



# Dynamic behavior of *Saccharomyces cerevisiae*, *Pichia kluyveri* and *Hanseniaspora uvarum* during spontaneous and inoculated cocoa fermentations and their effect on sensory characteristics of chocolate

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## ABSTRACT

The dynamic of *Saccharomyces cerevisiae*, *Pichia kluyveri* and *Hanseniaspora uvarum* during spontaneous and inoculated cocoa fermentations and their effect on sensory characteristics of chocolate were investigated. Yeast populations were assessed by qPCR. *S. cerevisiae* was predominant during spontaneous (average 5.4 log cell/g) and inoculated (average 7.2 log cell/g) fermentations. The *H. uvarum* seemed to be suppressed by the other two yeasts, as it showed similar population (approximately 4.0 log cell/g) even in the inoculated assay. Carbohydrates were consumed quickly at inoculated fermentation (68% and 42% were consumed in the inoculated and control assays respectively, at 24 h). Ethanol content was higher in the inoculated (8.3 g/kg at 48 h) than in the control (4.6 g/kg at 96 h) fermentation. Consumers did not report a significant preference for either chocolate ( $p < 0.5$ ). However, differences in the flavor attributes were noted, as consumers reported stronger coffee and sour attributes in the inoculated assay. This is the first time qPCR has been used to assess the dynamic of yeasts during the complex fermentation of cocoa beans. The inoculation accelerated the process. *S. cerevisiae* and *P. kluyveri* likely contributed coffee, sour and bitter flavors to the inoculated chocolate.

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## 1. Introduction

Cocoa beans (*Theobroma cacao* L.) are the principal raw material of chocolate manufacture. Fermentation of the beans is essential for removing the pulp that envelops the beans and developing precursors of chocolate flavor (Fowler, 2009). The cocoa fermentation consists of well-defined microbial succession that is initially dominated by yeasts and subsequently surpassed by lactic acid bacteria (LAB), acetic acid bacteria (AAB) and then aerobic spore forming bacilli (Ardhana & Fleet, 2003; Garcia-Armisen et al., 2010; Lima, Almeida, Nout, & Zwietering, 2011; Schwan & Wheals, 2004). According to Ho, Zhao, and Fleet (2014), the yeast growth and activity are essential for cocoa bean fermentation and the development of chocolate characteristics. The yeast metabolism results in ethanol production, which will be converted to acetic acid by AAB,

causing an increase in temperature during the fermentation, and a very strong vinegar-like aroma (Schwan & Wheals, 2004). *Saccharomyces*, *Hanseniaspora* (anamorph *Kloeckera*) and *Pichia* have been reported as the predominant genera found during cocoa fermentations (Jespersen, Nielsen, Hønholt, & Jakobsen, 2005; Moreira, Miguel, Duarte, Dias, & Schwan, 2013; Nielsen, Hønholt, Tano-Debrah, & Jespersen, 2005; Nielsen et al., 2007). The use of starter culture for better control of the fermentation process in order to improve the quality of the fermented cocoa have been proposed by some researchers (Crafack et al., 2013; Leal Jr. et al., 2008; Lefeber, Papalexandratou, Gobert, Camu, & De Vuyst, 2012; Schwan, 1998). In order to observe the dynamic of starter culture during fermentations, plating or denaturing gradient gel electrophoresis (DGGE) techniques have been used for evaluation of starter culture species (Crafack et al., 2013; Lefeber et al., 2012; Schwan, 1998). However, these methods may present some limitations. The plating method requires different types of media and different culture protocols that influence which species are recovered. The metabolic status of the cells also results in the presence of

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viable but non-culturable (VBNC) microbes, whose influence on fermentation can be underestimated because the population dynamics cannot be evaluated accurately (Cocolin & Ercolini, 2008). The DGGE serve as a fast source of information about the global population structure and may even detect the presence of the VBNC species (Giraffa & Neviani, 2001). However, it is not possible to quantify the population by using this technique. Quantitative real-time PCR (qPCR) is a faster and more reliable alternative to identify and quantify yeasts during fermentation (Hierro, Esteve-Zarzoso, González, Mas, & Guillamón, 2006) and is particularly advantageous for VBNC yeasts because of its sensitivity (Zott et al., 2010). Although the technique cannot distinguish living cells from intact dead cells, it remains the most widely used method for the evaluation of yeast dynamics during fermentation because VBNC cells may continue to influence the final product's characteristics regardless of their actual status (Cocolin & Ercolini, 2008). To our knowledge, qPCR has not been used to evaluate the yeast dynamic during the complex fermentation of cocoa. This work aimed to inoculate three yeast species (*Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Pichia kluyveri*) during cocoa fermentations via qPCR and evaluate their dynamic behavior. Further, the effect of these inoculations on the sensory qualities of chocolate was evaluated. The chemical parameters (carbohydrates, ethanol and organic acids) during fermentations were also studied.

## 2. Material and methods

### 2.1. Fermentation and sampling

The fermentation experiments were conducted at the Vale do Juliana cocoa farm in Igrapiúna, Bahia, Brazil. The ripe cocoa pods from PS1319 hybrid (Porto Seguro, Uruçuca, BA, Brazil) were harvested in November 2013. The cocoa pods were manually opened with a machete and the beans were immediately transferred to the fermentation house. The fermentation started approximately 4 h after the breaking of the pods and was performed in 0.06 m<sup>3</sup> wooden boxes. Each fermentation used 100 kg of cocoa beans. Fermentations were performed both with and without inoculation (control) of a mixed yeast starter culture containing *S. cerevisiae* UFLA CA11 (LNF- CA11, LNF Latino America, Bento Gonçalves, Rio Grande do Sul, Brazil), *Pichia kluyveri* UFLA YCH194 and *Hanseniaspora uvarum* UFLA YCH203 at the beginning of the process. The *P. kluyveri* and *H. uvarum* species were separately grown in YPD broth [10 g/L Yeast extract (Merck); 20 g/L Peptone (Himedia); 20 g/L dextrose (Merck)] at 30 °C and 150 rpm, and replicated every 24 h. The cells were recovered by centrifugation (7000 rpm, 10 min) and re-suspended in 1 L of sterile peptone water [1 g/L Peptone (Himedia)]. This solution was spread over the cocoa beans, reaching a concentration of approximately 10<sup>5</sup> cells/g of cocoa. The *S. cerevisiae* UFLA CA11 yeast, which is lyophilized by LNF, was weighed (as recommended by the manufacturer's instructions) and mixed in the solution with other yeasts to reach a population of approximately 10<sup>7</sup> cells/g of cocoa. All fermentations were evaluated over a period of 168 h, and samples of approximately 100 g each were withdrawn at 0, 24, 48, 72, 96, 120, 144 and 168 h of the process. The samples were taken approximately 40 cm from the surface of the center of the fermenting cocoa mass, placed in sterile plastic pots and transferred to the laboratory. The samples for chemical and culture-independent analyses were stored at −20 °C. All fermentations were performed in triplicate.

### 2.2. DNA extraction and qPCR reaction

The total DNA from the cocoa pulp was extracted from samples with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in

accordance with the manufacturer's instructions for DNA purification from tissues. The DNA was stored at −20 °C for further use.

Specific primers for the *S. cerevisiae*, *P. kluyveri* and *H. uvarum* yeast species used in this study were previously described by Díaz, Molina, Nöhling, and Fischer (2013) and are shown in Table 1. The specificity of each primer pair was confirmed by searching in GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Real-time PCR was carried out using the Rotor-Gene Q System (Qiagen, Hombrechtikon, ZH, Switzerland). Each reaction comprised 12.5 µL 2× Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Stockach, Konstanz, Germany), 0.8 µM of each primer (Invitrogen, São Paulo, SP, Brazil) and 1 µL template DNA extracted from cocoa pulp for a total volume of 25 µL. The mixture was heated to 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 15 s. The cycling temperature was then increased by 1 °C every 5 s from 50 °C to 99 °C to obtain the melting curve. All analyses were performed in triplicate. The DNA concentration in the samples was limited to 50 ng per analysis, except for standard curves prepared from samples containing a known number of yeast cells. For standard curves, all yeast species were cultivated in YPD agar at 30 °C for 24 h. The cells were counted using a Neubauer chamber. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and serially diluted (1:10) from 10<sup>8</sup>–10<sup>7</sup> down to 10 cell/mL. Each point on the calibration curve was measured in triplicate.

### 2.3. Carbohydrates, alcohols and organic acids analysis

The carbohydrates (glucose and fructose), organic acid (acetic, lactic, and citric acids) and alcohol (ethanol) from cocoa pulp and beans were extracted and analyzed using a liquid chromatography system (Shimadzu, model LC-10Ai, Shimadzu Corp., Japan) equipped with a dual detection system consisting of a UV–Vis detector (SPD 10Ai) and a refractive index detector (RID-10Ai). A Shimadzu ion exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm) was operated at 30 °C for carbohydrates and alcohols, and 50 °C for acids. Perchloric acid (100 mM) was used as the eluent at a flow rate of 0.6 mL/min. The acids were detected via UV absorbance (210 nm), while the alcohols and carbohydrates were detected via RID. All samples were analyzed in triplicate, and individual compounds were identified based on the retention time of standards injected using the same conditions. The sample concentrations were determined using an external calibration method. Calibration curves were constructed by injecting different concentrations of the standards under the same conditions of the samples analyses and the areas obtained were plotted a linear curve whose equation was used to estimate the concentration of the compounds in the sample (Ramos, Dias, Miguel, & Schwan, 2014).

### 2.4. Sensory analysis of chocolate

After fermentation, the beans were sun dried in drying greenhouses. Thereafter, the dried beans from the two different fermentation processes (control and inoculated) were sent for chocolate production at Sartori and Pedroso Alimentos Ltda. (São Roque, SP, Brazil). The chocolate contained 70% cocoa.

The sensory analyses of the two kinds of chocolates (from control and inoculated fermentations) were performed using a consumer acceptance test followed by a check-all-that-apply (CATA) question. The tests were conducted on 51 adults over 18 years of age. Participants were 19.3% male and 80.7% female and were consumers of dark chocolate. For the acceptance test, the consumers evaluated how much they liked each sample using a 9-

**Table 1**

Specific primers used for qPCR analysis and qPCR parameters of standard curves obtained from 10-fold dilution of yeast strains DNA by qPCR.

Species	Primers		Product size	qPCR parameters		
	Name	Sequence		R <sup>2</sup>	Slope	Efficiency (%)
<i>S. cerevisiae</i>	SC-5fw	5'-AGGAGTGC GGTTCTTTGTAAG-3'	215 bp	0.999	−3.420	96
	SC-3bw	5'-TGAAATGCGAGATTCCT-3'				
<i>P. kluyveri</i>	PK-5fw	5'-AGTCTCGGGTTAGACGT-3'	169 bp	0.998	−3.634	88
	PK-3bw	5'-GCTTTTCATCTTCTTCACA-3'				
<i>H. uvarum</i>	HU-5fw	5'-GGCGAGGATACCTTTCTCTG-3'	172 bp	0.998	−3.515	93
	HU-3bw	5'-GAGGCGAGTGCATGCAA-3'				

Primers were described by Díaz et al. (2013).

point hedonic scale (1 = dislike extremely; 2 = dislike very much; 3 = dislike moderately; 4 = dislike slightly; 5 = neither like nor dislike; 6 = like slightly; 7 = like moderately; 8 = like very much; 9 = like extremely) (Stone & Sidel, 1993). For the CATA question, the consumers were asked to evaluate seven sensory attributes and select those they considered appropriate to describe the chocolate. The attributes were sour, fruity, bitter, astringent, coffee, nutty and sweet.

The tests were performed in closed cabins with white illumination at the Sensory Analysis Laboratory, Food Science Department, Federal University of Lavras (Lavras, MG). The samples were labeled with three random digits on a white surface. These samples had a monadic form and followed a balanced order of presentation (Walkeling & Macfie, 1995). The chocolate was presented in 50 mL plastic cups containing approximately 2.5 g each. The subjects rinsed their mouths with water between tastings. The sensory analysis was performed with the approval of the local ethics committee (Federal Lavras University, Brazil).

### 3. Results

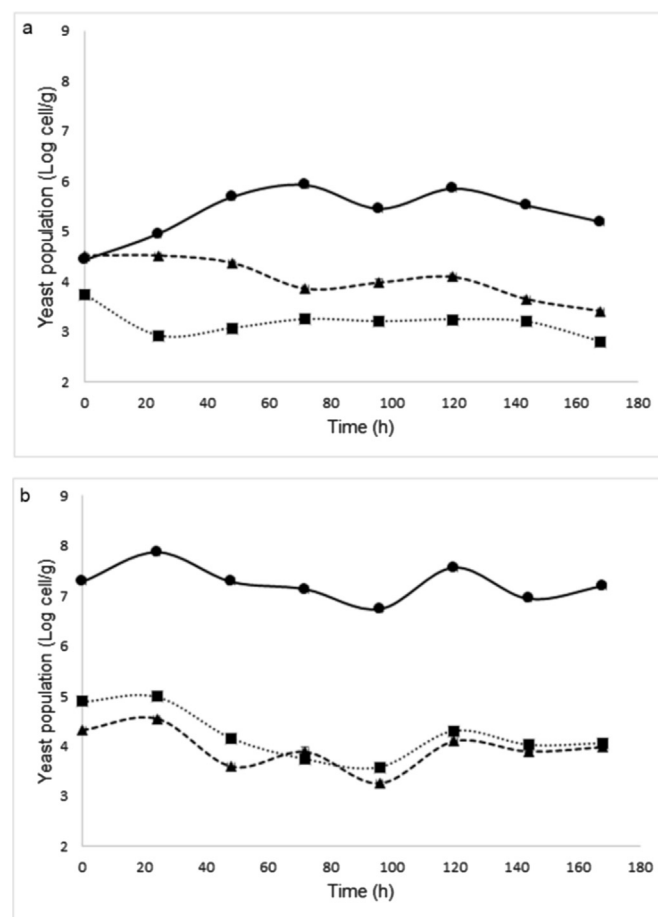
#### 3.1. qPCR analysis

Spontaneous and inoculated fermentations of cocoa in a farm scale were performed in this study. For inoculations, the starter culture containing *S. cerevisiae*, *P. kluyveri* and *H. uvarum* yeast species was used. The dynamic behavior of the population of the starter culture species during cocoa fermentations in the two assays (control and inoculated) was monitored by qPCR. The sequences and product sizes of the primers are summarized in Table 1, as well as the qPCR parameters obtained for standard curves. Standard curves were established for each primers set. The reaction efficiencies ranged between 88% (*P. kluyveri*) and 96% (*S. cerevisiae*) with high reproducibility. The lowest detection limit was  $10^2$  cells mL<sup>−1</sup>. The melt curve analysis for each PCR showed a single peak (data not shown).

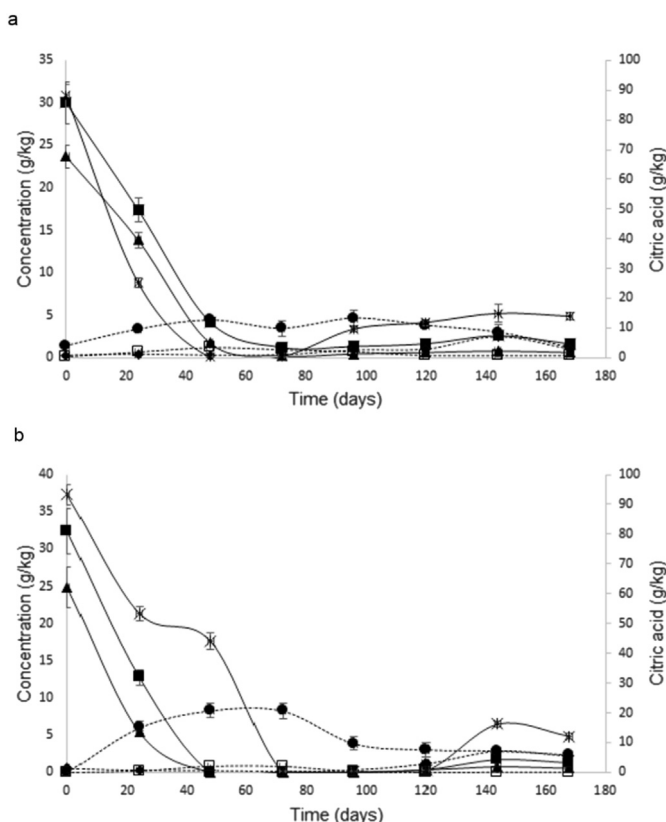
The yeasts *S. cerevisiae*, *P. kluyveri* and *H. uvarum* were detected and quantified during control and inoculated fermentations by qPCR (Fig. 1). *S. cerevisiae* was predominant in both fermentations; however, in the control the population was lower (ranging from 4.4 to 5.9 log cell/g) than in the inoculated fermentation (ranging from 6.7 to 7.9 log cell/g). In the control, the population of *H. uvarum* and *P. kluyveri* ranged from 3.4 to 4.5 log cell/g and 2.8 to 3.7 log cell/g, respectively. Whereas in the inoculated assay, *P. kluyveri* showed higher population than in the control (3.6–5.0 log cell/g) and *H. uvarum* showed similar population (3.6–4.5 log cell/g). It was expected that higher populations of these species would be found in the inoculated fermentation than in the control; however this was not the case for *H. uvarum*. It seems that the other yeasts, mainly *S. cerevisiae*, detected in highest numbers may inhibit the *H. uvarum* growth.

#### 3.2. Carbohydrates, ethanol and organic acids during cocoa fermentation

Carbohydrates, ethanol and organic acids were measured from the pulp and beans during the cocoa fermentations (Figs. 2 and 3). Their concentrations at 0 and 168 h are shown in Table 2. For the pulp, the initial concentrations of glucose, fructose and citric acid were approximately 25, 30 and 90 g/kg, respectively. The consumption of these compounds was observed at the initial time of fermentation (until around 70 h) and carbohydrates were consumed faster in the inoculated fermentation than in the control (Fig. 2). At 24 h of fermentation, glucose decreased to values of 13.8 g/kg (control) and 5.4 g/kg (inoculated), and fructose showed



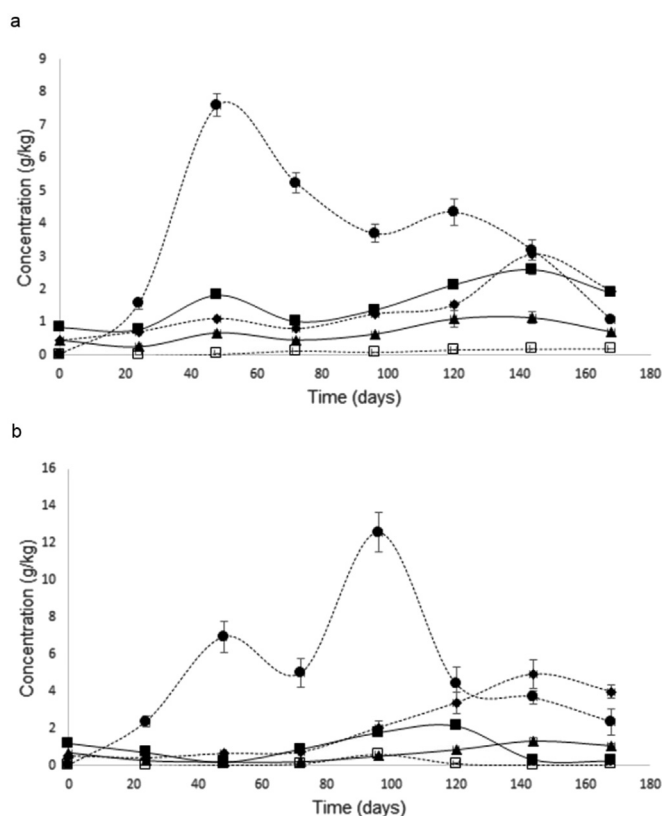
**Fig. 1.** Dynamic behavior of *S. cerevisiae* (●), *P. kluyveri* (■), and *H. uvarum* (▲) yeasts populations during the spontaneous (a) and inoculated (b) fermentations of cocoa, measured by qPCR. Standard deviation of mean ranged from 0.003 to 0.1 log cell g<sup>−1</sup>.



**Fig. 2.** Carbohydrates, organic acids and ethanol detected in the pulp during (a) spontaneous and (b) inoculated fermentations of cocoa. ▲ = glucose; ■ = fructose; ● = ethanol; ◆ = acetic acid; □ = lactic acid; \* = citric acid.

concentrations of 17.4 g/kg (control) and 12.8 g/kg (inoculated). From 72 h, the concentration of carbohydrates was lower than 3 g/kg for both assays. Sucrose was not detected at the beginning of the fermentation, likely because it was hydrolyzed into glucose and fructose given that the fruit harvest was performed three days before fermentation. This procedure is common on Brazilian cocoa farms because the producers have observed that it accelerates the fermentation process. Citric acid present in the pulp was also consumed; however, in the inoculated assay this acid showed concentrations lower than 1 g/kg only at 72 h, while for the control this concentration was reached at 48 h. As observed in Fig. 2, from 96 h for control and 144 h for inoculated fermentation, citric acid showed an increase, likely related to microbial metabolism. At the end of fermentation (168 h) the concentrations of citric acid detected were 14.0 and 12.1 g/kg for control and inoculated assays respectively.

As expected, the ethanol concentration in the pulp was higher in the fermentation inoculated with yeasts, reaching the peak of ethanol production (around 8 g/kg) from 48 to 72 h, compared to the control, which showed maximum concentration of 4.6 g/kg at 96 h. This fact may be due mainly to the *S. cerevisiae* inoculation, which was the predominant yeast detected by qPCR. For the acetic acid, the maximum concentrations were similar between the control and inoculated fermentations: 2.5 and 2.9 g/kg respectively, detected at 144 h. As ethanol was metabolized into acetic acid by acetic acid bacteria, it might have been expected that the highest concentrations of this acid would be in the inoculated assay; however, the concentrations of both fermentations were similar. This may be due to the high temperature of the fermentations' mass, around 45–50 °C (data not shown), which favors ethanol



**Fig. 3.** Carbohydrates, organic acids and ethanol detected in the beans during (a) spontaneous and (b) inoculated fermentations of cocoa. ▲ = glucose; ■ = fructose; ● = ethanol; ◆ = acetic acid; □ = lactic acid; \* = citric acid.

evaporation and did not affect in the acetic acid concentration at the end of the process. Lactic acid was produced in the middle of the fermentation process (between 48 and 72 h), showing maximum concentrations of 1.2 g/kg (control) and 0.8 g/kg (inoculated) at 48 h.

Fig. 3 shows the concentration of carbohydrates, ethanol and organic acids in the beans. The concentration of carbohydrates (lower than 2.7 g/kg) and lactic acid (lower than 0.2 g/kg) are similar in both experiments. Regarding ethanol, two peaks were observed in both assays. The first peak occurred at 48 h with concentrations of 7.6 and 6.9 g/kg for control and inoculated fermentations, respectively. The second peak for the control assay occurred at 120 h (4.3 g/kg) and for the inoculated assay at 96 h (12.6 g/kg). Acetic acid was detected in the beans primarily toward the end of fermentation. The highest concentration of this acid was observed at 144 h with values of 3.1 and 4.9 g/kg for control and inoculated assays respectively. These data show that the production of ethanol (between 48 and 72 h) as well as acetic acid (between 120 and 144 h), by microbial activities in the pulp caused penetration into the cotyledon and in combination with high temperature contributed to the death of the beans.

### 3.3. Sensory analysis

Sensory analyses were performed for both the chocolate produced from spontaneous fermentation and that produced by inoculated fermentation. The acceptance test and CATA question were given to untrained dark chocolate consumers. There were no significant differences ( $p < 0.5$ ) in acceptance between the two samples of chocolate. The result showed an average acceptance of



**Table 2**

Chemical compounds detected at 0 and 168 h in the control and inoculated fermentations.

Compounds	Pulp			Beans		
	0 h	Control (168 h)	Inoculated (168 h)	0 h	Control (168 h)	Inoculated (168 h)
Glucose	24.26 ± 2.02 <sup>b</sup>	0.60 ± 0.01 <sup>c</sup>	0.55 ± 0.04 <sup>c</sup>	0.58 ± 0.02 <sup>b</sup>	0.71 ± 0.00 <sup>b</sup>	1.08 ± 0.09 <sup>b</sup>
Fructose	31.20 ± 2.71 <sup>b</sup>	1.63 ± 0.10 <sup>b</sup>	1.28 ± 0.09 <sup>b</sup>	1.02 ± 0.09 <sup>a</sup>	1.89 ± 0.09 <sup>a</sup>	0.25 ± 0.00 <sup>b</sup>
Citric acid	90.66 ± 3.65 <sup>a</sup>	13.97 ± 1.32 <sup>a</sup>	12.15 ± 0.84 <sup>a</sup>	—	—	—
Lactic acid	0.03 ± 0.00 <sup>c</sup>	0.21 ± 0.21 <sup>c</sup>	0.05 ± 0.05 <sup>c</sup>	ND	0.20 ± 0.00 <sup>b</sup>	0.06 ± 0.00 <sup>c</sup>
Acetic acid	0.41 ± 0.00 <sup>c</sup>	1.00 ± 0.00 <sup>b</sup>	2.23 ± 0.10 <sup>b</sup>	0.48 ± 0.01 <sup>b</sup>	1.96 ± 0.02 <sup>a</sup>	3.98 ± 0.36 <sup>a</sup>
Ethanol	0.71 ± 0.04 <sup>c</sup>	1.22 ± 0.00 <sup>b</sup>	2.38 ± 0.10 <sup>b</sup>	ND	1.07 ± 0.00 <sup>a</sup>	2.35 ± 0.74 <sup>a</sup>

Mean values ± standard deviation within the same column followed by different superscript letters differ significantly ( $p < 0.05$ ) by Scott-Knott test. Citric acid were not detected in the beans.

6.5 and 6.4 for chocolates produced by control and inoculated assays respectively. This means that the consumers graded each of the chocolates “like slightly”. Answers to the CATA question show that the consumers found each of the chocolates to be mainly bitter (Fig. 4). However, a few differences were noted between the two chocolate samples. The chocolates produced by spontaneous fermentation were more related to sweetness than the chocolates produced by inoculated assay, while the chocolates produced by inoculated assay were more related to coffee and sour attributes than those from the control (Fig. 4).

#### 4. Discussion

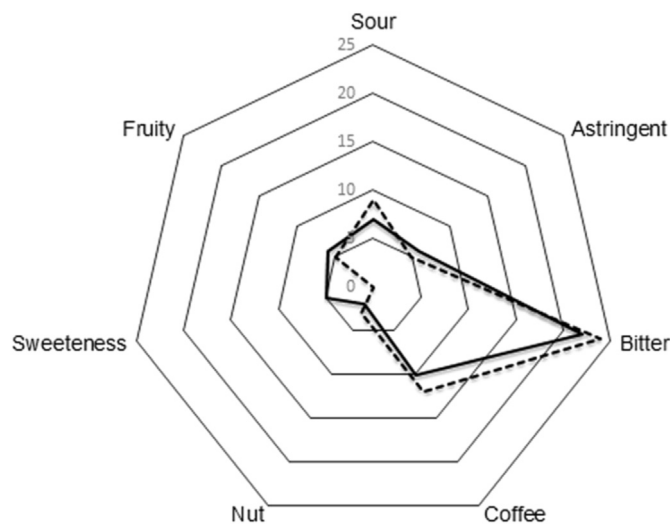
The dynamics of three yeasts (*S. cerevisiae*, *H. uvarum* and *Pichia kluyveri*) and their influence on sensory characteristics of chocolate were investigated in this work. Isolates of these yeasts were used as starter culture in order to observe their influence during the fermentation of cocoa beans and on the chocolate produced by these fermented beans. *S. cerevisiae* UFLA CA11 was previously used as starter culture in fermentations of different cocoa hybrids, including PS1319 (Ramos et al., 2014). According to Ramos et al., the inoculation of this strain accelerated the fermentation process. *H. uvarum* UFLA YCH203 and *P. kluyveri* YCH194 were isolated from cocoa fermentation (Moreira et al., 2013) and selected based on

their stress tolerance and fermentation performance in lab assays (data not shown). *H. uvarum* has been described as an important yeast species associated with *S. cerevisiae* detected during Brazilian cocoa fermentations (Moreira et al., 2013; Ramos et al., 2014). Regarding *P. kluyveri*, this species has never been reported as being the dominant yeast species, however it has previously been described in fermentations in Ghana and Brazil (Jespersen et al., 2005; Moreira et al., 2013; Nielsen et al., 2005, 2007). Craffack et al. (2013) studied the influence of *P. kluyveri* on cocoa flavor and observed that starter culture containing this yeast seemed to have a positive influence on the flavor profile of chocolate.

In this study, a culture-independent method (qPCR) was used to detect *S. cerevisiae*, *H. uvarum* and *P. kluyveri*, the starter culture species used for cocoa fermentation. The qPCR is a faster and more reliable alternative to identify and quantify yeasts during fermentation (Hierro et al., 2006) than the methods based on the culture. Further, VBNC yeasts may be detected (Zott et al., 2010). The qPCR has been employed to detect, identify and quantify either pathogens or beneficial populations such as fermenting microbes or probiotics in food products (Malorny, Lofstrom, Wagner, Kramer, & Hoorfar, 2008; Zott et al., 2010). However, studies using qPCR for microbial identification from complex environmental samples such as cocoa fermentation are still scarce.

The qPCR revealed that *S. cerevisiae* and *H. uvarum* populations showed similar values in the beginning of spontaneous (control) fermentation. However, after 24 h *S. cerevisiae* was predominant (average 5.4 log cell/g) among the three species. The stressor conditions during cocoa fermentation, including ethanol concentration and high temperature, influence the prevalence of adapted yeast, such as *S. cerevisiae*, that have been said to dominate fermentation (Andorrà, Berradre, Mas, Esteve-Zarzoso, & Guillamón, 2012; Ardhana & Fleet, 2003; Jespersen et al., 2005; Moreira et al., 2013; Ramos et al., 2014). The *S. cerevisiae* was followed by the *H. uvarum* population (average 4.0 log cell/g). *Hanseniaspora* genus and *S. cerevisiae* have been described as predominant yeasts during different laboratory and pilot-scale cocoa bean fermentations (Moreira et al., 2013; Nielsen et al., 2007; Pereira, Magalhães, Almeida, Coelho, & Schwan, 2013; Pereira, Miguel, Ramos, & Schwan, 2012; Ramos et al., 2014). The species *H. uvarum* was previously described as an important yeast during different Brazilian cocoa hybrid fermentations (Moreira et al., 2013; Ramos et al., 2014). As expected, the *P. kluyveri* yeast was detected in low numbers (average 3.1 log cell/g) during spontaneous fermentation.

When the yeast starter culture was employed, the yeast microbial profile showed modifications. As observed in the results, the *S. cerevisiae* was still predominant (average 7.2 log cell/g) among the three species, as it was inoculated in higher concentrations than the others. *P. kluyveri* showed its highest (average 4.2 log cell/g) population in the inoculated assay, however *H. uvarum* showed similar numbers between the assays (average 3.9 log cell/g). The



**Fig. 4.** Flavor profiles of the chocolates produced from cocoa beans spontaneously fermented (continuous line) and cocoa beans fermented with inoculations of yeasts starter culture (dotted line). The center of the diagram corresponds to the lowest flavor intensity and the perimeter to the highest flavor intensity.

highest populations of *S. cerevisiae* and *P. kluyveri* likely suppressed the *H. uvarum* growth in the inoculated fermentation. Although the inoculated yeasts were previously tested for some stressful conditions (data not shown), in a complex system containing different microorganisms a competition is an important issue that should be considerate. The increase in the *Saccharomyces* population matched a large decrease in the *Hanseniaspora* population, as has been widely reported (Hierro, Esteve-Zarzoso, Mas, & Guillamón, 2007; Nissen & Arneborg, 2003; Nissen, Nielsen, & Arneborg, 2003; Pérez-Nevado, Albergaria, Hogg, & Girio, 2006). The ability of *S. cerevisiae* to displace other microbial species during fermentations has always been attributed to its higher fermentative power and capacity to withstand the increasingly adverse conditions established in the medium as the fermentation progresses. These conditions include high levels of ethanol and organic acids, low pH values, scarce oxygen availability, high temperature and depletion of certain nutrients (Goddard, 2008; Hansen, Nissen, Sommer, Nielsen, & Arneborg, 2001). Further, species belonging to *Pichia* genera, including *P. kluyveri* as well as *S. cerevisiae*, have been reported as killer toxin-producing yeast (Branco et al., 2014; Santos, Marquina, Leal, & Peinado, 2000). These species produce toxins against other yeast genera including killer-sensitive strains of *H. uvarum* (Santos et al., 2000). All of these attributes probably favored the *S. cerevisiae* and *P. kluyveri* growth relative to *H. uvarum* during inoculated cocoa fermentation. Consequently, some characteristics observed during fermentation, including high ethanol concentration (Figs. 2b and 3b) and the sour, bitter and coffee flavors detected in the chocolate (Fig. 4), may be mainly related to the metabolism of these two yeasts' isolates.

According to the chemical results, carbohydrates were consumed faster (Fig. 2) in the inoculated assay. This is likely due to the higher population of *S. cerevisiae* in the inoculated assay than in the control. Further, higher ethanol concentrations (almost two times the concentration detected in the control) were observed in this assay. However, this was not the case for acetic acid. Similar results were found by Ramos et al. (2014) using *S. cerevisiae* UFLA CA11 as a single inoculant in cocoa fermentation. These authors suggested that ethanol was likely partially eliminated by evaporation and it did not affect in the acetic acid concentration at the end of the process, concluding that the yeast *S. cerevisiae* UFLA CA11 accelerated the fermentation, preventing undesirable microorganisms growth.

In order to investigate the influence of starter culture on the final product, two chocolates were produced and their sensory characteristics were evaluated. The consumers graded both chocolate samples "like slightly". Although there were no significant differences ( $p < 0.5$ ) in acceptance for chocolates produced by spontaneous and inoculated fermentations, other differences were observed. Bitter, sour and coffee flavor attributes were more strongly detected in the inoculated sample. It is known that the quantity, nature and distribution of the microorganisms present in the cocoa pulp will determine the speed and intensity of the fermentation as well as the quality of the fermented beans and the chocolate made from them (Camu et al., 2008). The inoculated yeasts *S. cerevisiae* UFLA CA11 and *P. kluyveri* UFLA YCH194, which showed higher populations in the inoculated assay, may contribute to the sensory characteristics observed in that assay. Further, the fermentation process seemed to be accelerated in this assay.

## 5. Conclusions

This study is the first to use the qPCR technique to assess the dynamic behavior of yeast during the complex fermentation of cocoa beans. A predominance of *S. cerevisiae* was observed during spontaneous fermentation of cocoa hybrid PS1319 compared to

*H. uvarum* and *P. kluyveri*. During inoculated fermentation, the population of *H. uvarum* seemed to be suppressed by the high populations of the other two inoculated yeasts, mainly *S. cerevisiae*, which was also predominant in the inoculated assay. Starter culture accelerated the consumption of carbohydrates, and thus the highest ethanol concentrations were observed. Further, the detection of highest and early ethanol and acetic acid into the cotyledon in the inoculated assay, may contribute to acceleration in the death of the beans and consequently the fermentation process. The acceptance of chocolate samples produced by the two different assays did not differ significantly; however, a few differences in the attributes related to chocolate flavor could be observed. The inoculation produced chocolate with stronger coffee and sour notes than the chocolate produced by spontaneous fermentations. However, studies regarding volatile compounds produced by fermentation and microbial diversity are important in order to better understand the role of these yeasts during cocoa fermentation and in the chocolate made from the fermented beans.

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