Research Article

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Influence of *S. cerevisiae* and *P. kluyveri* as starters on chocolate flavour

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Abstract

BACKGROUND: Fermented cocoa beans (*Theobroma cacao* L.) are a pivotal raw material for chocolate production. A cocktail yeast applied in the cocoa fermentation process can promote the formation of pleasant metabolites. *Saccharomyces*, *Pichia* and *Hanseniaspora* have been widely used in fermentation to improve the final product organoleptic profile, highlighting that fermentation is a critical point for chocolate flavour precursor production. This study aims to evaluate the impact of *Pichia kluyveri* and *Saccharomyces cerevisiae* strains as starter cultures on the fermentation for two cocoa hybrids, FA13 and CEPEC2002.

RESULTS: During fermentation processes, volatile organic compounds (VOCs) and protein profiles were assessed. Chocolates produced were also assessed regarding the presence of VOCs. Eighty VOCs were identified using gas chromatography coupled to mass spectrometry analysis. Mass spectrometry provided the protein profile evolution during fermentation and showed that the profiles changed with inoculation type (spontaneous *versus* inoculated fermentation). Chocolate obtained from FA13 inoculated with *S. cerevisiae* strain contained a greater amount of organics acids, being categorised as sourer than chocolate produced by spontaneous fermentation of FA13. CEPEC2002 inoculated with *S. cerevisiae* strain in co-culture with *P. kluyveri* strain generated less sour and sweeter chocolate than spontaneous fermentation only.

CONCLUSIONS: Chocolates from inoculated assays with starter cultures were more accepted by evaluators, highlighting that *P. kluyveri* and *S. cerevisiae* influence the composition of VOCs. Besides, protein profiles also changed throughout fermentation. Further investigation should be conducted to clarify protein degradation dynamics during inoculated fermentations to define which of the microbial cultures positively affect the chocolate sensory characteristics.
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Keywords: cocoa fermentation; GC–MS; MALDI-TOF MS; protein profile; yeast co-culture

INTRODUCTION

Cocoa beans are a pivotal raw material for many food products such as cocoa paste, butter, powder, liquor and chocolate.[1] Overall, global food industries direct the majority of cocoa bean yield to chocolate manufacture, which accounts for more than 45% of output.[2] This is due mainly to widespread chocolate consumption with large growth potential. In 2020, the global chocolate market was forecast to reach USD137.12 billion.[2] Moreover, by 2026, growth is expected to reach USD189.89 billion.[2] For that, it is of utmost necessity to improve and control the processing of each step of the chocolate production chain seeking to meet the market sensorial requirement demands.

High-quality chocolate production is directly reliant on an efficient fermentation process since flavour precursors are formed in this step. Before fermentation, cocoa bean is astringent and bitter, and therefore unfeasible for post-harvest processing (e.g. roasting and conching steps).[3]

Generally, cocoa fermentation is conducted spontaneously by the action of natural microbiota.[4] Under uncontrolled operating conditions, these microorganisms can be provided by contact with operators' hands and tools. Besides, microorganisms are naturally present on the surface of cocoa pods.[5] In spontaneous fermentation, a random fluctuation in inoculum is expected, since there is no real control of the natural microbiota composition harboured by each cocoa breed or of the microbiota that can be added by external factors. Hence, the fermentation process can be subjected to variations.[6]

Conversely, controlled fermentation processes use a defined cocktail of microorganisms, optimising the fermentation

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timeframe, and minimising the presence of spoilage organisms. The use of a standardised process provides a feasible condition to achieve a high-quality product.[6]

The microorganism dynamic of cocoa bean fermentation is complex, and thus is essential broaden knowledge of the role of each microbial group. The main microbial groups found in fermentation processes are yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB).[6] Yeasts are dominant in the early stages and, subsequently, are surpassed by LAB and AAB.[6–9] The functional roles of LAB and AAB in cocoa bean processing have been much discussed,[10–12] but are not the focus of this study.

Saccharomyces cerevisiae has been widely applied as a starter yeast in the fermentation of cocoa beans, either alone in the process[13] or in co-culture with other yeasts such as *Candida*, *Hanseniaspora* (anamorph *Kloeckera*), *Pichia* and *Torulaspora*.[14–17]

The real fermentation takes place in the mucilaginous fruit pulp that surrounds the cocoa beans. This sugar-rich substrate boosts yeast growth, triggering alcoholic fermentation of the pulp sugars into ethanol and also pectinolytic enzymes.[6]

Proteolytic reactions inside the beans during fermentation are responsible for releasing small peptides and free amino acids considered as flavour precursors of the final produced chocolate.[18] In addition, esters and higher alcohols produced by yeast during fermentation have been reported, potentially contributing to the volatile compound admixture.[19]

Various volatile organic compounds (VOCs) such as carboxylic acids, esters, aldehydes, alcohols and ketones that are produced during fermentation influence directly the chocolate sensory attributes.[18,20] Microbiota strains involved, proteins and VOC profiles released during the fermentation process will be crucial factors for the production of chocolate of high quality.[13,15,16,18,20]

Previous studies have investigated the influence of yeast inoculation on the volatile and non-volatile composition, microbial community and sensory profile of chocolate.[8,9,20,21] A proper understanding of the microbial dynamic in cocoa fermentation, mainly the role of yeasts, must be broadened. Despite this, up to now a microbial cocktail suitable as a starter culture for application in chocolate production has not been defined and marketed.[10]

The study presented here aimed to use *Pichia kluyveri* CCMA-UFLA 0237 and *Saccharomyces cerevisiae* CCMA-UFLA 0200 strains as starters for fermentation of cocoa beans from the hybrids FA13 and CEPEC2002, and to evaluate the sensorial characteristics of chocolate produced in relation to the physicochemical changes, such as protein profile combined with VOCs.

MATERIALS AND METHODS

Sampling, inoculation and fermentation

The field experiments were conducted at Igrapiúna, BA, Brazil. Ripe cocoa beans (*Theobroma cacao* L.) from CEPEC2002 and FA13 hybrids were harvested from September to December 2014. Physical characteristics of each hybrid are presented in Table S1 (supporting information).

After harvesting, cocoa pods from CEPEC2002 and FA13 hybrids were manually opened with a machete, and the beans were transferred to the fermentation site. The fermentation began approximately 3 h after the pods were broken and was performed in 0.06 m^3 wooden boxes; this point was considered as T0 of fermentation.[22] Each fermentation batch included 100 kg of cocoa beans. From these, 25 g of each hybrid was analysed in triplicate.

Fermentations were assessed using inoculation of CEPEC2002 with *S. cerevisiae* CCMA-UFLA 0200 in co-culture with *P. kluyveri* CCMA-UFLA 0237 and FA13 with *S. cerevisiae* CCMA-UFLA 0200 as single inoculum. Four fermentations were conducted: CEPEC2002 SP (*S. cerevisiae* and *P. kluyveri*), CEPEC2002 (control/spontaneous fermentation), FA13 S (*S. cerevisiae*) and FA13 (control/spontaneous fermentation).

Both *S. cerevisiae* and *P. kluyveri* strains were obtained from CCMA-UFLA Culture Collection (Lavras/Brazil, WDCM 1083). *S. cerevisiae* CCMA-UFLA 0200 is commercialised by LNF (CA11) and was assessed according to the manufacturer's instructions. Based on this, *S. cerevisiae* was mixed in solution to reach a population of approximately 10^7 cells per gram of cocoa. *P. kluyveri* was grown on yeast extract–peptone–dextrose broth (10 g L^{-1} yeast extract; 20 g L^{-1} peptone; 20 g L^{-1} dextrose) at 30 °C and replicated every 24 h. The cells were recovered by centrifugation (11 000 × *g*, 10 min) and re-suspended in 1 L of sterile peptone water (1 g L^{-1} peptone; Himedia, Mumbai, India). This solution was applied to cocoa fermentation, reaching a concentration of approximately 10^5 cells per gram of cocoa.[23]

The fermentations were performed in triplicate, with the analyses being performed every 24 h during 6 consecutive days (144 h). All samples were taken approximately 40 cm from the surface of the centre of the fermenting cocoa mass, placed in sterile plastic pots and stored at −20 °C. The fermentation results represent the mean ± standard error of three independent experiments.

Analysis of VOCs using gas chromatography coupled to mass spectrometry

The headspace–solid-phase microextraction (HS–SPME) technique combined with gas chromatography–mass spectrometry (GC–MS) was used for the analysis of VOCs from cocoa samples.[7,23] Analyses were performed for each sample from the beginning (0 h) and at the end of the fermentation process (144 h). Extraction and analysis conditions were applied as previously described by Moreira *et al.*[21].

VOCs from each headspace analysis were defined by integrating the peak areas of all the identified compounds. The relative percentages of individual compounds were calculated from the total contents of VOCs in the chromatograms.[24]

Determination of protein profiles

Purification of protein samples

Before conducting matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS analysis, samples from the beginning and end (0 and 144 h) of each fermentation were subjected to extraction of lipids and polyphenols. This purification allowed removal of interfering substances such as lipids or phenolic compounds. Lipids were extracted from cocoa beans (*ca* 6.0 g) that were crushed with liquid nitrogen and then extracted with *n*-hexane in a Soxhlet system as previously described by Voigt and Biehl.[25] After 6 h, lipid fraction was obtained using a rotary evaporator.

For polyphenol extraction, an acetone powder of defatted cocoa beans was obtained as previously reported.[21] Each sample (3 g) was successively treated with 70% and 80% aqueous acetone, and 60 mL of neat acetone (sufficient to cover the sample). In each step, acetone suspensions were then supplemented with ascorbic acid (5 mmol L^{-1}). Each mixture was stirred for 20 min using a vortex and then centrifuged at 30 000 × *g* for 20 min at 4 °C for recovering the pellet.

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MALDI-TOF MS analyses of proteins

After the lipid and polyphenol extraction, each sample (0.5 g) was crushed with a mortar and pestle using liquid nitrogen until a fine powder was obtained. Each sample was transferred to tubes containing 2 mL of solution A composed of water–acetonitrile–trifluoroacetic acid (50:47.5:2.5 by volume) that were immediately stirred using a vortex for 10 min. To improve protein extraction, glass beads were used. Tubes were centrifuged at 17 000 × *g* for 2 min at 4 °C and protein concentration in the supernatant was assessed by Bradford's method.[26]

Suspensions (1 μL) were transferred to the MALDI-TOF sample plate (Bruker Daltonics, Bremen, Germany) and 1 μL of saturated matrix solution of α-cyano-4-hydroxycinnamic acid prepared with solution A described above was added and mixed.[27–29] Spectra were acquired with a MALDI-TOF Microflex LT (Bruker Daltonics, Bremen, Germany) using the mass range from 2000 to 20 000 Da.

Calibration of the MALDI-TOF equipment was performed using freshly *in situ* extracted ribosomal proteins of *E. coli* strain DH5-alpha, as previously described by Matos *et al.*[30] and Paziani *et al.*[31] Cells of *E. coli* DH5-alpha were grown on nutrient agar medium (Merck) at 37 °C for 20 h. Briefly, fresh *E. coli* DH5-alpha biomass was transferred to MALDI sample plate and MALDI matrix solution was added and mixed. To evaluate reproducibility, each sample was applied in triplicate.

Raw spectra mzXML data were pre-processed and analysed using Mass-up software, following a previously published protocol.[32] Treatment of spectral data involved establishing parameters as previously described by Moreira *et al.*[33]

Sensorial analysis

Dried cocoa beans from different hybrids were used for chocolate production and the final moulded chocolate composed of 70% cocoa was wrapped and kept at 4 °C until sensory analysis. Sensory analyses of chocolates obtained from controls and inoculated fermentations were evaluated using a consumer acceptance test followed by a check-all-that-apply (CATA) questionnaire. Seventy-one people over 18 years old (59% female and 41% male), non-professional tasters, participated in the CATA test. Participants were often untrained consumers of dark chocolate.

Consumers indicated in the acceptance test their satisfaction regarding each sample using a 9-point hedonic scale (1 = dislike extremely and 9 = like extremely).[34] In the CATA questionnaire the consumers evaluated seven chocolate sensorial attributes (astringent, bitter, coffee, fruity, nutty, sour and sweetness) and selected those considered appropriate to describe each chocolate sample. Tests were carried out as previously described by Batista *et al.*[19]

Statistics

All statistical analyses were performed using SISVAR 5.1 software (Federal University of Lavras, Department of Statistics, Lavras, Brazil). Analysis of variance (ANOVA) was used for the data obtained from both the acceptance testing and protein quantification analysis. Test of Tukey (5% significance level) was employed to compare the means.

To establish the agglomerative hierarchical clustering (AHC), XLSTAT software (version 7.5.2) was used. AHC was obtained using results of Biomarker Discovery analysis (presence and absence of ion peaks). Pearson correlation coefficient was used for showing similarities among samples.

RESULTS

Chemical changes during fermentation

Temperatures of inoculated fermentations increased rapidly over time, ranging from 25 °C at 0 h to 48 °C at 96 h (Fig. 1). A sharp increase in temperature occurred between the second and third days. The FA13 inoculated fermentation (FA13 S) showed a maximum temperature at 96 h (48.1 °C). After 72 h, a continues and slower rise of temperature was observed in all fermentation treatments, mainly for CEPEC2002 SP and FA13 S. All fermentations exhibited a plateau (120 h) with temperatures up to 47.5 °C.

Volatile organic compounds

A total of 80 different VOCs were identified using HS–SPME GC–MS analysis. These compounds were identified as 9 acids, 22 alcohols, 26 aldehydes and ketones, 19 esters and 4 other compounds (including pyrazines, pyrroles and terpenes) (Table 1).

The distribution of the 80 VOCs varied according to the sample evaluated. At the beginning of fermentation, a total of 31 compounds were identified in both spontaneous and inoculated fermentations of CEPEC2002 hybrid. The profiles of aldehydes, ketones, pyrazines and other esters were closely similar for both treatments with CEPEC2002 and FA13 hybrids.

Among the VOCs, some of the common compounds identified were flavour-desirable ones, such as 3-methyl-1-butanol, 2,3-butanediol and 2-heptanol. Off-flavour acid compounds such as isobutyric acid, isovaleric acid and propanoic acid were also identified at the end of both fermentation processes. Conversely, phenylethyl alcohol, 1-butanol and ethyl benzoate were observed exclusively in CEPEC2002 SP. Other volatiles such as acetic acid were detected in the chocolates as well in T0 of CEPEC2002 SP and FA13 S fermentations.

FA13 S presented 40 compounds at the beginning, while in the FA13 control were found 48 compounds. However, the numbers of VOCs at the end of the fermentation process (144 h) increased for all samples. In detail, 46 compounds for CEPEC2002 SP, 43 for CEPEC2002 control, 42 for FA 13 S and 58 for FA13 control were found.

Regarding both hybrids, the profiles of aldehydes, ketones, pyrazines and esters were very similar. Despite the similarity in the presence/absence profiles, the percentages of volatile compounds were different, fluctuating throughout the fermentation. Acid and ester groups increased, while alcohols, aldehydes and

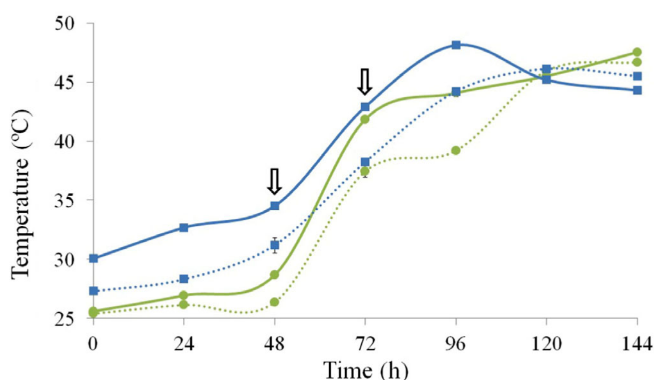

Figure 1. Evolution of temperature during fermentation under different conditions. CEPEC2002 (■), FA13 (●). Inoculated (CEPEC2002 SP and FA13 S, continuous line) and spontaneous (CEPEC2002 and FA13, dotted line) fermentations. Mixing (⇩) of the mass was performed at 48 and 72 h.

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Table 1. VOCs identified by HS–SPME GC–MS during cocoa beans fermentation from 0 h to 144 h, and in chocolate samples (Ch), and reference flavour for compounds. Fill box means presence and open box absence of the compound

Compounds	CEPEC2002 SP			CEPEC2002			FA13 S			FA13			Flavour description[a]
	0 h	144 h	Ch	0 h	144 h	Ch	0 h	144 h	Ch	0 h	144 h	Ch	
Acids													
Acetic acid	■	□	■	□	□	■	■	□	■	□	■	■	Sour, astringent
Caproic acid	□	■	■	□	■	■	■	■	■	■	■	■	Sweat, pungent
Caprylic acid	□	■	■	□	■	■	□	■	■	□	■	■	Sweaty, fatty
Isobutyric acid	□	■	■	□	■	■	■	■	■	■	■	□	Rancid, butter, cheese
Isovaleric acid	■	■	■	■	■	■	■	■	■	□	■	■	Sweat, rancid
Propanoic acid	□	■	□	□	■	■	■	■	■	■	■	■	
Valeric acid	□	■	□	□	□	□	□	■	■	■	■	□	Sweat, acid, rancid
Caprinic acid	□	□	□	□	□	■	□	□	■	□	■	■	
Phenylacetic acid	□	□	■	□	□	■	□	□	■	□	□	■	
Alcohols													
1-Butanol	□	■	□	□	□	□	■	□	□	□	□	□	
1-Hexadecanol	□	□	□	□	□	■	□	□	■	□	□	□	Fruity, green
1-Pentanol	■	■	□	■	■	□	■	■	□	■	■	□	
1-Phenylethanol	■	■	□	■	■	□	■	■	□	■	■	□	
2,3-Butanediol	□	■	■	□	■	■	□	■	■	■	■	■	Cocoa butter
2-Ethyl-1-hexanol	■	■	□	■	■	□	■	■	□	■	■	□	
2-Heptanol	■	■	■	■	■	□	■	■	■	■	■	□	Sweet, citrusy
2-Hexanol	■	■	□	■	■	□	■	□	■	■	■	□	Fruity, green
2-Methyl-1-butanol	□	□	□	■	■	□	■	■	□	■	■	□	Malty, chocolate
2-Methyl-3-buten-2-ol	■	■	□	■	■	□	■	■	□	■	■	□	
2-Nonanol	□	□	□	□	□	□	□	□	□	■	■	□	
2-Pentanol	■	■	□	■	■	□	■	■	□	■	■	□	
2-Phenylethanol	■	■	□	■	■	□	■	■	□	■	■	□	
3-Methyl-1-butanol	■	■	■	■	■	■	■	■	■	■	■	■	
4-Methyl-1-pentanol	□	□	□	□	□	□	□	□	□	■	□	□	
Benzyl alcohol	■	■	□	■	■	□	■	■	□	■	■	□	Sweet, flower
Ethyl alcohol	■	■	■	■	■	□	■	■	■	■	■	□	
Furfuryl alcohol	□	□	■	□	□	□	□	□	■	■	■	□	
Guaiacol	□	■	■	□	■	□	□	■	■	■	■	□	Smoke, sweet
Isobutyl alcohol	■	■	□	■	■	□	■	■	□	□	■	□	
Phenethyl alcohol	□	■	■	□	□	■	□	□	■	□	□	■	Honey, rose, caramel
α-Terpineol	□	□	□	□	■	□	□	□	■	□	□	■	

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Table 1. Continued

Compounds	CEPEC2002 SP			CEPEC2002			FA13 S			FA13			Flavour description[a]
	0 h	144 h	Ch	0 h	144 h	Ch	0 h	144 h	Ch	0 h	144 h	Ch	
Aldehydes and Ketones													
(E)-2-butenal	■						■			■			
1-phenylethanone	■	■		■	■		■	■		■	■		
2,3-Butanedione		■			■		■	■		■	■		
2,3-Pentanedione											■		
2-Acetylfuran										■	■		
2-Furaldehyde			■			■			■			■	
2-Heptadecanone			■			■			■			■	
2-Heptanone	■	■		■	■		■	■		■	■		
2-Methyl-2-butenal	■									■	■		
2-Octanone	■	■	■	■	■					■	■		
2-Pentanone	■	■		■	■		■	■		■	■		
2-Pentylfuran										■	■		
2-Propanone	■	■		■	■		■	■		■	■		
2-Pyrrolidone			■			■			■			■	
2-Vinylfuran	■	■		■	■		■	■		■	■		
3-Methyl-2(5H)-furanone	■									■	■		
3-Methyl-2-butenal										■			
3-Methylbutanal	■	■	■	■	■		■	■		■	■		
5-Methyl-2-hexanone	■	■		■	■		■	■		■	■		
Acetaldehyde	■	■		■	■		■	■		■	■		
Acetoin	■	■	■	■	■	■	■	■		■	■	■	Butter, cream
Benzaldehyde	■	■	■	■	■	■	■	■	■	■	■	■	Bitter
Benzeneacetaldehyde		■	■	■	■		■	■		■	■		
Butyrolactone									■				
Hexanal	■			■						■	■		
Pentanal		■			■		■	■		■	■		
Esters													
2-Pentyl acetate		■			■			■			■		Fruity
2-phenylethyl isobutyrate		■			■		■	■		■	■		
Allyl acetate	■	■		■	■		■	■		■	■		Fruity, banana
Amyl acetate									■		■		
Ehtyl acetate	■			■						■	■		
Ethyl benzoate		■									■		
Ethyl caproate							■				■		
Ethyl caprylate		■			■		■	■			■		Fruity, flowery

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Table 1. Continued

Compounds	CEPEC2002 SP			CEPEC2002			FA13 S			FA13			Flavour description[a]
	0 h	144 h	Ch	0 h	144 h	Ch	0 h	144 h	Ch	0 h	144 h	Ch	
Esters													
Ethyl myristate		■	■		■	■	■	■			■	■	
Ethyl phenylacetate		■			■			■		■	■		
Ethyl pyruvate		■	■	■	■	■	■	■	■	■	■	■	
Isoamyl acetate		■			■			■			■		
Dibutyl phthalate						■			■			■	Waxy, green
Diisobutyl phthalate			■			■			■			■	
Ethyl caprate			■			■			■			■	Pear, grape
Ethyl laurate			■			■			■			■	Fruity, floral
Ethyl palmitate			■			■			■			■	
Methyl palmitate			■			■			■			■	Fruity, sweet
Phenylethyl acetate			■			■			■			■	
Others (Pyrazines, pyrroles and terpenes)													
Tetramethyl-pyrazine			■			■			■			■	Chocolate, coffee
Trimethyl-pyrazine			■			■			■			■	Cocoa, rusted nuts
2-Acetyl pyrrole			■			■			■			■	Chocolate, hazelnut
Linalool		■		■	■		■	■		■	■		Flower, lavender

[a]Obtained from literature.

ketones decreased in different proportion for all samples, as shown in Fig. 2.

Concerning the chocolate samples, 29 compounds were identified in spontaneous fermentations (CEPEC2002 and FA13). Chocolates from CEPEC2002 inoculated with a mix of the two yeasts showed 30 identified compounds, while chocolates produced from inoculated FA13 presented a total of 35 VOCs. The occurrence of acids, esters and other compounds (pyrazines, pyrroles and terpenes) gradually increased during the whole process, from fermentation to chocolate, for all samples; while the occurrence of alcohols, aldehydes and ketones decreased, as observed in Fig. 2.

MALDI-TOF MS: protein degradation kinetics and clustering

Protein extracts were quantified using Bradford's method and the results are presented in Table 2. For all fermentations, protein concentrations decreased approximately half way from the beginning to the end of fermentation. The greatest protein degradation was observed in the CEPEC2002 spontaneous fermentation, a reduction of 0.42 mg of protein per millilitre of extract. Protein samples were then analysed using the MALDI-TOF MS technique. Mass spectra obtained for each sample allowed the determination of minimum and maximum molecular masses (*m/z*) and the total mass peaks for each sample (Table S2, supporting information).

The peak matching operation in Mass-up software generated a list with 131 ion peaks of different *m/z* values (data not shown). Only 3 out of 131 peaks were common among all samples (*m/z* 2818.716, 4195.56 and 9538.966). Ion peaks with mass values *m/z* 2723.138, 3127.244 and 3735.043 were found only in CEPEC2002 inoculated with *S. cerevisiae* and *P. kluyveri*.

A greater number of ion peaks with different *m/z* values was observed for FA13 S (2103.681, 2302.024, 2879.490, 2929.855, 3256.854, 3421.062 and 4797.277). In Fig. 3 can be observed a clustering in terms of similarity of the samples using data of presence and absence of the generated peak list in Mass-up software.

Sensorial analysis

Both the acceptance test and CATA questionnaire allowed differentiation between the chocolates produced from spontaneous and inoculated fermentations. The parameters used in CATA questionnaire analysis were focused for determining if the flavours of the chocolates were bitter, sweet, coffee and nutty. Judges indicated which attributes better described the chocolate samples, and the results are shown in Fig. 4.

Although there were no significant differences ($P < 0.5$, by Tukey's test) between samples from inoculated (CEPEC2002 SP and FA13 S) and non-inoculated fermentations (CEPEC2002 and FA13), pleasant notes were predominantly perceived in chocolates from CEPEC2002 SP and FA13 S.

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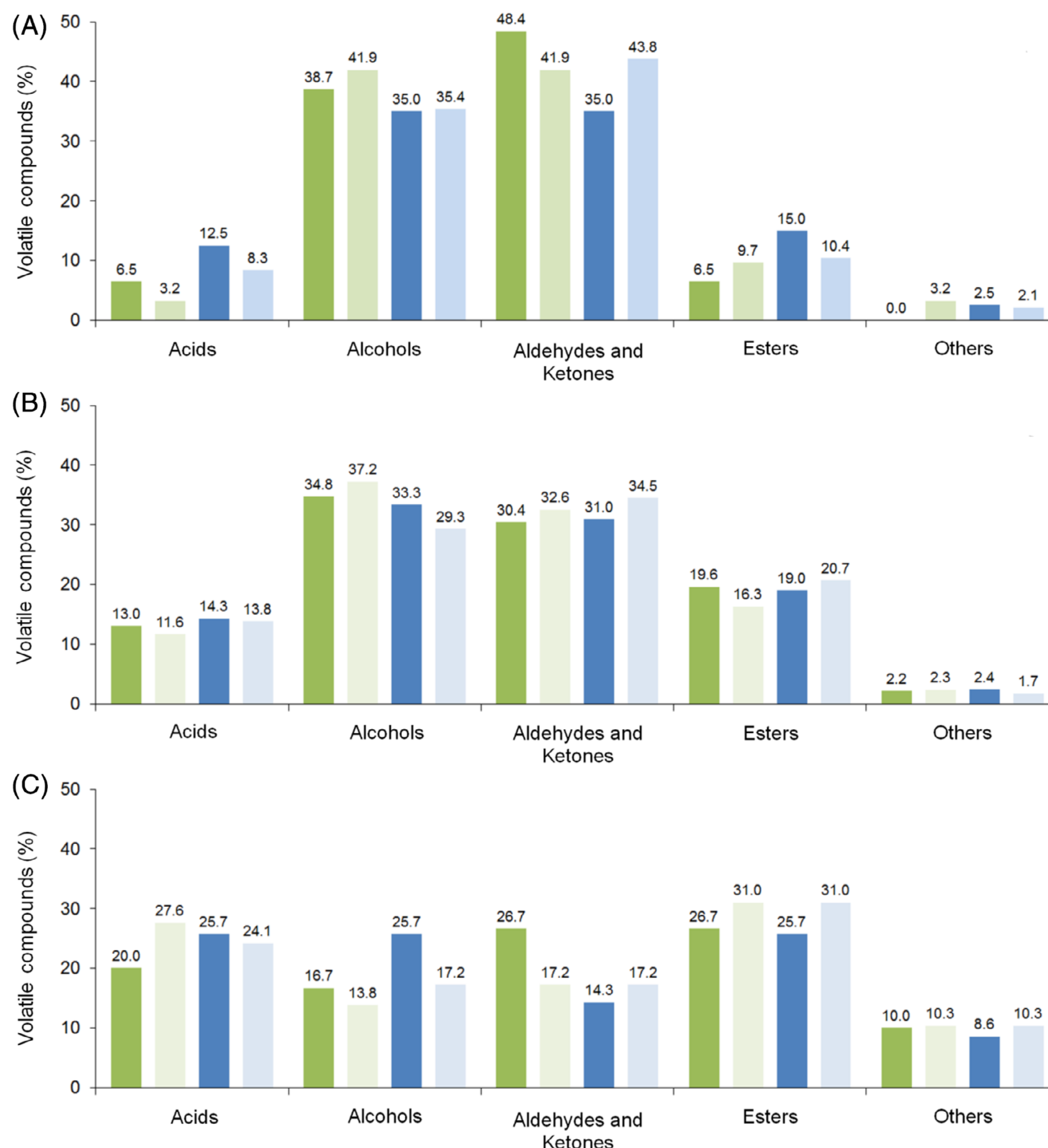

Figure 2. VOCs identified using HS–SPME GC–MS during cocoa bean fermentations for (A) 0 h and (B) 144 h and chocolate samples (C) of CEPEC2002 SP (■), CEPEC2002 control (■), FA13 S (■) and FA13 control (■).

Table 2. Protein quantification by Bradford's method for inoculated and spontaneous fermentations at beginning and at end of process

Sample	Extract (mg mL^{-1})
CEPEC2002 SP 0 h	0.71 ± 0.01[a]
CEPEC2002 SP 144 h	0.39 ± 0.01[b]
CEPEC2002 0 h	0.76 ± 0.05[a]
CEPEC2002 144 h	0.34 ± 0.01[b]
FA13 S 0 h	0.73 ± 0.00[a]
FA13 S 144 h	0.38 ± 0.05[b]
FA13 0 h	0.64 ± 0.03[a]
FA13 144 h	0.33 ± 0.03[b]

In each hybrid, values followed by different letters are different at the 5% level of significance by ANOVA test.

Samples from CEPEC2002 SP were described as bitter and sweeter, with a lower perception of an undesirable sour taste. In contrast, the chocolates from FA13 S were perceived as sourer, fruity, sweetness and less astringent than chocolates from spontaneous fermentation.

DISCUSSION

Temperature is a driving factor for chocolate manufacture. This parameter may fluctuate due to metabolic reactions carried out by microbiota harboured in cocoa beans.[35] Temperature changes were detected for all the various fermentations performed in the present study. However, a considerable increase in temperature was noted between 48 and 72 h in inoculated fermentations.

The addition of a yeast cocktail may have potentiated alcoholic fermentation of the pulp sugars, producing ethanol. This consequently potentiated the next reaction in which AAB oxidize the alcohol formed, first into acetic acid and then to CO_2 and H_2O.[6]

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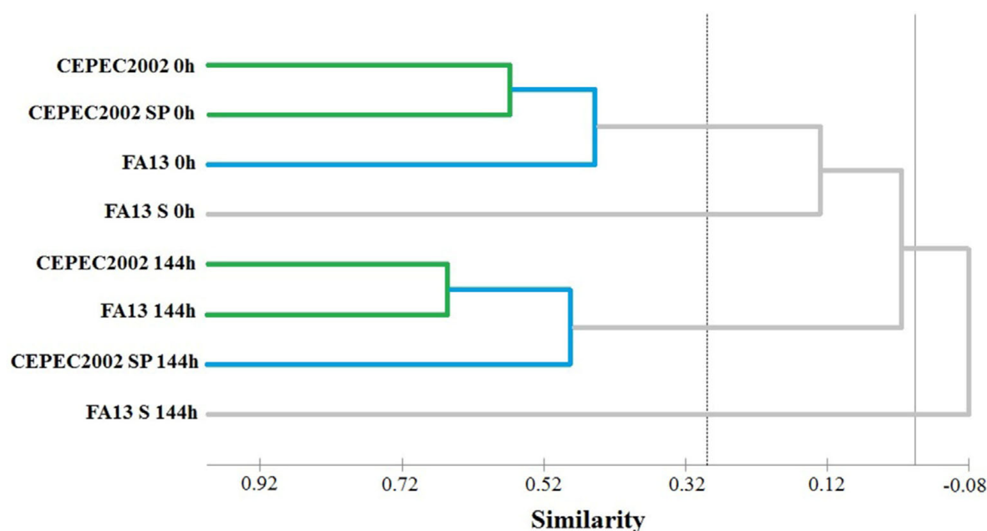

Figure 3. AHC based on presence and absence of mass peaks with different *m/z* values.

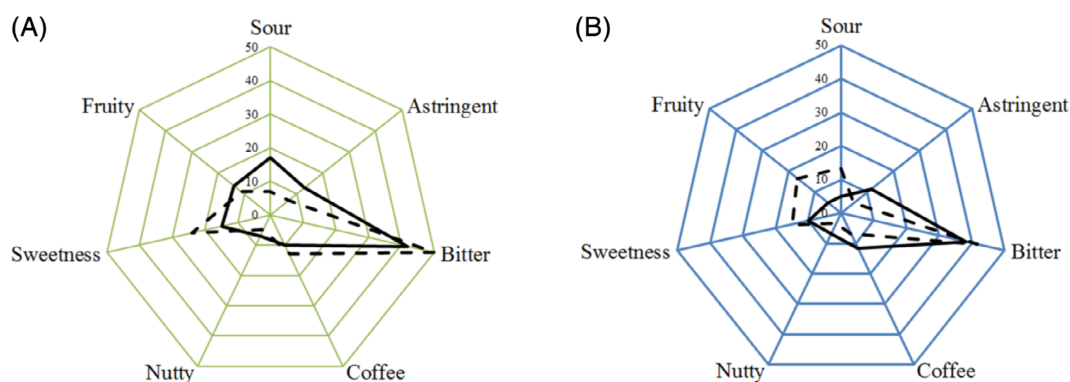

Figure 4. Results of CATA questionnaire analysis regarding the flavour of chocolates produced with spontaneous fermentation (solid line) and fermentation with a yeasts starter culture (dashed line). (A) CEPEC2002 and (B) FA13.

Table 3. Scores of acceptance test for chocolate samples of each fermentation, with and without inoculation

Chocolate sample	Acceptance test
CEPEC2002 SP	7.27[a]
CEPEC2002	6.79[b]
FA13 S	7.32[a]
FA13	6.83[b]

Values followed by the same letter in the same row are not different at the 5% level of significance by Tukey's test.

This exothermic reaction can lead to rising cocoa bean mass heap temperature.[6] Also, a temperature increase occurred simultaneously with performing a turning ofbean mass (e.g. 48 and 72 h). Aeration stimulated the exothermic reactions carried out by aerobic AAB.[36]

Addition of a starter yeast cocktail can drive pleasant metabolites in raw cocoa. Some of the volatiles found in inoculated samples have been assigned to *P. kluyveri* metabolism (e.g. benzaldehyde, 1-butanol, phenylethyl alcohol) and *S. cerevisiae* metabolism (3-methyl-1-butanol, 2-phenylethanol, 2-pentanone).[13]

As is evident from Table 1, some identified alcohols are responsible for conferring sweet, fruity, malty, honey and caramel flavours to the cocoa final product.[7,37,38] In the present study alcohols with pleasant notes, such as 2-phenylethanol, 2,3-butanediol, 2-heptanol and benzyl alcohol, have been detected.

Conversely, the occurrence of some VOCs (e.g. phenol, 2-ethyl-3,5-dimethylpyrazine, ethyl heptanoate and so forth) that may confer unpleasant flavour must be avoided.[7] Increasing acid concentration during the fermentation process can generate perceptions of sour taste (Fig. 2). Chocolate from FA13 S contained a higher number of organic acids identified by GC–MS (Table 1), which may have given rise to the sour characteristic described by judges (Fig. 4). On the other hand, chocolate from CEPEC2002 SP had a decreased amount of acids, which could be reflected in the sour taste perception (Fig. 4). Overall, the inoculation process improved the chocolate sensorial qualities, as translated in chocolates being judged more acceptable (Table 3).

Several studies showed that yeast inoculation in cocoa fermentation modifies the chemical composition of fermented beans and consequently the chocolate sensorial profiles.[21,39,40] Batista *et al.*[19] reported a yeast cocktail applied in cocoa fermentation (*S. cerevisiae*, *P. kluyveri* and *Hanseniaspora uvarum*). Yeast inoculation accelerated carbohydrate consumption and ethanol

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production during the process. Also, judges reported stronger coffee and sour attributes of chocolate produced from the inoculated assay.[19]

According to previous work, *S. cerevisiae* and *P. kluyveri* probably inhibit the growth of another yeast (*H. uvarum*) used as inoculum. This was justified based on the ability of *S. cerevisiae* and *P. kluyveri* in producing toxins against other yeast genera.[41] Furthermore, other studies have mentioned that VOCs play a key role in microbial interaction avoiding a growth of competitor microorganisms.[42,43] Understanding VOCs released during cocoa fermentation would also enlighten microbial succession that occurs during this process.

Crafack *et al.*[40] analysed the impact of starter cultures on sensory attributes of chocolate. Fermentation inoculated with *P. kluyveri* in association with *Kluyveromyces marxianus* produced chocolates characterised as fruity, acid and bitter. *P. kluyveri* is considered a highly aromatic yeast, while *S. cerevisiae*, besides producing aromatic compounds, is considered a large alcohol producer and highly resistant to environmental conditions (e.g. pH, temperature, ethanol concentration).[4]

The proper performance of the fermentative process is pivotal for downstream steps (e.g. drying, roasting and conching) and hence for flavour compound formation.[44] Pyrazine compounds are formed mainly during the cocoa bean roasting process due to Maillard reaction. Moreover, pyrazine production has also been linked to *Bacillus* strains.[45]

Tetramethylpyrazine and trimethylpyrazine have been detected in all chocolate samples, although during fermentation (0 and 144 h) these compounds were not detected (Table 1). Tetramethylpyrazines have been reported in cocoa aroma, being related to pleasant flavour notes (e.g., nutty, roasted and chocolate).[39] The absence of *Bacillus* spp. and the temperatures reached in the fermentation have been reported as possible reasons for the absence of these compounds.[46]

The aroma admixture which characterises chocolate is not formed only by the presence of volatile compounds. As mentioned previously, the onset of proteolytic processes inside the beans occurs after embryo death due mainly to yeast action. Some studies confirmed the presence of endoprotease and carboxypeptidase.[47,48] Both these proteases are composed mainly of albumin and vicilin-class (7S) globulin, which can degrade the majority of protein content inside beans during fermentation.[49,50]

Free amino acids and hydrophilic peptides derived from the vicilin-class (7S) globular storage protein are necessary for the formation of the cocoa-specific flavours during the roasting process.[51,52]

In the present study, as an effect of the proteolytic processes inside the beans, the content of total protein decreased during all fermentations, both inoculated and spontaneous ones (Table 2). Furthermore, initial and final times of fermentation showed different protein profiles, as presented in Table 2.

Using the MALDI-TOF MS technique, it was possible to determine more precisely the pool of different peptide molecular masses and generate more adequate protein profiles. The MALDI-TOF MS technique delivered only qualitative information about proteins. Hence the lack of quantification of each ion peak did not allow an in-depth determination of the protein degradation dynamics. However, in agreement with sensorial analysis results, the yeast inoculation triggered modification in protein profiles.

According to the results observed in the AHC graph (Fig. 3), it can be possible to identify similarities among protein profiles of the inoculated fermentations at the end of the process (144 h). In addition, differences between the fermentation at 0 h can be explained by microbiota intrinsic to each hybrid. Overall, to clarify the influence of the protein/peptide profiles and VOCs on the final chocolate flavour, a clear need for exhaustive analysis with complementary techniques such as MALDI-TOF MS/MS is here highlighted.

This study showed that inoculation with *P. kluyveri* and *S. cerevisiae* yeast starter can influence the composition of VOCs and sensory profile of chocolates, enhancing the cocoa quality. Moreover, MALDI-TOF MS results corroborated that protein profiles also changed with fermentations.

Regarding the results of the CATA questionnaire, use of *S. cerevisiae* CCMA-UFLA 0200 as single inoculum showed a greater effect on changing chocolate sensory attributes. Although, based on the scores of the acceptance test, both chocolates produced from inoculated fermentations were most appreciated by judges.

A further in-depth investigation would be necessary to understand the modulation of each VOC throughout the fermentation process, as well as the protein degradation dynamics during inoculated fermentation.

AUTHOR CONTRIBUTIONS

R.S., N.L. and C.S. developed the project, planned the experiments and drafted the paper and made the final revision of the paper; I.M. performed the experiments; I.M., J.C. and L.V. analysed the data and wrote the paper.

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CONFLICTS OF INTEREST

Nothing to declare.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Vásquez ZS, Carvalho Neto DP, Pereira GVM, Vandenberghe LPS, de Oliveira PZ, Tiburcio PB *et al.*, Biotechnological approaches for cocoa waste management: a review. *Waste Manag* **90**:72–83 (2019). https://doi.org/10.1016/j.wasman.2019.04.030.

2 Voora V, Bermúdez S, Larrea C, Global Market Report. Sustainable Commodities Marketplace Series 62 (2019). http://www.jstor.com/stable/resrep22025.

3 Muñoz MS, Cortina JR, Vaillant FE and Parra SE, An overview of the physical and biochemical transformation of cocoa seeds to beans and to chocolate: flavor formation. *Crit Rev Food Sci Nutr* **60**: 1593–1613 (2020). https://doi.org/10.1080/10408398.2019.1581726.

4 Schwan RF and Fleet GH eds, *Cocoa and Coffee Fermentations*. CRC Press, Boca Raton, FL (2014).

5 Alayo EMC, Vásquez GI, Siche R and Toro FPC, Formation of aromatic compounds precursors during fermentation of Criollo and Forastero cocoa. *Heliyon* **5**:e01157 (2019). https://doi.org/10.1016/j.heliyon.2019.e01157.

© 2021 Society of Chemical Industry

6 Schwan RF and Wheals AE, The microbiology of cocoa fermentation and its role in chocolate quality. *Crit Rev Food Sci Nutr* **44**:205–221 (2004). https://doi.org/10.1080/10408690490464104.
7 Campos JR, Buendía HBE, Avila IO, Cervantes EL and Flores MEJ, Dynamics of volatile and non-volatile compounds in cocoa (*Theobroma cocoa* L.) during fermentation and drying process using principal components analysis. *Food Res Int* **44**:250–258 (2011). https://doi.org/10.1016/j.foodres.2010.10.028.
8 Ho VTT, Zhao J and Fleet G, Yeasts are essential for cocoa bean fermentation. *Int J Food Microbiol* **174**:72–87 (2014). https://doi.org/10.1016/j.ijfoodmicro.2013.12.014.
9 Serra JL, Moura FG, Pereira GVDM, Soccol CR, Rogez H and Darnet S, Determination of the microbial community in Amazonian cocoa bean fermentation by Illumina-based metagenomic sequencing. *LWT Food Sci Technol* **106**:229–239 (2019). https://doi.org/10.1016/j.lwt.2019.02.038.
10 Ho VTT, Fleet G and Zhao J, Unravelling the contribution of lactic acid bacteria and acetic acid bacteria to cocoa fermentation using inoculated organisms. *Int J Food Microbiol* **279**:43–56 (2018). https://doi.org/10.1016/j.ijfoodmicro.2018.04.040.
11 Ouattara HD, Ouattara HG, Droux M, Reverchon S, Nasser W and Niamke SL, Lactic acid bacteria involved in cocoa beans fermentation from Ivory Coast: species diversity and citrate lyase production. *Int J Food Microbiol* **256**:11–19 (2017). https://doi.org/10.1016/j.ijfoodmicro.2017.05.008.
12 Viesser JA, Pereira GVM, Neto DPC, Vandenberghe LP, Azevedo V, Brenig B *et al.*, Exploring the contribution of fructophilic lactic acid bacteria to cocoa beans fermentation: isolation, selection and evaluation. *Food Res Int* **136**:109478 (2020). https://doi.org/10.1016/j.foodres.2020.109478.
13 Clair BJA, Koné MK, Kouamé K, Lahon MC, Berthiot L, Durand N *et al.*, Effect of aroma potential of *Saccharomyces cerevisiae* fermentation on the volatile profile of raw cocoa and sensory attributes of chocolate produced thereof. *Eur Food Res Technol* **245**:1459–1471 (2019). https://doi.org/10.1007/s00217-018-3181-6.
14 Gutierrez JM, Botta C, Ferrocino I, Giordano M, Bertolino M, Dolci P *et al.*, Dynamics and biodiversity of bacterial and yeast communities during fermentation of cocoa beans. *Appl Environ Microbiol* **84**:1–17 (2018). https://doi.org/10.1128/AEM.01164-18.
15 Romanens E, Näf R, Lobmaier T, Pedan V, Leischtfeld SF, Meile L *et al.*, A lab-scale model system for cocoa bean fermentation. *Appl Microbiol Biotechnol* **102**:3349–3362 (2018). https://doi.org/10.1007/s00253-018-8835-6.
16 Hamdouche Y, Christophe J, Lebrun M, Guehi T, Boulanger R, Teyssier C *et al.*, Impact of turning, pod storage and fermentation time on microbial ecology and volatile composition of cocoa beans. *Food Res Int* **119**:477–491 (2019). https://doi.org/10.1016/j.foodres.2019.01.001.
17 Hernández CF, Gutierrez JM and Ferrocino I, The challenges and perspectives of the selection of starter cultures for fermented cocoa beans. *Int J Food Microbiol* **301**:41–50 (2019). https://doi.org/10.1016/j.ijfoodmicro.2019.05.002.
18 John WA, Böttcher NL, Aßkamp M, Bergounhou A, Kumari N, Ho P *et al.*, Forcing fermentation: profiling proteins, peptides and polyphenols in lab-scale cocoa bean fermentation. *Food Chem* **278**:786–794 (2018). https://doi.org/10.1016/j.foodchem.2018.11.108.
19 Batista NN, Ramos CL, Ribeiro DD, Pinheiro ACM and Schwan RF, Dynamic behavior of *Saccharomyces cerevisiae*, *Pichia kluyveri* and *Hanseniaspora uvarum* during spontaneous and inoculated cocoa fermentations and their effect on sensory characteristics of chocolate. *LWT Food Sci Technol* **63**:221–227 (2015). https://doi.org/10.1016/j.lwt.2015.03.051.
20 Gutierrez JM, Pereira LB, Ferrocino I and Cocolin L, Traceability of functional volatile compounds and its potential health benefits. *Nutrients* **11**:2–23 (2019). https://doi.org/10.3390/nu11040884.
21 Moreira IMV, Vilela LF, Miguel MGCP, Santos C, Lima N and Schwan RF, Impact of a microbial cocktail used as a starter culture on cocoa fermentation and chocolate flavour. *Molecules* **22**:766 (2017). https://doi.org/10.3390/molecules22050766.
22 Ramos CL, Dias DR, Miguel MG d CP and Schwan RF, Impact of different cocoa hybrids (*Theobroma cacao* L.) and *S. cerevisiae* UFLA CA11 inoculation on microbial communities and volatile compounds of cocoa fermentation. *Food Res Int* **64**:908–918 (2014). https://doi.org/10.1016/j.foodres.2014.08.033.
23 Moreira N, Lopes P, Cabral M and Pinho PG, HS-SPME/GC-MS methodologies for the analysis of volatile compounds in cork material. *Eur Food Res Technol* **242**:457–466 (2016). https://doi.org/10.1007/s00217-016-2636-x.
24 Petisca C, Palacios TP, Farah A, Pinho O and Ferreira I, Furans and other volatile compounds in ground roasted and espresso coffee using headspace solid-phase microextraction: effect of roasting speed. *Food Bioprod Process* **91**:233–241 (2013). https://doi.org/10.1016/j.fbp.2012.10.003.
25 Voigt J and Biehl B, The major seed proteins of *Theobroma cacao* L. *Food Chem* **47**:145–151 (1993). https://doi.org/10.1016/0308-8146(93)90236-9.
26 Bradford MM, A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254 (1976). https://doi.org/10.1016/0003-2697(76)90527-3.
27 Miguel MGCP, Reis VVC, Efraim P, Santos C, Lima N and Schwan RF, Cocoa fermentation: microbial identification by MALDI-TOF MS and sensory evaluation of produced chocolate. *LWT Food Sci Technol* **77**:351–362 (2017). https://doi.org/10.1016/j.lwt.2016.11.076.
28 Neto RL, Santos C, Lima N, Sampaio P, Pais C and Neves RP, Application of MALDI-TOF MS for requalification of a Candida clinical isolates culture collection. *Braz J Microbiol* **45**:515–522 (2014). https://doi.org/10.1590/s1517-83822014005000044.
29 Oliveira M, Santos C, Sampaio P, Romeo O, Paes RA, Pais C *et al.*, Development and optimization of a new MALDI-TOF protocol for identification of the Sporothrix species complex. *Res Microbiol* **166**:102–110 (2015). https://doi.org/10.1016/j.resmic.2014.12.008.
30 Matos AMF, Moreira LM, Barczewski BF, Matos LX, Oliveira JBV, Pimentel MIF *et al.*, Identification by MALDI-TOF MS of *Sporothrix brasiliensis* isolated from a subconjunctival infiltrative lesion in an immunocompetent patient. *Microorganisms* **8**:22–33 (2020). https://doi.org/10.3390/microorganisms8010022.
31 Paziani MH, Carvalho LT, Melhem MSC, Almeida MTG, Silva MENB, Martinez R *et al.*, First comprehensive report of clinical *Fusarium* strains isolated in the state of Sao Paulo (Brazil) and identified by MALDI-TOF MS and molecular biology. *Microorganisms* **8**:66 (2020). https://doi.org/10.3390/microorganisms8010066.
32 Fernández HL, Santos HM, Capelo JL, Riverola FF, Peña DG and Jato MR, Mass-up: an all-in-one open software application for MALDI-TOF mass spectrometry knowledge discovery. *BMC Bioinf* **16**:1–12 (2015). https://doi.org/10.1186/s12859-015-0752-4.
33 Moreira IMV, Vilela LF, Santos C, Lima N and Schwan RF, Volatile compounds and protein profiles analyses of fermented cocoa beans and chocolates from different hybrids cultivated in Brazil. *Food Res Int* **109**:196–203 (2018). https://doi.org/10.1016/j.foodres.2018.04.012.
34 Stone HS and Sidel JL eds, *Sensory Evaluation Practices*. Academic Press, Los Angeles, CA (2004).
35 Rodriguez-Campos J, Escalona-Buendía HB, Contreras-Ramos SM, Orozco-Avila I, Jaramillo-Flores E and Lugo-Cervantes E, Effect of fermentation time and drying temperature on volatile compounds in cocoa. *Food Chem* **132**:277–288 (2012). https://doi.org/10.1016/j.foodchem.2011.10.078.
36 Passos FML, Lopez AS and Silva DO, Aeration and its influence on the microbial sequence in cacao fermentations in Bahia, with emphasis on lactic acid bacteria. *J Food Sci* **49**:1470–1474 (1984). https://doi.org/10.1111/j.1365-2621.1984.tb12823.x.
37 Camu N, de Winter T, Addo SK, Takarama JS, Bernaert H and de Vuyst L, Fermentation of cocoa beans: influence of microbial activities and polyphenol concentrations on the flavour of chocolate. *J Sci Food Agric* **88**:2288–2297 (2008). https://doi.org/10.1002/jsfa.3349.
38 Marseglia A, Musci M, Rinaldi M, Palla G and Caligiani A, Volatile fingerprint of unroasted and roasted cocoa beans (*Theobroma cacao* L.) from different geographical origins. *Food Res Int* **132**:101–109 (2020). https://doi.org/10.1016/j.foodres.2020.109101.
39 Rottiers H, Sosa DAT, de Winne A, Ruales J, de Clippeleer J, de Leersnyder I *et al.*, Dynamics of volatile compounds and flavor precursors during spontaneous fermentation of fine flavor Trinitario cocoa beans. *Eur Food Res Technol* **245**:1917–1937 (2019). https://doi.org/10.1007/s00217-019-03307-y.
40 Crafack M, Keul H, Eskildsen CE, Petersen MA, Saerens S, Blennow A *et al.*, Impact of starter cultures and fermentation techniques on the volatile aroma and sensory profile of chocolate. *Food Res Int* **63**:306–316 (2014). https://doi.org/10.1016/j.foodres.2014.04.032.
41 Branco P, Francisco D, Chambon C, Hébraud M, Arneborg N, Almeida MG *et al.*, Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in

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wine microbial interactions. *Appl Microbiol Biotechnol* **98**:843–853 (2014). https://doi.org/10.1007/s00253-013-5411-y.

42 Ruiz-Moyano S, Hernández A, Galvan AI, Córdoba MG, Casquete R, Serradilla MJ *et al.*, Selection and application of antifungal VOCs-producing yeasts as biocontrol agents of grey mould in fruits. *Food Microbiol* **92**:103556 (2020). https://doi.org/10.1016/j.fm.2020.103556.

43 Contarino R, Brighina S, Fallico B, Cirvilleri G, Parafati L and Restuccia C, Volatile organic compounds (VOCs) produced by biocontrol yeasts. *Food Microbiol* **82**:70–74 (2019). https://doi.org/10.1016/j.fm.2019.01.008.

44 Jinap S, Ikrawan Y, Bakar J, Saari N and Lioe HN, Aroma precursors and methylpyrazines in underfermented cocoa beans induced by endogenous carboxypeptidase. *J Food Sci* **73**:H141–H147 (2008). https://doi.org/10.1111/j.1750-3841.2008.00858.x.

45 Oracz J and Nebesny E, Effect of roasting parameters on the physicochemical characteristics of high-molecular-weight Maillard reaction products isolated from cocoa beans of different *Theobroma cacao* L. groups. *Eur Food Res Technol* **245**:111–128 (2019). https://doi.org/10.1007/s00217-018-3144-y.

46 Jinap S, Harun SM and Ghazali NM, Formation of methyl pyrazine during cocoa bean fermentation. *Int J Trop Agric Sci* **17**:27–32 (1994).

47 Voigt J, Biehl B, Heinrichs H, Kamaruddin S, Marsoner GG and Hugi A, In-vitro formation of cocoa-specific aroma precursors: aroma-related peptides generated from cocoa-seed protein by co-operation of an aspartic endoprotease and a carboxypeptidase. *Food Chem* **49**:173–180 (1994). https://doi.org/10.1016/0308-8146(94)90155-4.

48 Brunetto MR, Gallignani M, Orozco W, Clavijo S, Delgado Y, Ayala C *et al.*, The effect of fermentation and roasting on free amino acids profile in Criollo cocoa (*Theobroma cacao* L.) grown in Venezuela. *Braz J Food Technol* **23**:1–12 (2020). https://doi.org/10.1590/1981-6723.15019.

49 Voigt J and Lieberei R, Biochemistry of cocoa fermentation, in *Cocoa and Coffee Fermentation*, ed. by Schwan RF and Fleet GH. CRC Press, Boca Raton, FL, pp. 193–227 (2014).

50 D'Souza RN, Grimbs A, Grimbs S, Behrends B, Corno M, Ullrich MS *et al.*, Degradation of cocoa proteins into oligopeptides during spontaneous fermentation of cocoa beans. *Food Res Int* **109**:506–516 (2018). https://doi.org/10.1016/j.foodres.2018.04.068.

51 Scalone GLL, Taube KT, de Meulenaer B, de Kimpe N, Wöstemeyer J and Voigt J, Cocoa-specific flavor components and their peptide precursors. *Food Res Int* **123**:503–515 (2019). https://doi.org/10.1016/j.foodres.2019.05.019.

52 Voigt J, Janek K, Taube KT, Niewienda A and Wöstemeyer J, Partial purification and characterization of the peptide precursors of the cocoa-specific aroma components. *Food Chem* **192**:706–713 (2016). https://doi.org/10.1016/j.foodchem.2015.07.068.

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