} in the first 12 h of fermentation and lower reductions in glucose (γ_{glu}) and fructose (γ_{fru}) after 6 h of fermentation. It is also possible to infer the preferential consumption of glucose by *S. cerevisiae* L63, since γ_{fru} remained higher than γ_{glu} until the end of fermentation. In the CH fermentation evaluated by Leite *et al.* (*17*), for example, the total sugar content was reduced from 186.78 g/L to 5.02 g/L after 240 h of fermentation with a residual fructose content of 4.21 g/L. In this present work, the φ_{CH}=90 % (*V/V*) must result in a total reduction of 54 % in *SS*, a better result than with φ_{CH}=80 % (*V/V*), whose total reduction was 38 %. In addition, the greatest reduction (91 %) in *A* occurred in the first 3 h of fermentation with φ_{CH}=80 % (*V/V*) but the lowest *A* value was

achieved with φ_{CH}=90 % (*V/V*) (greatest clarification) at the end of 24 h (Fig. 2b and 2d). Considering the variability, the increase in *N* once again indicated to be more favorable with higher proportion of CH than CP. Also, the *pH* and citric acid (γ_{cit}) profiles presented little variation over time (Fig. 2b and 2d) and methanol, acetic acid and lactic acid were not identified in any of the fermentations.

Insert Fig. 2

Still considering the 24 h of fermentation, the φ_{CH}=90 % (*V/V*) wine (Table 3) presented a higher alcohol content but the φ_{CH}=80 % (*V/V*) wine resulted in a higher concentration of residual sugar (79.06 g/L) which is in accordance with the classification of “sweet wine” based on the Brazilian legislation (*27*). Regarding the fruit wine fermented with the commercial strain for 24 h, compositions were similar, especially in relation to φ_{etol}, however, higher levels of residual sugar (> 88 g/L) were obtained (Table 3).

Insert Table 3

For the fermentations conducted for 48 h with γ_{suc}=100 g/L, it was possible to observe similar trends. In these cases, sucrose was almost entirely consumed around 24 h and the profiles of γ_{glu} and γ_{fru} showed a decay from 12–15 h (Fig. 3a and 3c). The φ_{CH}=90 % (*V/V*) wine also presented a reduced γ_{glu} at the end of fermentation (Table 3). For both fruit wines with 48 h of fermentation, the γ_{etol} profiles were increased with time (Fig. 3a and 3c) and the final values of φ_{etol} (Table 3) were similar. Once again, the φ_{CH}=80 % (*V/V*) wine resulted in a higher concentration of residual sugar (79.83 g/L). The *SS* profiles (Fig. 3b and 3d) indicate a significant increase in *SS* values in the first 12 h of fermentation (as a reflex of the sugar profiles) followed by a constant decay, representing 65–67 % of reduction until the end of fermentation. Clarification was also achieved since the *A* profiles showed reductions between 94–96 % at the end of fermentation (Fig. 3b and 3d). In comparison with the fruit wine fermented for 48 h using the commercial strain CA-11 (Table 3), it is also possible to observe similar levels of alcohol but much lower values of residual sugars (< 11 g/L).

Insert Fig. 3

The citric acid (γ_{cit}) profiles showed increases throughout the fermentations times of 24 h (Fig. 2a and 2c) and 48 h (Fig. 3a and 3c); for 24 h the increments were between 15–25 % and for 48 h, larger increments (87–94 %) were observed. In general, lactic acid, acetic acid and methanol were not detected, except for the small concentrations of acetic acid in the φ_{CH}=80 % (*V/V*) wine with the strain L63, and lactic acid in the φ_{CH}=90 % (*V/V*) wine with the strain CA-11 and 48 h of fermentation (Table 3). The organic acids naturally found in fruit wines are fundamental to the unique sensory quality of each beverage (*16*). These acids can come

from the fruit pulps used or they can be produced during fermentation; citric acid is an example that fits these two situations and its presence is generally valued for sensory aspects. However, the presence of lactic and/or acetic acid, for example, can indicate contamination of the fermentation process and is a good indicator of quality (*29*) or it can be detected as a result of fermentation. For example, the CP wine obtained by Dias *et al.* (*14*) presented contents of 5.5 g/L of citric acid, 1.1 g/L of acetic acid and an absence of lactic acid. Longer fermentations of fruit pulps generally require the addition of sulfur dioxide in the form of potassium metabisulfite (up to 100–200 mg/L of free SO_2) to inhibit bacterial growth (*14,16,17*); this was not performed in the present study but it is also recommended for scaled up fermentations.

For fruit wine production, the presence of glycerol also contributes to sensory characteristics such as sweetness and fullness, as mentioned by Dias *et al.* (*14*) who obtained glycerol concentrations of 5.53–9.0 g/L with a CP wine. In the present study, the glycerol profiles were increased with time (Fig. 2a, 2c, 3a and 3c) and the final concentrations obtained for the wines fermented for 48 h (Table 3) are within the range declared by Gamella *et al.* (*30*) for dry wines (4–10 g/L); the wines fermented for 24 h had lower levels of glycerol (Table 3). Interestingly, different *Saccharomyces* strains have been investigated in order to obtain wines with low-ethanol content, higher smoothness and a greater capacity to produce glycerol (*31*).

CONCLUSIONS

From the data presented, it can be concluded that *S. cerevisiae* L63 demonstrated not only good fermentative performance in musts composed of cocoa honey and cocoa pulp, but also a good ability to promote clarification during fermentation, thus resulting in fruit wines with a crystalline aspect and basic chemical composition in accordance with the current Brazilian legislation. The results obtained also confirm the versatility and the potential when using a volumetric proportion between cocoa honey and cocoa pulp. It is important to carry out future studies (different strains, sensorial analysis, *etc*.) to deepen the knowledge about the use of these pulps to obtain alcoholic beverages, such as fruit wines as well as distilled spirits. Nonconventional fruit pulps, as substrate for fermentation and distillation, can definitely contribute to obtain promising beverages by adding valuable sensorial aspects to the final product when in comparison to sugarcane or apple or grape, for example.

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CONFLICT OF INTEREST

There are no conflicts of interest.

SUPPLEMENTARY MATERIAL

All supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

BTAK: data collection, data analysis and interpretation, performing the analysis and drafting the article. SMMS: data collection, data analysis and interpretation, performing the analysis and drafting the article. AMC: data analysis and interpretation, drafting the article and final approval of the version to be published. EAO: conception or design of the work, data analysis and interpretation, drafting the article and final approval of the version to be published.

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Table 1. Coded Placket Burman matrix (PB8) for the study of fermentation and clarification of cocoa honey (CH) and cocoa pulp (CP) by *S. cerevisiae* L63 with the factors: volumetric proportion of cocoa honey φ_{CH} (% *V/V*) (total 200 mL), sucrose concentration (γ_{suc}, g/L), temperature (*T*, °C) and inoculum (N_o, cell/mL) and the responses: alcohol content (φ_{etol}, % *V/V*), cell count (*N*, cell/mL), pH (*pH*), soluble solids content (*SS*, °Brix) and absorbance at 600 nm (*A*). Real values of factors are presented in parentheses.

	Factors				Responses											
					φ_{etol} (%, *V/V*)			*N* (cell/mL)			pH		*SS* (°Brix)		*A*	
Runs	φ_{CH} (% *V/V*)	γ_{suc} (g/L)	*T* (°C)	N_o (cell/mL)	24 h	48 h	72 h	24 h	48 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
1	+1 (100)	-1 (0)	-1 (28)	+1 (10^8)	8.30	9.00	9.10	$3.0{\cdot}10^8$	$2.3{\cdot}10^8$	$2.5{\cdot}10^8$	3.73	3.40	14.4	4.8	1.165	0.084
2	+1 (100)	+1 (100)	-1 (28)	-1 (10^6)	3.70	9.60	13.60	$1.0{\cdot}10^8$	$1.6{\cdot}10^8$	$1.2{\cdot}10^8$	3.73	3.38	23.3	8.6	0.957	0.081
3	+1 (100)	+1 (100)	+1 (36)	-1 (10^6)	5.00	10.20	12.60	$5.7{\cdot}10^7$	$8.6{\cdot}10^7$	$1.1{\cdot}10^8$	3.71	3.44	22.9	9.2	1.040	0.113
4	-1 (80)	+1 (100)	+1 (36)	+1 (10^8)	4.90	9.80	12.30	$3.3{\cdot}10^7$	$6.8{\cdot}10^7$	$5.7{\cdot}10^7$	3.53	3.57	23.2	8.9	1.786	0.082
5	+1 (100)	-1 (0)	+1 (36)	+1 (10^8)	8.90	9.00	9.10	$1.9{\cdot}10^8$	$1.8{\cdot}10^8$	$1.7{\cdot}10^8$	3.63	3.50	15.6	4.0	1.165	0.092
6	-1 (80)	+1 (100)	-1 (28)	+1 (10^8)	9.50	13.50	14.00	$1.1{\cdot}10^8$	$1.8{\cdot}10^8$	$1.6{\cdot}10^8$	3.73	3.60	19.8	6.2	1.786	0.073
7	-1 (80)	-1 (0)	+1 (36)	-1 (10^6)	1.80	6.70	8.50	$1.2{\cdot}10^7$	$3.6{\cdot}10^7$	$1.6{\cdot}10^7$	3.59	3.72	15.0	5.0	2.154	0.080
8	-1 (80)	-1 (0)	-1 (28)	-1 (10^6)	0.00	0.50	6.50	$1.8{\cdot}10^6$	$1.3{\cdot}10^7$	$7.0{\cdot}10^7$	3.54	3.66	15.1	7.2	2.150	0.066
9	0 (90)	0 (50)	0 (32)	0 (10^7)	10.50	11.10	11.20	$1.9{\cdot}10^8$	$1.9{\cdot}10^8$	$2.2{\cdot}10^8$	3.60	3.71	19.2	5.2	1.370	0.068

Table 2. Effect Analysis of the factors: volumetric proportion of cocoa honey φ_{CH} (% *V/V*), sucrose concentration (γ_{suc}, g/L), temperature (*T*, °C) and innoculum (N_o, cell/mL) for the responses: alcohol content (φ_{etol}, % *V/V*) and cell count (*N*, cell/mL)

Response: φ_{etol}		24 h				48 h				72 h		
Term	Effect	s.d.	*t*(4)	*p*	Effect	s.d.	*t*(4)	*p*	Effect	s.d.	*t*(4)	*p*-value
Mean	4.79	0.90	5.35	0.0059 *	8.82	0.93	9.52	0.0007 *	10.77	0.34	31.92	<0.0001 *
φ_{CH}	2.43	1.90	1.28	0.2710	1.83	1.96	0.93	0.4055	0.78	0.72	1.08	0.3396
γ_{suc}	1.03	1.90	0.54	0.6183	4.48	1.96	2.28	0.0850 *	4.83	0.72	6.74	0.0025 *
T	-0.23	1.90	-0.12	0.9115	0.78	1.96	0.39	0.7134	-0.18	0.72	-0.24	0.8188
N_o	5.28	1.90	2.78	0.0500 *	3.58	1.96	1.82	0.1429 *	0.83	0.72	1.15	0.3131
Response: *N*		24 h				48 h				72 h		
Term	Effect	s.d.	*t*(4)	*p*	Effect	s.d.	*t*(4)	*p*	Effect	s.d.	*t*(4)	*p*-value
Mean	$9.9\cdot10^7$	$2.2\cdot10^7$	4.44	0.0113 *	$1.0\cdot10^8$	$2.2\cdot10^7$	4.85	0.0083 *	$1.1\cdot10^8$	$2.7\cdot10^7$	4.38	0.0119 *
φ_{CH}	$9.7\cdot10^7$	$4.7\cdot10^7$	2.06	0.1085 *	$5.0\cdot10^7$	$4.7\cdot10^7$	1.05	0.3534	$5.7\cdot10^7$	$5.6\cdot10^7$	1.01	0.3714
γ_{suc}	$-7.5\cdot10^7$	$4.7\cdot10^7$	-1.59	0.1861	$-3.0\cdot10^7$	$4,7\cdot10^7$	-0.64	0.5539	$-4.4\cdot10^7$	$5.6\cdot10^7$	-0.78	0.4770
T	$-3.0\cdot10^7$	$4.7\cdot10^7$	-0.64	0.5594	$-1.3\cdot10^7$	$4.7\cdot10^7$	-0.28	0.7894	$-3.2\cdot10^7$	$5.6\cdot10^7$	-0.56	0.6022
N_o	$1.4\cdot10^8$	$4.7\cdot10^7$	2.95	0.0418 *	$1.3\cdot10^8$	$4.7\cdot10^7$	2.73	0.0526 *	$1.0\cdot10^8$	$5.6\cdot10^7$	1.94	0.1246 *

*statistically significant effects with 85 % of confidence ($p < 0.15$)

s.d. = standard deviation

Table 3. Final compositions in 200 mL of fruit wines obtained from 90 and 80 % (*V/V*) of cocoa honey complemented with cocoa pulp fermented by *S. cerevisiae* strains L63 and CA-11 at 28 °C for 24 h (with 50 g/L of sucrose) and for 48 h (with 100 g/L of sucrose).

	S. cerevisiae L63				*S. cerevisiae* CA-11			
	90 %	80 %	90 %	80 %	90 %	80 %	90 %	80 %
Composition	24 h		48 h		24 h		48 h	
Ethanol (%, *V/V*)	10.20	8.20	14.40	14.60	10.70	8.10	15.90	14.50
pH	3.89	3.85	3.69	3.98	3.85	3.90	3.74	3.76
Soluble solids (Brix°)	8.7	11.8	8.7	10.9	13.0	12.8	8.4	7.1
Absorbance (at 600 nm)	0.161	0.218	0.110	0.105	0.139	0.076	0.096	0.066
Sucrose (g/L)	0.58	n.d.	0.74	1.01	1.24	1.56	0.88	n.d.
Fructose (g/L)	27.67	54.90	23.68	63.74	65.23	62.34	9.31	8.48
Glucose (g/L)	4.60	24.16	2.39	15.08	22.36	34.23	0.90	0.78
Acetic acid (g/L)	n.d.	n.d.	n.d.	0.97	n.d.	n.d.	n.d.	n.d.
Citric acid (g/L)	5.66	6.86	6.63	8.04	6.0	5.14	6.52	6.0
Lactic acid (g/L)	0.21	n.d.	n.d.	n.d.	n.d.	n.d.	1.00	n.d.
Methanol (g/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycerol (g/L)	3.70	2.72	4.88	4.77	5.14	3.25	6.36	4.81

n.d. = not detected

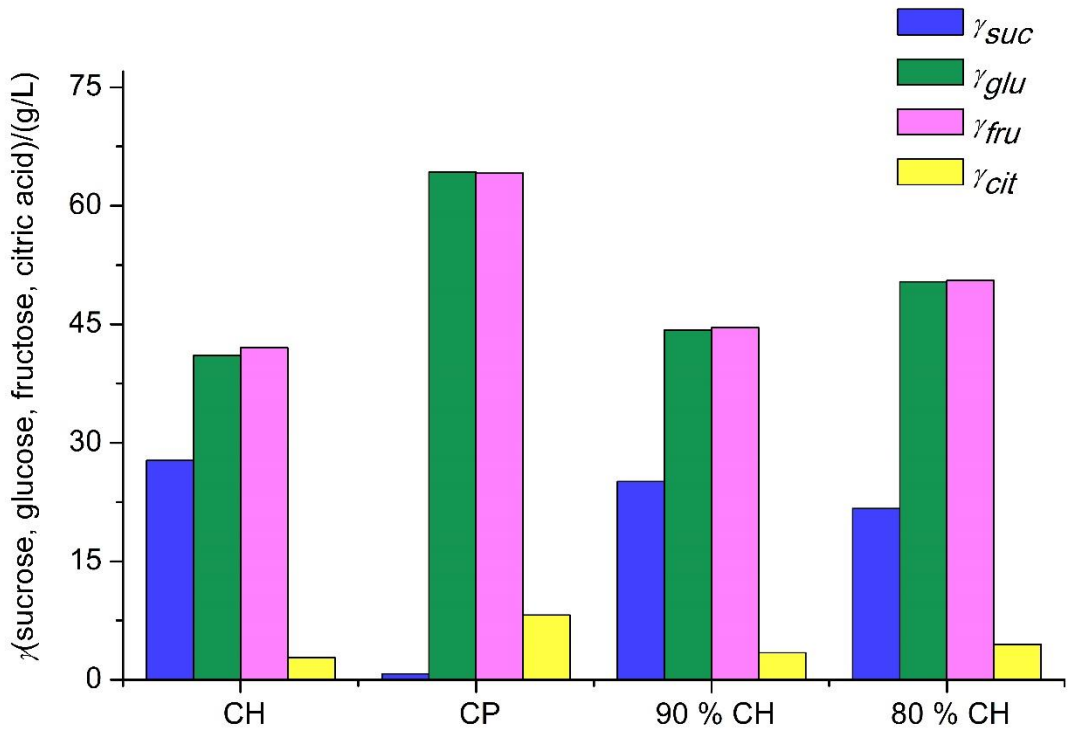

Fig. 1. Concentrations (g/L) of sucrose (γ_{suc}), glucose (γ_{glu}), fructose (γ_{fru}) and citric acid (γ_{cit}) for cocoa honey (CH), cocoa pulp (CP) and the volumetric proportions of 90 % and 80 % (*V/V*) of CH added of CP.

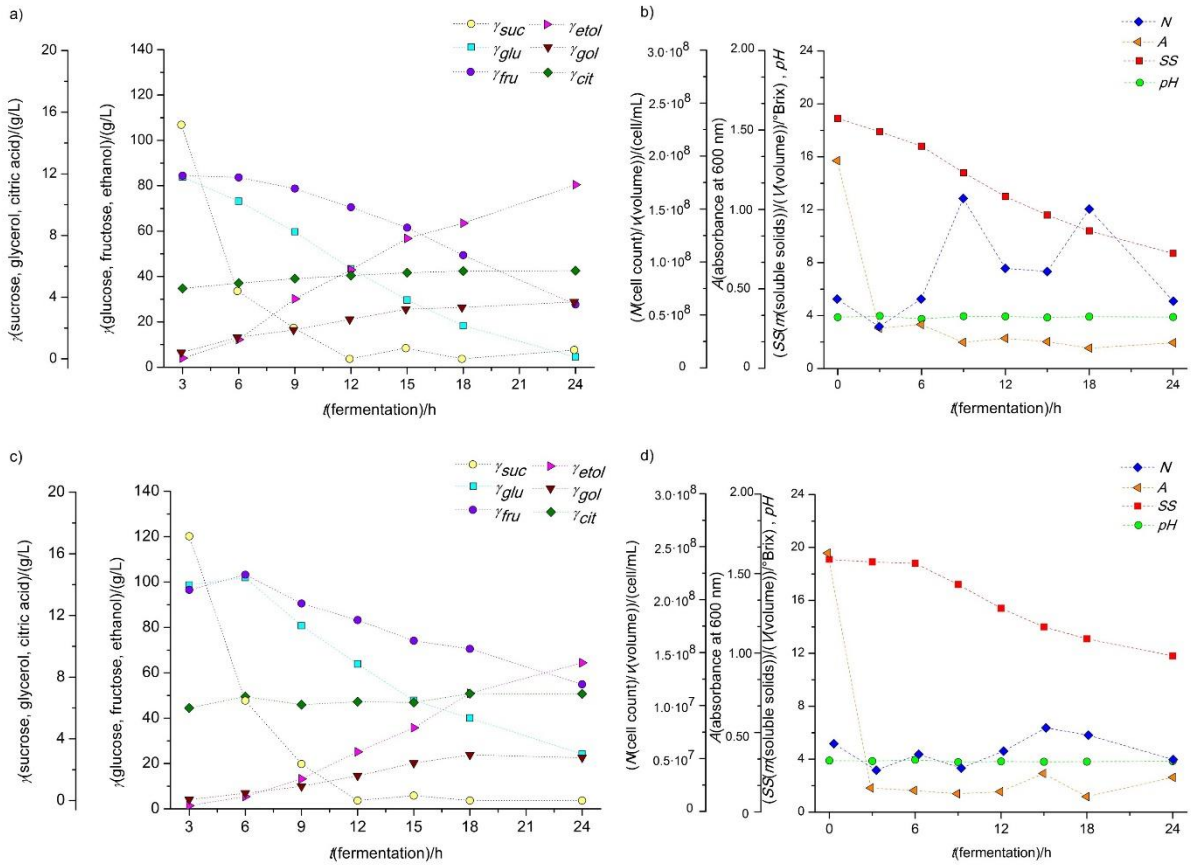

Fig. 2. Fermentative profiles of *S. cerevisiae* L63 in cocoa honey at a) and b) 90 % (*V/V*) and c) and d) 80 % (*V/V*) added of cocoa pulp, for 24 h and with 50 g/L of sucrose. Concentrations (g/L) of sucrose (γ_{suc}), glucose (γ_{glu}), fructose (γ_{fru}), glycerol (γ_{gol}), citric acid (γ_{cit}) and ethanol (γ_{etol}) are presented in a) and c). Cell count (*N*, cell/mL), absorbance (*A*), soluble solids (*SS*, °Brix) and pH (*pH*) are presented in b) and d).

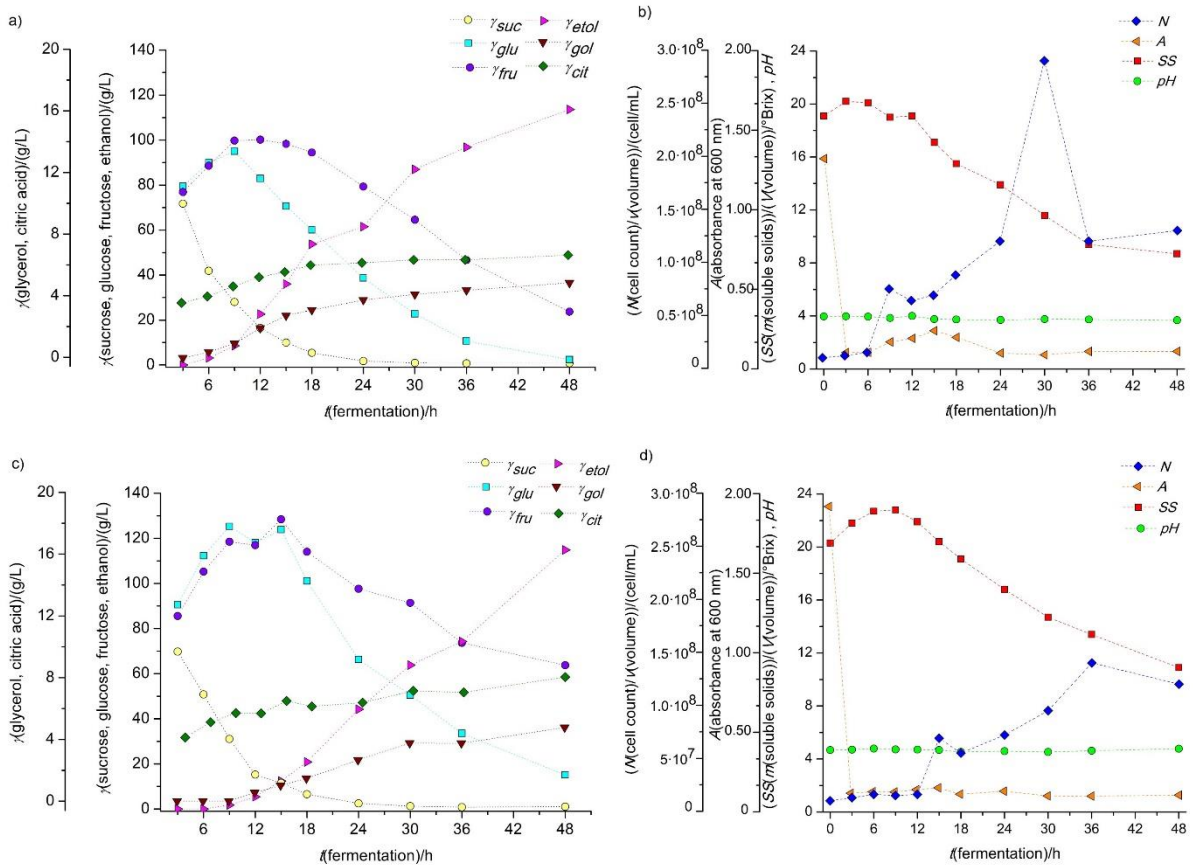

Fig. 3. Fermentative profiles of *S. cerevisiae* L63 in cocoa honey at a) and b) 90 % (*V/V*) and c) and d) 80 % (*V/V*) added of cocoa pulp, for 48 h and with 100 g/L of sucrose. Concentrations (g/L) of sucrose (γ_{suc}), glucose (γ_{glu}), fructose (γ_{fru}), glycerol (γ_{gol}), citric acid (γ_{cit}) and ethanol (γ_{etol}) are presented in a) and c). Cell count (*N*, cell/mL), absorbance (*A*), soluble solids (*SS*, °Brix) and pH (*pH*) are presented in b) and d).

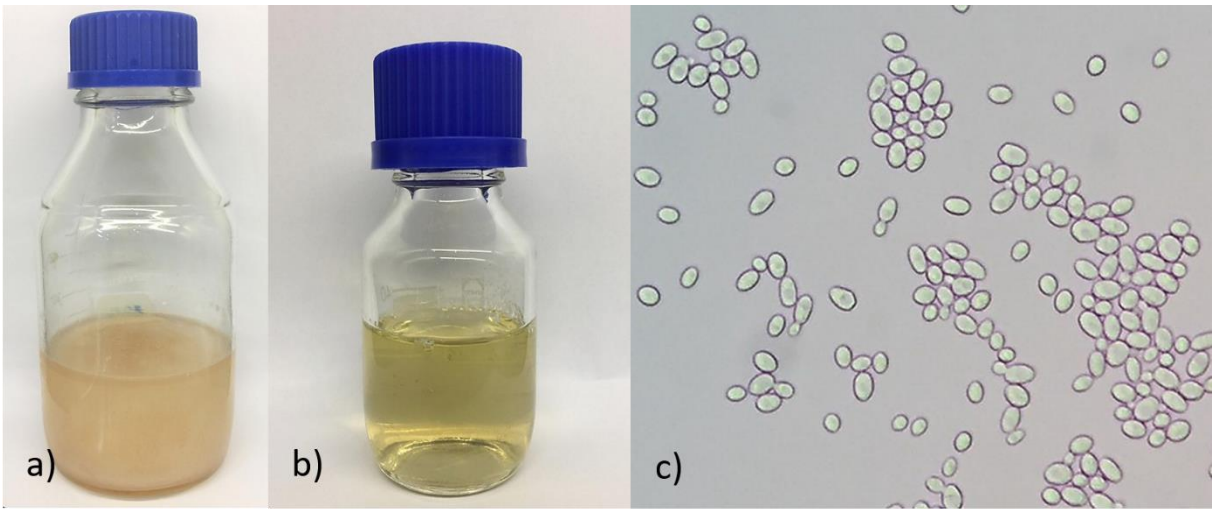

Fig. S1. Demonstration of cocoa honey a) before (absorbance at 600 nm = 1.165) and b) after (absorbance at 600 nm = 0.084) fermentation by c) *S. cerevisiae* L63, observed under a microscope at 400x magnification.