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Fermentation Progression and Quality Attributes of Trinitario and Refractario Cacao (*Theobroma cacao* L.) Hybrid Groups at the International Cocoa Genebank Trinidad (ICGT) – Opportunities for Genetic Branding

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Abstract

Theobroma cacao L. is an important tropical tree crop that is grown for its beans used in the food, beverage, cosmetic and pharmaceutical industries. There are ten genetic clusters and two hybrid groups (Trinitario and Refractario) originating from the admixing of several of the genetic clusters recognised within cacao. Although the two hybrid groups are highly regarded in the fine or flavour market segment of the cocoa industry, their fermentation behaviour and distinctive flavour/nutraceutical attributes are not fully understood. The aim of this study was to determine the fermentation behaviour, flavour, nutraceutical profiles and the market potential of these two hybrid groups grown in the same environment at the International Cocoa Genebank Trinidad (ICGT). The beans from the two groups were separately fermented with replications over three years using a small-scale box fermentation method. The samples collected at 0, 2, 4, 6 and 8 days of fermentation were sun-dried to 6.5-7.0% moisture content and analysed chemically and sensorially. The temperature and pH trends measured during fermentation were different but congruent with proper fermentation. Sensory assessment identified higher intensities of floral flavour in Trinitario samples fermented for 6 and 8 days. Overlaying chromatograms obtained via Gas Chromatography–Mass Spectrometry of samples belonging to both hybrids fermented for 6 days showed that Trinitario exhibited peaks not present in the chromatograms for Refractario, namely for 2-Heptanone (fruity, flowery), 2-Heptanol (sweet, citrus) and 2-Nonanone (fruity). Analysis via High-Performance Liquid Chromatography of samples fermented for 8 days show that Trinitario beans contained more theobromine, caffeine and (-)-epicatechin while Refractario beans had higher quantities of (+)-catechin and procyanidin B2. The study demonstrated the uniqueness of the two hybrids in relation to sensory characteristics and nutraceuticals; and underscored the importance of genetics and fermentation time on flavour diversity, which could be exploited through genetics-based branding.

Introduction

Distinct flavour profiles, health benefits and mood enhancing effects of cocoa are becoming increasingly important considerations in marketing. There is also heightened interest in origin specific- and genetics-based branding (Filou 2016). In cocoa, the harnessing of genetic diversity towards market differentiation requires a clear demonstration of genetic distinctiveness of groups for market attributes as well as technology toolkits and organisation systems to exploit these commercially, as suggested by Elliot (2013).

The International Cocoa Genebank Trinidad (ICGT), a field collection of 2400 accessions planted in plots of 8-16 trees per accession in a single site and is an ideal resource to assess the genetic influences on marketable attributes without the confounding effects of the growing environment. Bekele et al. (2010) estimated that there were approximately 236 Trinitario accessions and 716 Refractario accessions held at ICGT. Trinitario cacao from Trinidad has been theorised to have arisen out of hybridisation between an existing Criollo cacao base with introductions from the lower Amazon Forastero following a calamity in 1727 (Motilal and Sreenivasan 2012). A number of studies have subsequently confirmed the genetic architecture of the Trinitario group (Yang et al. 2013).

The Refractario group was selected from farmers' fields in Ecuador based on their putative resistance to Witches' Broom Disease caused by *Moniliophthora perniciosa* and introduced into Trinidad in 1937 by F.J. Pound. They have a genetic composition that suggests hybrid origin - an admixture of the genetic cluster 'Nacional' with others of upper Amazon origin (Zhang et al. 2008). Bartley (2001) indicated that there were approximately six types within Refractario each with distinct origins. Although both Trinitario and Refractario groups are of hybrid origin, they are quite distinctive, not only genetically, but also phenotypically based on pod index, bean size and flower morphology (Bekele et al. 2010), with the

Trinitario group having larger fresh bean weight, lower pod index and shorter style lengths compared to the Refractario group.

Although both hybrid groups are highly valued in the fine or flavour segment of the cocoa bean market (ICCO 2017), there have been no studies on the distinctive quality attributes when grown in the same location and processed separately using the same method. The overall objective of the present study is to understand the fermentation behaviour, flavour and nutraceutical profiles of these two distinctive groups sans effects of growing and processing location, as these have been shown to have a significant effect on final quality contributing to a "terroir" effect in Cocoa (Sukha et al. 2014). From the data obtained, potentially marketable traits can be identified and exploited in a genetic branding strategy.

Methodology

Location, trees and season

The study was conducted over a 3-year period utilising a random sample of beans obtained from 30 year old cocoa trees belonging to the Trinitario and Refractario groups held at the ICGT, Centeno (10°34' N latitude and 61°18'W longitude, at an altitude of 15 m above sea level). The study was conducted each year during the main harvesting season December to February. Accessions and trees belonging to these groups have been correctly identified in a previous study (Motilal et al. 2013).

Harvest, fermentation and drying

The pods were carefully harvested, cracked within 3 days and subjected to fermentation and drying at a single location *viz.* the fermentation and drying facility at Cocoa Research Centre, The University of the West Indies. Approximately 30kg of pulp-covered beans were collected from each genetic hybrid group under standardised conditions and fermented in separate customised Styrofoam boxes for 8 days with turning on days 3 and 5 (Ali et al. 2014). During fermentation sampling was done on days 0, 2, 4, 6 and 8 and beans dried on separate, labelled, slatted wooden trays, (L [76cm] × W [56cm]; 1cm between slats), arranged in ridges and furrows and turned regularly, according to standard drying protocols.

Measurements, sample analyses and assessments

Temperature (Digi-Sense 94460-40 thermometer) readings were taken on each day (0-8 days) of fermentation in 3 zones (10-16cm depth; western end of box, left corner; middle of box; eastern end of box, right corner). pH of testa and cotyledon (Oakton® Acorn pH meter, featuring pH5 meter, Model no. WD-35613-70) were measured for samples withdrawn from the same 3 zones and a composite derived for testing (in triplicate) on days 0, 2, 4, 6 and 8. Composite samples were fermented for 4, 6 and 8 days and dried to a 6.5-7% moisture range (Burrows Digital Moisture Computer; model 700, serial no: 24542) and bean parameters measured. Bean count and individual bean weights were determined (in triplicate) using a top loading balance (Navigator, Ohaus) as specified by End and Dand (2015). Bean length, width and thickness were determined (10 replicates) using an electronic digital caliper (Neiko 01407A) as specified by Bart-Plange and Baryeh (2003). All measurements were collected over a 3-year period.

Theobromine, caffeine, procyanidin B2, (+)-catechin and (-)-epicatechin levels (3 replicates) were generated from dry composite samples of the two hybrids fermented for 0, 4, 6 and 8 days for 2 crop years at Hamburg School of Food Science, Institute of Food Chemistry, University of Hamburg using High-Performance Liquid Chromatography (HPLC). Gas Chromatography-Mass Spectrometry (GC-MS) was carried out on similar dried composite samples fermented for 0, 4, and 6 days at Hamburg University of Applied Sciences to identify flavour volatiles.

Composite samples fermented for 4, 6 and 8 days and dried to a 6.5-7% moisture range were converted to liquors as outlined by Sukha et al. (2008). Sensory assessment of coded, randomised samples with two replicates was carried out by a trained panel of six using the protocol and sensory evaluation template of ESSeguine-DASukha Cocoa and Chocolate Flavour Evaluation by ES Seguine and DA Sukha licensed under a Creative Commons Attribution-ShareAlike 4.0 International License. This was done for 3 years, however only year 1 data was analysed. Statistical analyses (Analysis of Variance [ANOVA], Newman-Keuls Multiple Comparison [N-KMC] and Fisher's Least Significance Difference [LSD] Tests) of data were done using NCSS Version 07.1.19, Hintze, J. (2009), NCSS, LLC, Kaysville, Utah, USA.

Results

Fermentation monitoring

a. Temperature

Both genetic hybrids produced fermentation temperatures greater than 44°C required for bean death and thorough fermentation. Table 1 summarises the significant effects for selected factors analysed using ANOVA.

Table 1. Summary of significant effects for fermentation temperature for group, year and day from ANOVA

Factor	F Value	Significance
Group	34.97	***
Year	53.37	***
Day	61.32	***

NS, *, **,***Non-significant or significant at P≤ 0.05, 0.01 and 0.001.

Zone differences were not significant and there was a significant group × day interaction. Both hybrid groups exhibited temperature peaks on day 4 (Trinitario - 46.5°C and Refractario - 48.0°C). On day 6 Refractario exhibited a second larger temperature peak (48.9°C) and Trinitario temperature was similar to what it was on day 5 (42.3°C). Applying Fisher's Least Significance Difference (LSD) Test revealed that temperature peaks for hybrids on day 4 were not significantly different however, peaks on day 6 were significantly different, with Refractario having a higher mean temperature (Figure 1). According to Newman-Keuls Multiple Comparison (N-KMC) Test, means for years 1 and 3 were similar.

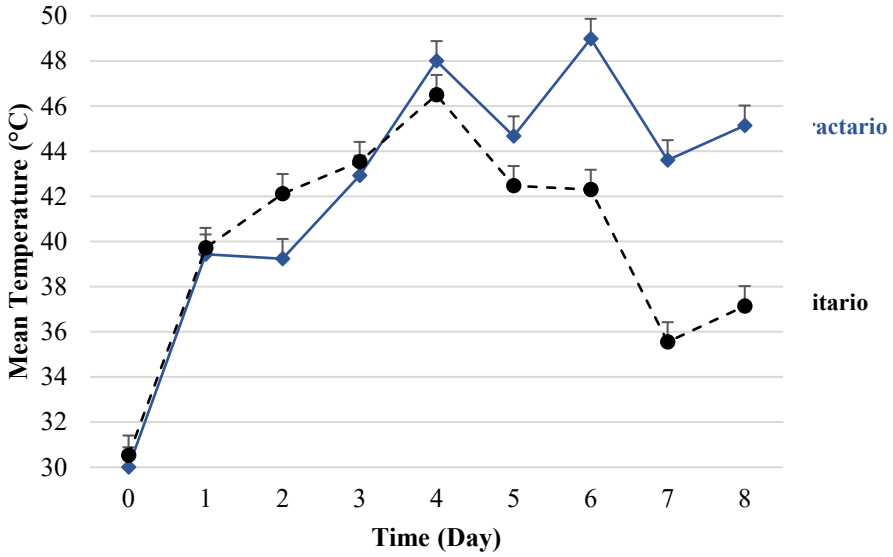

Figure 1. Temperature changes exhibited by Hybrids during fermentation

b. pH of testa and cotyledon

Results revealed that differences between pH values of testing replicates were not significant, however, there was significant group × day interactions. Though pH readings were significantly different, both hybrid groups showed similar pH trends, that is, a decrease in cotyledon pH and an increase in testa pH as fermentation progressed until day 4; by day 6 Trinitario cotyledon pH started increasing and Refractario pH readings were remaining consistent, see Figure 2. Applying Fisher's LSD Test revealed both groups were significantly different in terms of testa and cotyledon pH on day 6. According to N-KMC Test, means for years 2 and 3 were similar.

Table 2. Summary of significant effects for testa and cotyledon pH for group, year and day from ANOVA

	Testa pH		Cotyledon pH	
Factor	**F Value**	**Significance**	**F Value**	**Significance**
Group	131.92	***	23.94	***
Year	10.41	***	3.70	*
Day	66.81	***	42.54	***

NS, *, **,***Non-significant or significant at P≤ 0.05, 0.01 and 0.001.

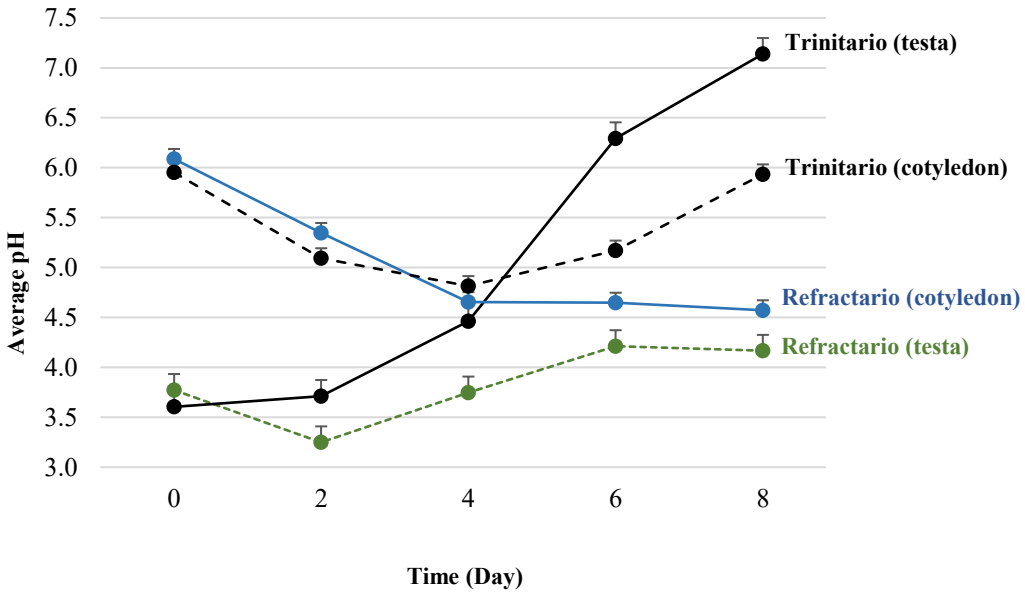

Figure 2. pH changes during fermentation

Physical attributes

a. Bean width, length and thickness

Data analysis revealed that only group effect was significant with respect to both bean width and thickness (Table 3) with the means for Trinitario higher than those for Refractario. There were no significant differences between years, replicates or fermentation days.

Table 3. Summary of significant effects for dried cocoa bean measurements for width, length and thickness from ANOVA

	Bean width		Bean length		Bean thickness	
Factor	**F Value**	**Sig.**	**F Value**	**Sig.**	**F Value**	**Sig.**
Group	17.96	***	18.35	***	10.69	**

NS, *, **,***Non-significant or significant at P≤ 0.05, 0.01 and 0.001.

b. Bean count and individual bean weight

Trinitario beans had lower bean counts and higher individual bean weights than Refractario. Average bean count and individual bean weight ranged from 68 - 72 and 1.39 - 1.47g respectively for Trinitario beans and 79 - 84 and 1.1 9- 1.26g for Refractario beans.

Chemical Attributes

a. Polyphenol and alkaloid measurements using HPLC

Overall, means for Trinitario were higher than Refractario for the polyphenol and alkaloid compounds of interest. Means for year 1 were larger for caffeine and (-)-epicatechin. There were no significant differences between testing replicates. In terms of interactions, genetic group × year interaction was significant for all chemical attributes assessed except (+)-catechin. Genetic group × fermentation time interaction was significant for all chemical attributes except caffeine and year × fermentation interaction was significant for all chemical attributes assessed. Table 4 summarises significant effects for samples fermented for 0, 4, 6 and 8 days over a 2-year period.

Table 4. Summary of significant effects for alkaloids and polyphenols for group, year and day from ANOVA

Factor	Theobromine		Caffeine		Procyanidin B2		(+)-catechin		(-)-epicatechin	
	F Value	Sig.	F Value	Sig.	F Value	Sig.	F Value	Sig.	F Value	Sig.
Group	26.00	***	146.70	***	14.83	***	27.3	***	0.92	NS
Year	0.69	NS	14.75	***	1.18	NS	0.16	NS	17.75	***
Day	57.06	***	5.88	***	19.47	***	41.17	***	782.82	***

NS, *, **,***Non-significant or significant at P≤ 0.05, 0.01 and 0.001.

Graphs comparing levels of alkaloids and polyphenols on days 6 and 8 of fermentation revealed higher levels of procyanidin B2, (+)- catechin and (-)- epicatechin on day 6 compared to day 8 for Trinitario beans. Refractario beans also exhibited decreases in these compounds on day 8, see Figures 3 and 4.

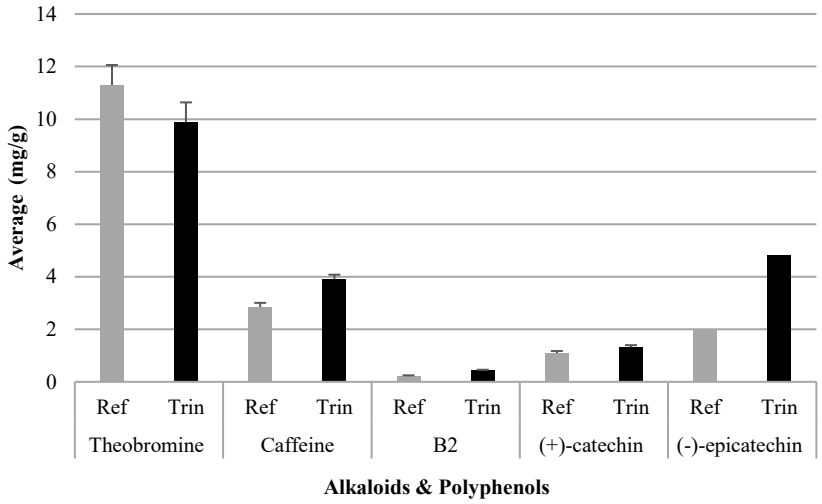

Figure 3. Alkaloid and polyphenol profile for beans fermented for 6 days

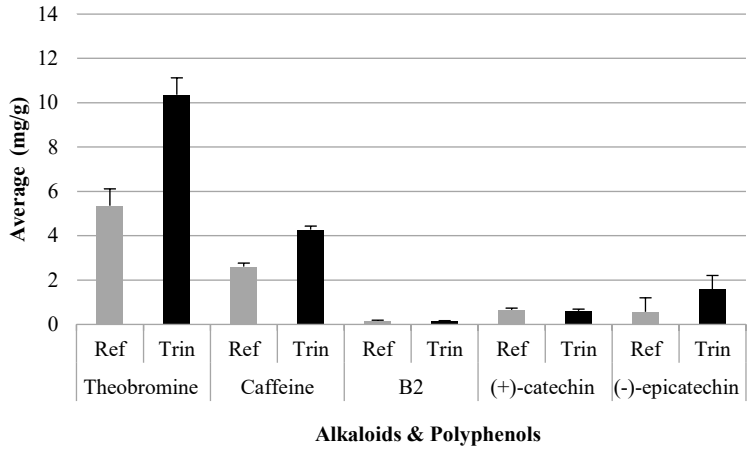

Figure 4. Alkaloid and polyphenol profile for beans fermented for 8 days

b. Flavour volatile identification using GC-MS

Samples fermented for 6 days had the highest number of positively perceived compounds identified. Overlay of chromatograms generated using GC-MS data for both hybrids at 6 days of fermentation (Figure 5) showed aroma volatiles that were identified. Peaks in continuous black lines represent Trinitario and peaks in grey slashed lines represent Refractario. Labelling is also colour coordinated in black and blue respectively. In cases where both samples possessed the same compound and peaks are overlapping the label is in the colour of the higher (more dominant) peak.

Some volatile compounds identified, and their associated sensory attributes based on literature include, in Trinitario, 2-Heptanol- citrusy (odour quality), fruity (sensory perception); 2-Heptanone- fruity, floral (odour quality), fruity, floral (sensory perception). In Refractario, ethyl acetate- pineapple (odour quality), fruity (sensory perception) was observed (Rodriguez-Campos et al. 2012).

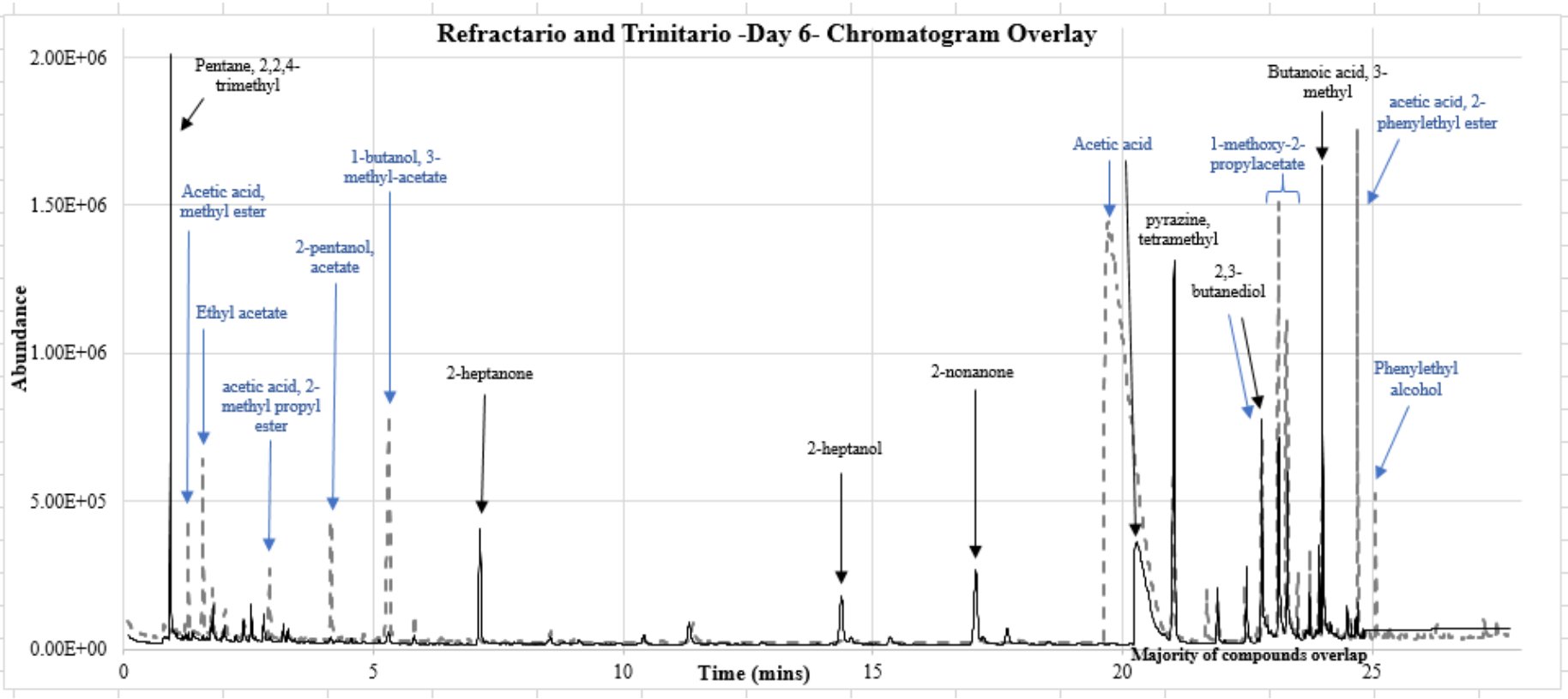

Figure 5. Overlay of chromatograms from GC-MS analyses for Refractario and Trinitario bean samples fermented for 6 days

*Trinitario and peaks in grey slashed lines represent Refractario. Labelling is also colour coordinated in black and blue respectively. In cases where both samples possessed the same compound and peaks are overlapping the label is in the colour of the higher (more dominant) peak.

Sensory attributes

ANOVA revealed that the hybrids were significantly different in terms of acidity scores ($P \leq 0.01$; F value =6.43). Applying Fisher's LSD Test revealed that the hybrids were significantly different in terms of scores for spice on day 6 and on day 8. Fisher's LSD Test also confirmed a significant decrease of fresh fruit in Refractario from day 6 to day 8 and in fresh fruit and spice notes in Trinitario from day 6 to day 8 (Figures 6 &7). Both hybrids were scored for desirable floral, fruity, spice and nutty notes. However, Trinitario beans fermented for 4 and 6 days had a spice note present and by day 8 this was not perceived. Refractario however, maintained higher woody and nutty notes as fermentation time progressed and was scored for a spice note on day 8.

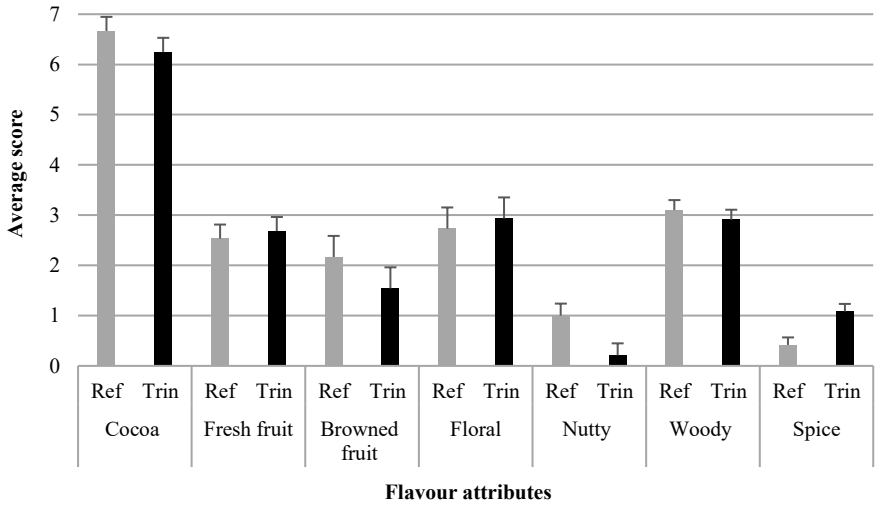

Figure 6. Flavour profile for beans fermented for 6 days

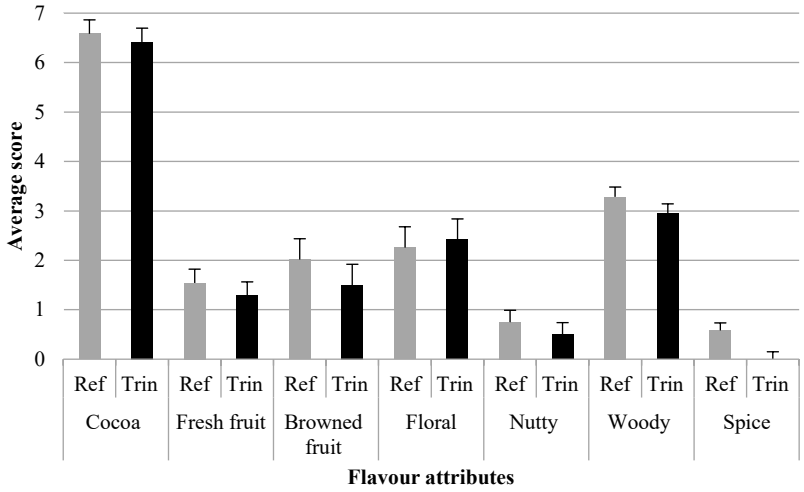

Figure 7. Flavour profile for beans fermented for 8 days

Discussion

The results show that the optimum time to end fermentation for Trinitario (6 days) was shorter than for Refractario for which a longer fermentation was suggested by the temperature and testa and cotyledon pH data. While the cotyledon pH started increasing after day 4 in Trinitario, which is an indication of over fermentation, the pH was consistent for Refractario. Based on pH readings alone, day 6 would have been the time to terminate fermentations for Trinitario. Furthermore, the fact that the testa pH was significantly higher during 2-8 days of fermentation in Trinitario compared to Refractario suggests possible differences

among genetic groups with regards to pulp quantity or quality that warrants further investigation. The results suggest that the conventional 7-8 day fermentation (Schwan 1998) would have resulted in some level of over fermentation in Trinitario but not in Refractario.

The bean size was significantly larger for Trinitario compared to Refractario which resulted in a smaller bean count for Trintario than Refractario, which is in conformity with the findings of Bekele et al (2010). Bean size and bean uniformity (dried beans) are important physical attributes, as large beans consist of more cotyledon, the economically valuable part of the bean, whereas small beans consist of more shell. Bean uniformity impacts roasting, as large beans are likely to under-roast and small beans over-roast, thus resulting in improper expression of beans' flavour potential. (End and Dand 2015). Although both hybrid groups produced uniform beans, Trinitario beans were better in terms of individual bean weight and bean count.

At the optimum fermentation time, the Trinitario (6 days) showed a significantly higher level of fresh fruit, floral and spice notes compared to Refractario (8 days) based on the sensory analysis. In contrast the level of nutty and brown fruit notes were more prominent in Refractario than Trinitario.

In cocoa beans, alkaloids comprise theobromine and caffeine; and polyphenols comprise catechins (flavan-3-ols), anthocyanins and proanthocyanidins. Catechins are represented by (-)-epicatechin, (+)-catechin, (+)-gallocatechin and (-)-epigallocatechin. The most important proanthocyanidins are B1, B2, B3, B4, B5, C1 and D (Aprotosoaie at al. 2016). The chemical analyses revealed higher levels of procyanidin B2, (+)-catechin and (-)- epicatechin on day 6 compared to day 8 for Trinitario beans, which is similar the optimum times found with the sensory results. These together suggest that ending fermentation at 6 days is beneficial with respect to both sensory and nutraceutical levels for Trinitario. With regards to Refractario, although 7-8 day fermentation was suggested, this showed decreases in the level of all nutraceutical compounds. This is expected (Afoakwa et al. 2012), these compounds decrease with increase in fermentation time. Caffeine content varied most significantly among groups and (-)-epicatechin content decreased as fermentation progressed.

Kadow et al. (2013) reported differences in pulp and cotyledon aroma volatiles in different genotypes which undoubtedly can impact final flavour. The GC-MS overlay obtained in this study revealed differences, among the two hybrid groups in aroma volatiles perceived. Many of these compounds correspond to the fresh fruit and floral scores perceived in the sensory assessment. Refractario, maintained higher woody and nutty notes as fermentation time progressed (perhaps associated with pyrazine compound identified via GC-MS). Refractario also scored higher for a spice note on day 8. According to sensory scores obtained, it appears as though a shorter fermentation time for Trinitario beans may facilitate better expression of fruity, floral and spice notes. Kadow et al. (2013) also concluded that 2-heptanol, 2-heptanone and 2-nonanone significantly contribute to "fine/ flavour". Interestingly, these compounds have also been found in cocoa liquor by Counet et al. (2004) and were found to be distinct to the Trinitario beans in this study.

A study by Frauendorfer and Schieberle (2008) explored the changes in volatiles with roasting and concluded that the same compounds were present in the unroasted and roasted cocoa beans, respectively, although they differed in their intensity. These findings substantiate the findings of this study where the aroma volatiles identified in unroasted beans corresponded to flavour notes scored in cocoa liquors produced from roasted beans. This information can be leveraged when marketing beans.

Conclusion

Based on temperature, pH, chemical and sensory analyses data it was established that 6 days would be the best fermentation time for Trinitario. However, temperature and pH trends indicated that Refractrio could benefit from a longer fermentation time (7-8 days), which can negatively impact the development of fresh fruit notes.

The study has therefore demonstrated the potential marketable uniqueness of the hybrids in relation to variability in physical, sensory and chemical characteristics and the results underscore the importance of genetics and fermentation time on quality, which can be exploited to develop genetic branding strategies. The study builds on the genetic and morphological distinctions noted previously and can be used as a basis for further research and eventual application of genetic group-specific postharvest protocols.

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