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Linking cocoa varietals and microbial diversity of Nicaraguan fine cocoa bean fermentations and their impact on final cocoa quality appreciation

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ABSTRACT

Nicaraguan cocoa bean fermentations of several single local cocoa varieties originating from the same region (North Highlands of Nicaragua, San Jose de Bocay/El Cuá) were compared to fermentations of blended cocoa varietals from other producing regions of the country (Waslala and Nueva Guinea) making use of High Throughput Sequencing techniques, metabolite target analysis and sensory evaluation of cocoa liquor samples. A succession of the important cocoa-related yeasts *Hanseniaspora uvarum/opuntiae*, *Saccharomyces cerevisiae* and/or *Pichia kudriavzevii* was seen for single varietals and Nueva Guinea fermentations, while *Kazachstania humilis* dominated the mid and end phase of the Waslala cocoa fermentations. *Tatumella* species (mainly *Tatumella terrea* and *Tatumella punctata*) predominated the bacterial community at the onset of all fermentations followed by unusually late (generally 2 days into the fermentations) appearance of *Lactobacillus fermentum* relative to fermentations in other parts of the World. *Acetobacter* spp. were the main acetic acid bacteria during all fermentations, but also *Gluconobacter* spp. were involved in some single-variety fermentations. All fermentations proved complete as determined by metabolite analysis with bean sucrose being fully depleted and pulp sugars exhausted after 48–72 h of fermentation. From an organoleptic point of view, all Nicaraguan cocoas of this study reflected fine fruity (citrus or berry-like) flavours with distinct herbal or caramel notes. Floral notes were associated with the cases where *P. kudriavzevii* was involved in the later stages of fermentation. Intense citrus/fruity character was related to high pulp and bean citrate concentrations. Off-notes were found in some over-fermented batches where *Bacillus* spp. was detected. No relation between cut-test results and organoleptic appreciation was seen.

1. Introduction

Cacao was domesticated in Mesoamerica 3000 years ago (McNeil, 2009). The crop was widely spread in Central and South America even before the Spanish arrived, but the commercial production began in the 18th century (Coe, 2007; Cruz et al., 1995). Although Central America represents nowadays only 0.2–0.3% of the global cocoa production (https://www.icco.org), this region has during the last decade attracted the interest of the fine cocoa market. Genetic studies have shown that several fine cocoa varieties, mainly Trinitario- and Acriollado-type hybrids, can still be found in most of the countries of this region (Ji et al., 2013; Motilal et al., 2010; Trognitz et al., 2011, 2013). Therefore, during recent years a range of projects have focused on reproduction of the precious original germplasm aided by improved farm management. High-quality cocoa from Central American countries can enjoy premium prices in the international markets which can be as high as 3 times the stock market prices for bulk cocoa.

The raw cocoa beans, once scooped out of freshly harvested cocoa pods, need to undergo a complex fermentation and drying process before they develop the typical “chocolate” flavours consumers are seeking for. The fresh pulp-bean mass is naturally inoculated with microbes from the surrounding environment, namely yeasts, Enterobacteriaceae, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (Camu et al., 2007; Nielsen et al., 2007; Papalexandratou et al.,

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2011b, 2011c). The initial populations of the different microbial groups vary from place to place and growth kinetics depend on the composition of the fresh pulp, as well as the practices applied during fermentation (Nielsen et al., 2013; Papalexandratou and Nielsen, 2016). At the onset of the process, yeasts consume sugars and produce ethanol as well as aromatic secondary metabolites that may contribute to the final flavour of cocoa (Ho et al., 2014). They also produce pectinolytic enzymes which break down the viscous pulp and allow for oxygen penetration into the fermenting mass. Lactic acid bacteria grow in parallel with yeasts and assimilate the citric acid of the fresh pulp causing a pH increase (De Vuyst and Weckx, 2016). Also, LAB produce lactic acid as well as acetic acid, ethanol, CO_2 and mannitol (from fructose) (De Vuyst and Weckx, 2016; Nielsen et al., 2013).

Enterobacteriaceae have been found in most recent studies to be part of the microbial community the first 24 h of fermentation. Their role is not yet studied in detail but it has been suggested to be contaminants from the soil or phytopathogens of other tropical crops with small/no role as they disappear quickly, though a putatively positive cannot be ruled out (Ostovar and Keeney, 1973; Schwan and Fleet, 2014). As more pulp drains away and more oxygen is injected into the mass with periodical mixing of the fermenting mass for homogenization, AAB start oxidizing the ethanol into acetic acid and they may oxidize further lactic acid and acetic acid into CO_2 and water. The metabolism of AAB is highly exothermic causing temperature increase to > 45 °C (Pothakos et al., 2016). Ethanol and acetic acid diffuse into the bean and in combination with the heat kill the embryo. Then, enzymes are activated and cotyledon cells break open allowing interaction of different bean compounds and formation of flavour and aroma precursors.

The spontaneous fermentation of bulk cocoa like in the Ivory Coast, Ghana, and Brazil has been studied over the last decade (Camu et al., 2007; Crafack et al., 2013; Nielsen et al., 2013; Papalexandratou et al., 2011a, 2011b, 2011c). Making use of either classic microbiological or DNA-based methodologies, the main conclusion has been that a restricted microbial diversity dominates the cocoa bean fermentations of these regions. *Hanseniaspora opuntiae* and *Saccharomyces cerevisiae* are the most predominating yeasts, while *Lactobacillus fermentum* is the main lactic acid bacteria contributing from the onset of these fermentations (Papalexandratou et al., 2013). *Acetobacter pasteurianus* has been shown to be the main species of acetic acid bacteria at the later stages of bulk cocoa bean fermentations resulting in high temperatures after 3 days of fermentation. Also, Enterobacteriaceae have been found during the first 24 h of cocoa bean fermentations in West Africa as well as Malaysia and Brazil, but no further studies have been carried out to unravel their exact role during the fermentation. However, Enterobacteriaceae are very important for coffee fermentations of the gourmet *Arabica* type (Silva, 2014) and they might play a positive role during cocoa fermentations as well.

Only little is known about the microbiology of fine cocoa fermentations. Gálvez et al. (2007) studied fermentations in Dominican Republic and although the study is based only on traditional microbiological techniques it showed that the most abundant yeast species was *Candida insconspicua*, a species that did not contribute to ethanol production nor had pectinolytic activity but instead contributed to lactic acid assimilation. Also, lactic acid bacteria were not detected the first 12 h of fermentation. *Lactobacillus plantarum* and *Acetobacter lovaniensis* were the dominant bacterial species of this fermentation. Fine cocoa bean fermentations in Ecuador showed wide microbial diversity the first three days and yeasts were abundant during the entire fermentation process (Papalexandratou et al., 2011b). *Tatumella* species were present in high numbers during the first 48 h of all fermentations; also *Pichia* sp. and *Fructobacillus tropaeoli* were abundant in the process with *Lactobacillus fermentum*, *Acetobacter lovaniensis/fabarum* and *Acetobecter pasteurianus* similarly playing notable roles. Further, a number of recent studies have investigated the use of starter cultures on the fermentation of Brazilian cocoa varieties/hybrids. Inoculation with *Saccharomyces cerevisiae* either alone of combined with *Torulaspora delbrueckii*. In general the use of starter cultures improved the aroma/sensorical properties of the cocoa, even though the effect seemed bigger for some hybrids (Magalhaes da Veiga Moreira et al., 2017; Menezes et al., 2016; Visintin et al., 2017).

Driven by technological developments and dropping prices, high throughput sequencing (HTS) based techniques have become widely used for the study for complex fermentations (Ercolini, 2013). However, cocoa fermentations have only been sparsely characterized by HTS, with a study by Illeghems et al. (2012) investigating one sample from a Brazilian cocoa bean fermentation through HTS. The study revealed wider bacterial (mainly c-Proteobacteria) and fungal diversity than previously found. Recently Mota-Gutierrez et al. (2018) used high throughput amplicon sequencing to study Forastero cocoa bean fermentations, showing among other things that fermentation method (box vs. heap) influence the relative abundance of several yeast and bacterial species throughout the fermentations.

In 2007, a group of cocoa geneticists and chocolate experts screened Nicaragua for heirloom cocoa trees that illustrated potential for fine flavour development (Ji et al., 2013). Six fine cocoa varieties, namely Chuno®, Rugoso®, Nicalizo®, Johe®, Barba®, and Medalla™, possible grandfathers to the Criollo trees of Venezuela or Ocumare, Mexico (https://www.c-spot.com/atlas/chocolate-strains/cultivar-strains/chuno/), were chosen for reproduction in a central nursery and distributed to approximately 300 small and medium-size farmers around the country. To fully exploit the potential of these noble cocoas, optimized primary processing becomes paramount. One such system is developed by the Danish-Nicaraguan cocoa producer Ingemann Fine Cocoa where a centralized and optimized cascade-type box fermentation system is used.

To date, no study has investigated the impact of plant genetics on the microbial species diversity succession during spontaneous cocoa bean fermentations or compared fermentations carried out with cocoa harvested from different locations of one fine cocoa origin. Therefore, the purpose of this study was to investigate the microbial succession and sensorial characteristics developing during Nicaraguan single-variety cocoa bean fermentations originating from the same region (North Highlands of Nicaragua, San Jose de Bocay/El Cuá) and compare these to fermentations of blended cocoa types from other producing regions of the country (Waslala and Nueva Guinea) making use of HTS techniques and metabolite target analysis. The outcome could help improve post-harvest practices locally but also get a better understanding of fine cocoa bean fermentations.

2. Materials and methods

2.1. Field trials

Five spontaneous cocoa bean fermentations were carried out following in-house practices and supervision at the beginning of the harvest cycle of 2014-2015 (December 2014); four single-variety fermentations (Chuno®, Rugoso®, Nicalizo®, and Johe® 2014) with cocoa originating from affiliated and trained farmers of San José de Bocay and El Cuá (Jinotega) and one regional cocoa-blend fermentation with cocoa from the region of Waslala (O'Payo™ 2014; North Caribbean Coast Autonomous Region). The same experimental set-up was performed in January 2015 repeating the single-variety fermentations (Chuno®, Rugoso®, Nicalizo®, and Johe® 2015) in parallel to one cocoa-blend fermentation from the region of Nueva Guinea (Nugu™ 2015; South Caribbean Coast Autonomous Region).

The pods were broken open by the farmers with wooden machetes and the mass was collected into clean buckets or nylon bags. In the case of single varieties, pods were first piled per phenotype according to in-house protocols. Only high-quality freshly harvested wet mass was collected at the farm gate. Sugar content (°Brix) and initial pulp pH were measured as indicators of mass freshness making use of a

Table 1
Summary of fermentation practices and the respective physical changes and microbial counts (CFU/g pulp).

	Chuno® 2014							Chuno® 2015					
Volume of wet mass (kg)	535.8 kg							1377. 5 kg					
Duration & turns (hours in BOLD)	0 h	24 h	**48 h**	72 h	**96 h**	120 h	140 h	0 h	24 h	**48 h**	72 h	**96 h**	120 h
°Brix	15.5							15.5					
pH pulp	3.54	4.08	3.89	3.97	4.34	4.43	4.48	3.52	3.31	3.38	4.07	4.66	4.7
pH cotyledon	5.68	5.73	5.71	5.89	4.66	4.79	4.48	5.69	5.83	5.58	5.88	4.92	4.5
Temperature (°C)	24.0	24.8	30.0	35.2	37.5	46.6	44.6	22.0	23.6	27.3	38.4	42.5	48.9
Yeasts log CFU/g pulp	6.44	6.76	7.32	7.22	6.94	4.26	4.00	6.96	8.02	7.31	7.53	6.80	3.28
LAB log CFU/g pulp	6.53	7.32	7.94	7.79	8.08	6.92	7.23	6.58	3.00	3.00	7.45	8.05	6.24
AAB log CFU/g pulp	4.88	5.70	6.76	6.15	8.13	6.08	7.86	3.51	4.55	5.15	5.40	7.08	7.02
	Rugoso® 2014							Rugoso® 2015					
Volume of wet mass (kg)	197.5 kg							423.3 kg					
Duration & turns (hours in BOLD)	0 h	24 h	**48 h**	72 h	**96 h**	120 h	140 h	0 h	24 h	**48 h**	72 h	**96 h**	120 h
°Brix	15.7							15.1					
pH pulp	3.41	3.71	3.89	4.1	4.3	4.68		3.39	3.41	3.5	3.7	3.75	4.70
pH cotyledon	5.73	5.45	5.74	4.82	4.52	4.58		5.61	5.55	5.39	4.74	4.75	4.60
Temperature (°C)	25.0	27.2	33.6	49.8	43.9	49.7		23.0	22.7	30.7	49.5	48.7	44.9
Yeasts log CFU/g pulp	5.66	7.83	7.25	6.04	3.13	5.47		7.51	7.62	8.10	5.83	7.14	5.06
LAB log CFU/g pulp	4.70	3.78	7.15	7.13	6.78	7.93		5.00	7.18	6.90	6.60	7.48	6.51
AAB log CFU/g pulp	3.45	4.27	6.68	6.09	6.17	7.82		3.51	3.38	5.65	6.65	8.45	8.71
	Nicalizo® 2014							Nicalizo® 2015					
Volume of wet mass (kg)	416.6 kg							775.0 kg					
Duration & turns (hours in BOLD)	0 h	24 h	**48 h**	72 h	**96 h**	120 h	140 h	0 h	24 h	**48 h**	72 h	**96 h**	120 h
°Brix	15.5							15.5					
pH pulp	3.45	3.5	3.63	3.86	4.51	4.30		3.54	3.24	3.54	3.56	4.49	4.23
pH cotyledon	5.71	5.61	5.64	4.64	4.95	4.47		5.73	5.6	5.47	5.08	4.74	4.36
Temperature (°C)	24.0	25.5	29.7	44.4	47.0	48.7		23.0	24.5	30.2	41.4	46.7	51.1
Yeasts log CFU/g pulp	7.62	6.66	7.39	6.34	3.78	4.72		7.42	6.87	7.59	6.98	3.85	2.60
LAB log CFU/g pulp	5.60	5.63	7.66	7.52	6.34	4.11		5.00	6.30	4.48	6.48	6.56	5.51
AAB log CFU/g pulp	4.70	3.68	6.54	6.71	5.26	6.09		3.20	5.00	5.50	6.60	5.22	6.41
	Johe® 2014							Johe® 2015					
Volume of wet mass (kg)	152.5 kg							359.1 kg					
Duration & turns (hours in BOLD)	0 h	24 h	**48 h**	72 h	**96 h**	120 h	140 h	0 h	24 h	**48 h**	72 h	**96 h**	120 h
°Brix	14.9							15.0					
pH pulp	3.62	3.5	3.82	3.91	4.13	4.23		3.46	3.52	3.68	3.82	4.67	4.5
pH cotyledon	5.62	5.61	5.56	5.61	4.94	4.49		5.69	5.59	5.35	5.37	5	4.6
Temperature (°C)	25.0	25.7	29.8	45.3	45.4	50.5		24.0	24.7	33.4	47.4	46.1	51.2
Yeasts log CFU/g pulp	6.09	7.17	7.61	7.29	3.95	3.97		7.65	7.31	7.56	6.85	7.37	4.70
LAB log CFU/g pulp	5.41	6.60	7.34	7.89	5.56	6.35		7.26	6.00	6.00	7.08	7.65	6.78
AAB log CFU/g pulp	4.15	4.68	6.23	6.90	6.04	7.62		4.85	4.70	4.30	6.97	8.74	8.48
	O'PAYO™ box 1							NUGU™ box 1					
Volume of wet mass (kg)	627.2 kg							579.9 kg					
Duration & turns (hours in BOLD)	0 h	24 h	**48 h**	**72 h**	**96 h**	120 h	140 h	0 h	24 h	**48 h**	72 h	**96 h**	120 h
°Brix	17.5							17.4					
pH pulp	3.63	3.59	3.69	4.08	4.19	4.56	4.83	3.62	3.67	3.82	3.89	4.19	4.64
pH cotyledon	5.83	5.53	5.5	5.42	5.29	4.47	4.43	5.67	5.67	5.48	5.08	4.47	4.56
Temperature (°C)	24.0	24.4	27.9	32.4	43.2	45.4	43.5	25.0	26.7	29.3	43.3	44.9	50.1
Yeasts log CFU/g pulp	6.31	7.34	7.89	6.64	4.90	3.68	2.48	7.65	7.78	6.20	6.68	4.22	2.98
LAB log CFU/g pulp	4.88	6.06	7.98	8.21	6.51	7.66	6.18	3.70	7.11	6.30	6.85	5.04	6.36
AAB log CFU/g pulp	4.66	5.40	5.70	7.41	7.04	7.99	6.51	3.32	4.24	6.32	6.86	5.94	7.23

refractometer Brix RHB-32ATC and portable pHmeter (pH Testr 30; Oakton, IL, USA), respectively. A layer of banana leaves was placed at the bottom of each box made of "Cedro macho" wood (*Carapa guianensis*). Once revised, the wet mass was immediately placed in the corresponding fermentation boxes and covered with an extra layer of banana leaves and jute sacks to control heat escape. The volume of wet mass per fermentation as well as fermentation practices are reported in Table 1. The size and dimensions of the fermentation recipient differed according to the total volume per fermentation (0.50 m × 0.70 m × 0.50 m for 100–180 kg; 0.70 m × 0.70 m × 0.60 m for 200–280 kg; 0.70 m × 0.90 m × 1.20 m for 300–500 kg; 1.0 m × 1.0 m × 1.20 m for 600–1400 kg). The filled fermentation boxes were transported on a closed (pickup) truck to the centralized fermentation centre within 6–10 h. Once at the fermentation centre, the filled fermentation boxes were placed with a forklift at the top level of an open-air cascade-type infrastructure under roof. Homogenization of the fermenting mass took place periodically (Table 1) by vigorous mixing of the mass and its shovelling to an identical box (thereafter referred to as turn). Termination of the fermentation was based on pH and temperature changes; smell and appearance of the mass were also taken into account. The fermented mass was spread for sun-drying on stainless steel surfaces in a layer of 3–4 cm. The drying mass was mixed with rubber rakes every 1.5 h the first three days of drying and every 3 h from day 4 onwards with a total exposure between 08:00 am and 04:00 pm. The surfaces were covered under a tent roof on wheels overnight and under rainy conditions. All lots were dried to a final moisture content of 7.0% (approximately 7–8 days drying) and stored in jute bags.

During all cocoa bean fermentations, temperature (of the environment and inside the fermenting mass) and pH (of the pulp and the cotyledon) were registered. Temperature and pH were measured in the middle of the fermenting mass with a digital pH 340i sensor (WTW GmbH, Weilheim, Germany). To measure the pH inside the beans, 10 g of fermented cocoa beans were collected every 24 h. The beans were peeled manually with a blade and 5 g of peeled beans were grounded in a porcelain mortar in the presence of 45 mL of distilled water. The pH of this aqueous solution was measured using a bench pH-meter (FE20; Mettler Toledo, Hialeah, FL, USA).

Samples of 300 g were collected once per day and before the mixing step when applied. Sampling was always done at the same depth of the fermenting mass (approx. 30 cm from the upper surface) but in different points of the fermenting mass. All samples were divided in two; an aliquot was used for immediate culture-dependent microbiological analysis locally, while the rest of the sample was stored at −20 °C before being transported to Denmark on dry ice, followed by storage at −60 °C until further analysis.

2.2. Microbial counts and selective isolate identification

Fresh samples were transported to the laboratory for immediate plating on several selective agar media. Therefore, 10 g of pulp-bean mass were mixed with 90 mL of peptone water (0.1%, *w/v*, bacteriological peptone; Oxoid, Basingstoke, United Kingdom) in a sterile stomacher bag for homogenization of the material during 2–3 min by manual washing.

Series of 10-fold dilutions of each sample were prepared using 0.1% (*w/v*) peptone water and the appropriate dilutions, depending on the expected microbial counts, were plated on yeast-glucose (YG) agar for yeast enumeration, modified de Man-Rogosa-Sharpe [m-MRS, lactic acid bacteria; MRS (Oxoid) enriched with 10 g/L fructose] agar, and Acetic Acid Medium (AAM, acetic acid bacteria) agar (10 g/L D-glucose, 5 g/L ethanol, 3 g/L acetic acid, 15 g/L peptone, 8 g/L yeast extract, 18 g/L agar, pH 4.5). AAM and m-MRS media were supplemented with 400 mg of cycloheximide (Sigma-Aldrich, Steinheim, Germany) per litre of medium and 100 mg of oxytetracycline (Sigma-Aldrich) were added in every litre of YG medium to inhibit yeasts and bacteria, respectively. For enumeration of microbial species well-adopted to the environment of cocoa fermentation, all media were incubated at 30 °C for 2–4 days for samples collected during the first two days of fermentation and then the incubation temperature was increased to 37 °C for the plates of the rest of the fermentation samples. Plates with 30–300 colonies were used for counting. Catalase-positive colonies (non-lactic acid bacteria) on m-MRS agar as well as catalase-negative colonies (non-acetic acid bacteria) on AAM agar were excluded during colony counting by checking for catalase activity with H_2O_2 (20% aqueous solution) as reported before (Papalexandratou et al., 2011b). The morphology of the different colonies was noted too.

2.3. High-throughput amplicon sequencing analysis

2.3.1. DNA extraction from fermentation samples

DNA extraction was done according to a protocol described by Crafack et al. (2013). Following the extraction with phenol-chloroform-isoamyl alcohol the aqueous phase was purified by ethanol precipitation. The supernatant was removed without taking the phenol-chloroform mixture and transferred into a new Eppendorf tube. DNA is precipitated by adding 2 volumes of ice cold 96% ethanol and 1/10 volume of disodium acetate (3 M pH = 7.0). Samples were stored for at least 2 h or overnight at −20 °C, followed by a centrifugation at 21,000 × *g* for 20 min at 4 °C. The supernatant is carefully removed by leaving 50–100 µL of it on the pellet. 1 mL of ice cold 70% ethanol is added and samples centrifuged again at 21,000 × *g* for 20 min at 4 °C. The supernatant is removed with all the residual ethanol and 100 µL of Elution buffer (NE, Micherey-Nagel, Düren, Germany) is added. Samples are stored overnight at 4 °C to re-dissolve DNA and further stored at −60 °C until usage.

2.3.2. Library preparation for amplicon sequencing with Illumina

Bacteria and yeast composition was determined by MiSeq-based sequencing of the partial 16S rRNA gene and the internal transcribed spacer two (ITS2), respectively, using primers designed with adapters Nextera Index Kit® (Illumina, CA, USA). For profiling of the bacterial community 16S rRNA gene (V3-region) and for the fungal communities internal transcribed spacer 2 (ITS2) high throughput amplicon sequencing were used as described elsewhere (Haastrup et al., 2018; Krych et al., 2018).

2.3.3. Data analysis for sequencing results

The raw dataset containing pair-ended reads with corresponding quality scores were merged and trimmed using fastq_mergepairs and fastq_filter scripts implemented in the USEARCH v10 (Edgar, 2013). The max expected error was E = 2.0, and first truncating position with quality score $N \leq 4$, while the minimum length of merged reads was 130 bp. Purging the dataset from chimeric reads and constructing de novo Operational Taxonomic Units (OTU) were conducted using the UPARSE pipeline (Edgar, 2013). The Greengenes (v13.8) 16S rRNA gene collection was used as a reference database (McDonald et al., 2012) for bacteria and UNITE (v7.0) was used for yeasts (Kõljalg et al., 2013). Principal coordinate analysis (PCoA) based on Bray-Curtis distances were carried out on rarefied OTU-tables (9000 reads per sample) (Krych et al., 2018).

2.3.4. Metabolite target analysis

Metabolites were quantified according to a modified protocol described by Crafack et al. (2013). In brief, an aqueous extract for the analysis was prepared from frozen samples of cocoa beans. The pulp and cotyledon fractions were separated by manual peeling. Five grams of pulp or 10 g of cotyledon was mixed in a mortar with 4 parts of ultrapure water, followed by mixing with Ultra Turrax T25 lab scale homogenizer (IKA Labortechnik, Staufen, Germany). In case of pulp fractions larger pieces of the peel were removed beforehand. The homogenate was centrifuged at 5525 × *g* for 15 min. The supernatant

was mixed with HPLC-grade acetonitrile (Sigma-Aldrich) in the ratio of 1:1 and centrifuged at 5525 ×*g* for 15 min. The supernatant was filtered through 0.22 μm nylon filters (Frisenette, Denmark) and stored at −20 °C until further analysis. The concentrations of glucose, fructose, sucrose and mannitol were determined by High Performance Anion-Exchange Chromatography (HPAEC) and the concentrations of citric acid, lactic acid, acetic acid and ethanol were determined by High Performance Liquid Chromatography (HPLC) as described by Crafack et al. (2013). All substrate and metabolite analysis were carried out in duplicate.

2.3.5. Quality assessment of dried beans

Hundred dried cocoa beans were weighed on a digital balance to evaluate bean size (g per bean). The cut-test technique was used to assess defects and degree of fermentation based on cotyledon's colour development (del Boca, 1962). For this test, a sample of 100 beans was randomly selected and split open lengthwise with the cocoa bean Magra cutting unit (Teserba, Rüti, Switzerland) to assess the internal colour. The cut surfaces were then examined and assessed based on the following criteria: (a) under-fermented%; (b) semi-fermented%; (c) well-fermented%; (d) over-fermented%; (e) slaty%; (f) mouldy%; (g) infested%. The genetic attributes of the dry cocoa beans of the samples were examined too. In detail, apart from the intensity and presence/absence of brown colour development (traditional "indicator" of fermentation progress), the original cotyledon colour was checked: (a) white%; (b) lilac%.

2.3.6. Roasting trials and sensory evaluation of liquor samples

According to the results of the cut-test, roasting trials were carried out using a digital convection oven (Black & Decker, New Britain, Connecticut, United States) at 130 °C for various roasting times (16 min, 18 min, 20 min, 22 min) so the optimal roasting duration could be chosen per cocoa type.

Three hundred grams of deshelled roasted cocoa beans of the selected roasting time were ground into liquor (< 300 μm; 9 min × 9000 rpm) using a knife mill Retsch GRINDOMIX GM 200 (Retsch, Haan, Germany).

Liquor samples (60 °C) were evaluated through tasting by a trained panel of six. The panelists had followed previously national training sessions by the International Institute of Cocoa and Chocolate Tasting (https://www.chocolatetastinginstitute.org/) as well as internal trainings for calibration and expansion of flavour pallet by the panel leader following the guidelines of the Cocoa of Excellence Program (http://www.cocoaofexcellence.org; Edition 2013). The liquor samples were evaluated for the following sensory descriptors: cocoa, acidic, bitter, astringent, floral, fruity, raw/green, nutty, off-flavours. Each sample was tasted once while average scores of the 6 panelists were plotted in spider charts and overall appreciation was reported (scale 0–6), including standard deviations. To neutralize the pallet between tastings, each panelist ate soda crackers and used still water to rinse the mouth.

3. Results

3.1. Physical changes

3.1.1. Single varietals

Table 1 shows physical changes during the single-variety fermentations. In general, similar evolution was seen between the two rounds of fermentations for each variety. At the onset of all single-variety fermentations, the fresh pulp sugar content was approximately 15.5 °Brix while the initial pulp pH was between 3.3 and 3.5 with very small variations between the different cocoa types. The fresh bean pH was between 5.6 and 5.7 for all cocoa types. In general, these fermentations were characterized by a slow start, meaning slow pH_{pulp} and temperature increase reaching 30–33 °C after 48 h. The first mixing of the fermentation mass boosted the temperature increasing to > 40 °C for most single-variety trials of both rounds (except Chuno® trials). The impact of mixing was more pronounced in the fermentations of Rugoso® and Johe® where temperature rose to 49.5 °C and 46 °C, respectively. Chuno® fermentations advanced slowly reaching maximum temperature after 5–6 days fermentation (47–49 °C). The highest temperatures were seen at the end of Nicalizo® 2015 and Johe® trials (~51 °C). The bean pH during Chuno® fermentations was almost stable during the first 72 h and it only started dropping after 96 h reaching a final value of 4.5. In the case of Rugoso® and Nicalizo® trials, bean pH dropped below 5.0 after 72 h and reached final values of 4.6 and 4.4 respectively. Despite the rapid temperature increase during Johe® fermentations, bean pH only dropped below 5.0 after 96 h of fermentation, reaching final average value of 4.55. The pulp pH increased after each mixing and then decreased again reaching final value of 4.2–4.7 for all trials.

3.1.2. Regional blends

For the regional blends (Table 1) the fresh pulp was characterized by higher sugar content in comparison to single-varietals (17.5 and 17.4 °Brix for O'Payo™ and Nugu™, respectively). Also, the pH of the fresh pulp was ~3.65 for both cases while the initial bean pH was 5.83 and 5.67 for O'Payo™ and Nugu™ blends, respectively. Despite the application of three turns, the O'Payo™ fermentation reflected the slowest progress in terms of temperature changes reaching a maximum temperature of 45.4 °C after 120 h. However, there was a quick pulp pH increase after 72 h and bean pH dropped to 4.47 after 120 h getting to a final value of 4.43 at the end of the process. In the case of Nugu™, the temperature increased to 43.3 °C after the first mixing and it reached 50.1 °C at 120 h. The bean pH slowly dropped and it reached its minimum after 96 h (4.47). The last 24 h of the process the bean pH slightly increased to the final value of 4.56.

3.1.3. Microbial dynamics during Nicaraguan cocoa fermentations: counts and species diversity

Principal coordinate analysis (PCoA) of 16S rRNA gene (bacteria) and ITS2 (yeast/fungi) amplicon high-throughput sequencing derived profiles revealed that based on yeast/fungi community structure the fermentations at 0–24 h and the 48–72 h generally clustered separately, while the 96–144 h samples do not show a clear pattern (Fig. 1A). For the bacterial community it went through 3 distinct phases, with 0–24 h, 48–72 h and 96–144 h clustering together (Fig. 2A). No clear separation between years (2014 and 2015) was observed, nor clustering based on cocoa varieties (those for consecutive years) were seen (Figs. 1A, 2A).

3.2. Yeasts

3.2.1. Single-varietals

The initial yeast counts on YG medium were relatively high in all cases starting at an average of 6.00 and 7.50 log CFU/g during the trials of 2014 and 2015, respectively (Table 1). In general, the yeast counts were maintained high throughout the entire fermentation process of the single-variety trials and they did not drop below 4.00–5.50 log CFU/g except for the case of the Chuno® 2015 (3.28 log CFU/g) and Nicalizo® 2015 (2.60 log CFU/g) trials. The highest yeast counts were seen at 24 h of Chuno® 2015 (8.01 log CFU/g) and 48 h of Rugoso® 2015 (8.10 log CFU/g).

The yeast species found during the different single-variety fermentations was quite similar with some differences mainly on the time of succession of some species. A common finding regarding the most predominant yeast species was the succession of Saccharomycetales members (Fig. 1B). *Hanseniaspora uvarum/opuntiae* (first 48 h), *Saccharomyces cerevisiae* (48–120 h), and *Pichia kudriavzevii* (72–144 h), but an overlap occurred in most cases. Both Chuno® fermentations showed the widest yeast species diversity but with the same species succession as mentioned above. *Pichia barkeri* was among the predominant yeasts found after 96 h of Rugoso® 2015, but also at 0 h of Chuno ® 2015

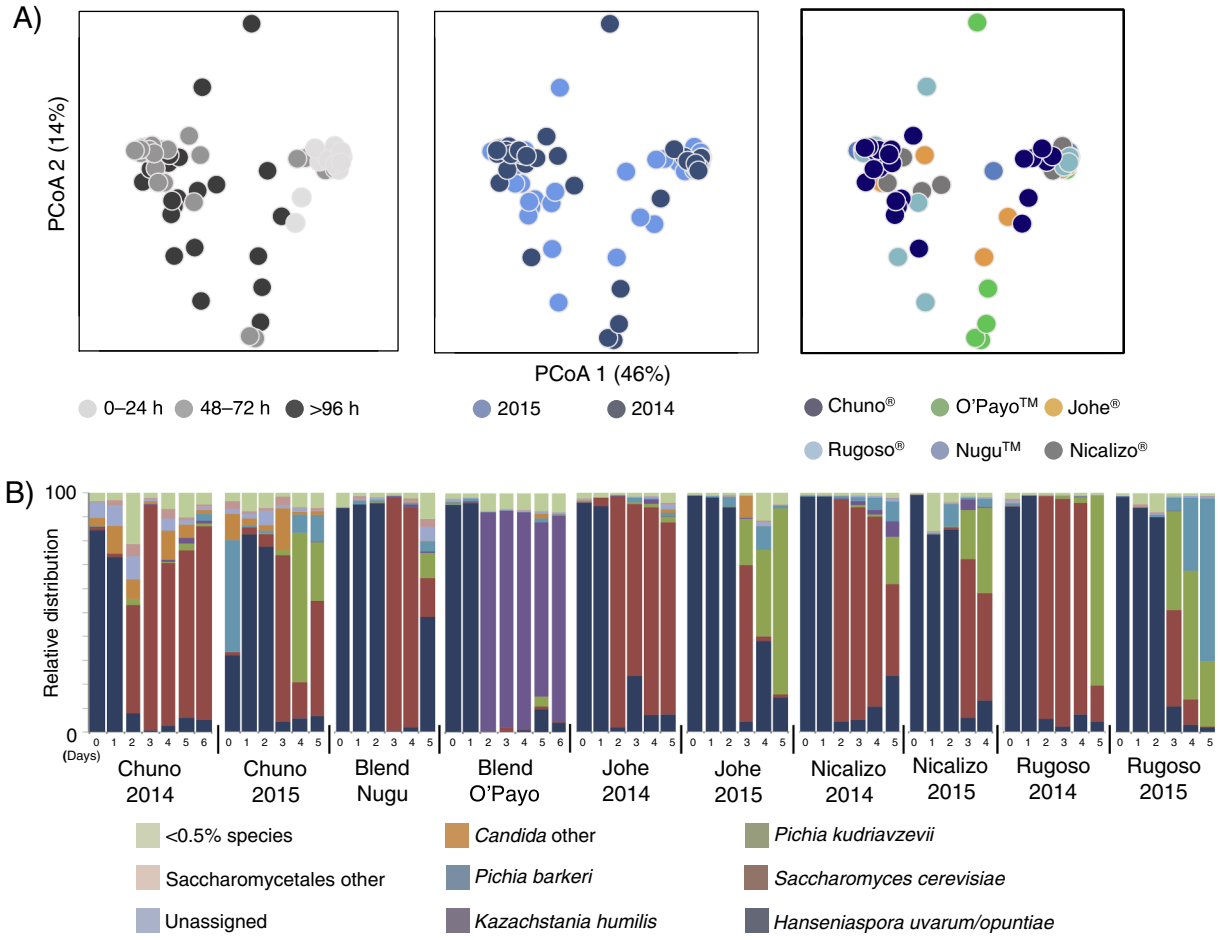

Fig. 1. Yeast community profile as determined by high throughput sequencing of ITS2 region amplicons.
A) Bray-Curtis distance based Principal Coordinate Analysis (PCoA) based on OTUs summarized to the species level. B) Relative distribution of yeast species (OTUs summarized to species level) with an accumulative relative abundance of ≥0.5%. All analyses and comparisons were based on tables rarefied to 9000 random sequences.

(Fig. 1B). Occasional presence of *Candida* was also found.

3.2.2. Regional blends

During the fermentations of the regional blends O'Payo™ 2014 and Nugu™ 2015 the yeast counts followed the same trend as for the single varietals, namely starting at 6.31 log CFU/g and 7.65 log CFU/g, respectively (Table 1). In both cases a maximum yeast population of approximately 7.80 log CFU/g was seen after 48 h and 72 h for O'Payo™ 2014 and Nugu™ 2015, respectively. After 72 h, the yeast counts started dropping to 2.50–2.90 log CFU/g at the end of the process.

While the Nugu™ 2015 fermentation showed similar yeast diversity as the single varietals (with *H. uvarum/opuntiae* appearing again at the last day of fermentation), the yeast species diversity of O'Payo™ 2014 was very distinct. *Hanseniaspora uvarum/opuntiae* members were the dominant yeast species at the onset of the fermentation, but after 48 h was succeeded by *Kazachstania humilis* which dominated the remaining process and seemed to have outcompeted completely *Saccharomyces* (Fig. 1B).

3.3. Bacteria

3.3.1. Single varietals

From the bacterial community, the populations of lactic acid bacteria (LAB) and acetic acid bacteria (AAB) were followed through plating on selective media as mentioned above. For precise counting of the LAB colonies, the catalase test was applied for exclusion of the catalase-positive colonies that were strongly growing on the m-MRS plates even in the presence of supplements against yeasts. In detail, only 10–20% of the colonies of the plates at 0 h represented LAB. After 24 h it increased to 50% of the colonies (although it dropped to 0% for Rugoso 2014) reaching 100% catalase-negative LAB colonies between 72 and 96 h. At the later stage 50% of the colonies were again catalase-positive, causing a bias for accurate LAB colony counting. The same goes for AAB counts on AAM plates, where the catalase test was used for exclusion of catalase-negative colonies. Also, growth of staphylococci on AAM media was among the biases of the culture-dependent method. The same biases have been encountered during Ecuadorian (Papalexandratou et al., 2011b) as well as Honduran (unpublished data) fermentations.

Low LAB and AAB counts were seen at the start of all fermentations with initial counts of 4.00–5.00 log CFU/g and 3.50–4.50 log CFU/g, respectively (Table 1). The Johe® 2015 fermentation was the exception showing higher LAB (7.28 log CFU/g) and AAB (4.85 log CFU/g) counts at 0 h. The LAB peaked after 72–96 h with an average maximum of 6.60–7.50 log CFU/g, with Chuno® LAB peaking at 8.10 log CFU/g after 96 h in both cases. The AAB counts increased throughout the fermentations reaching their peak after 96–120 h (approximately 7.00 log CFU/g) while the highest AAB counts were found during Chuno® 2014, Rugoso® 2015 and Johe® 2015 (> 8.00 log CFU/g).

The bacterial species succession did not have a clear pattern as seen with the yeast species diversity. 16S rRNA gene amplicon sequencing showed the high abundance of Enterobacteriaceae at the onset of all

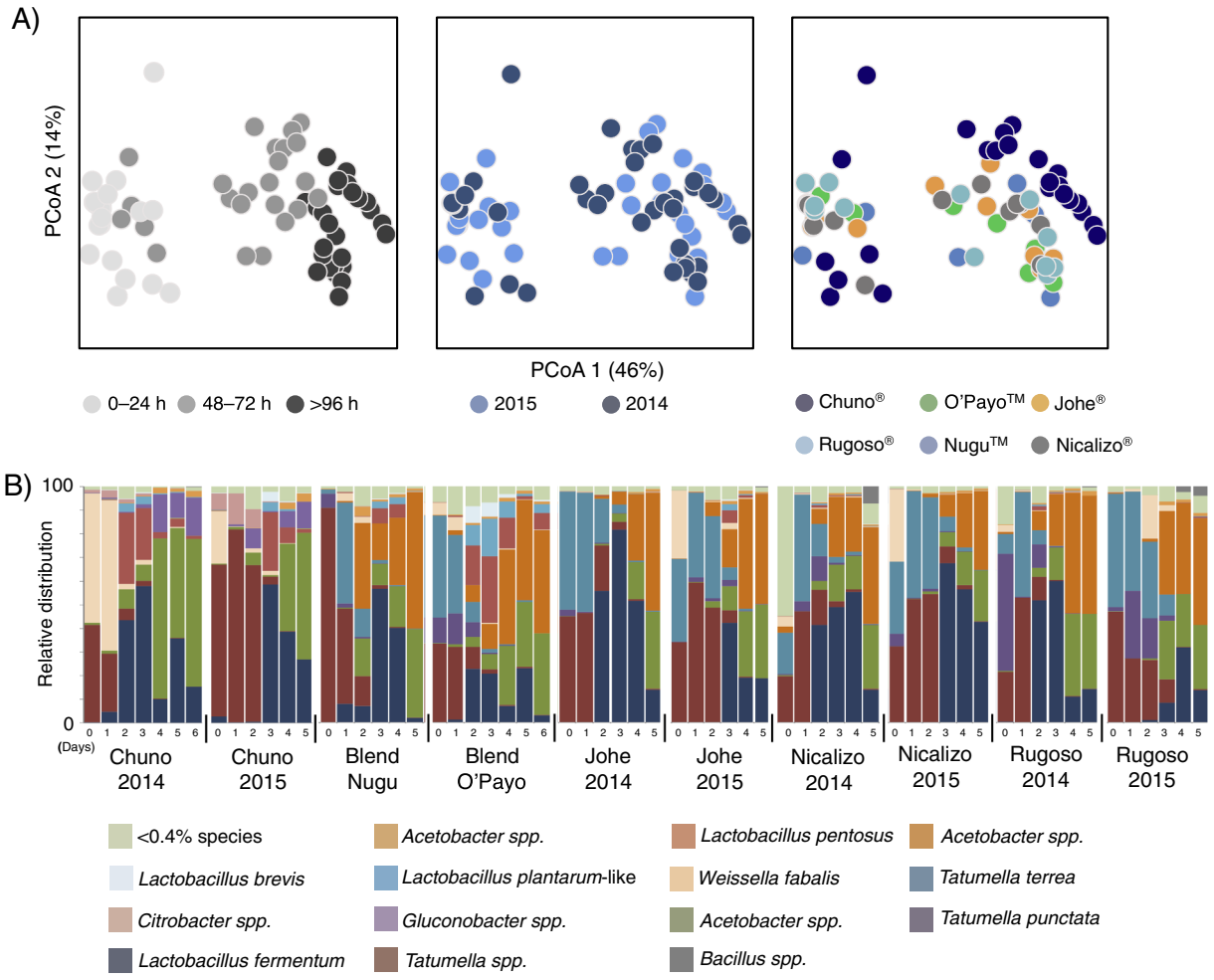

Fig. 2. Bacterial community profile as determined by high throughput sequencing of 16S rRNA gene (V3-region) amplicons.
A) Bray-Curtis distance based Principal Coordinate Analysis (PCoA) based on OTUs summarized to the species level. B) Relative distribution of bacterial species (OTUs summarized to species level) with an accumulative relative abundance of ≥ 0.4%. All analyses and comparisons were based on tables rarefied to 9000 random sequences.

fermentations (Fig. 2B). They were found to dominate the bacterial community the first 48 h (constituting > 60% of the bacterial relative abundance; mainly *Tatumella* spp., *Tatumella punctata*, and *Tatumella terrea*). When the pulp pH rose above 4.0 the relative abundance of *Tatumella* spp. generally dropped to < 5% of total bacterial relative abundance, but whether this reflects the influence of pH or other factors (e.g. nutrient depletion) remains to investigated.

The low LAB counts and therefore the absence/low participation of LAB at the start of the fermentations were confirmed by 16S rRNA gene amplicon sequencing as LAB were hardly detected in most cases during the first 48 h (Fig. 2B). *Lactobacillus fermentum* appeared to be among the dominant bacterial species after 48 h during 2014 trials but only after 72 h during 2015 trials (Fig. 2B). *Weissella fabalis* was found during the first 24 h of fermentations (mainly Chuno®). Further, *Lactobacillus pentosus* appeared during 48–72 h of the Chuno® fermentations.

The acetic acid bacteria (AAB) mainly appeared after 72–96 h of fermentation and were the main bacterial representatives after 96 h (Fig. 2B). In the case of Nicalizo® 2015, *Acetobacter* spp. represented 55% (relative abundance) of the bacterial community after 120 h while in all other cases they constituted up to 80% of the total bacterial community at the end of fermentation. During Chuno® trials, 16S rRNA gene amplicon sequencing showed that *Gluconobacter* spp. could be distinguished from the AAB community and represented 10–15% of the bacterial community after 72–120 h of fermentation (Fig. 2B).

Bacillus spp. were also detected after 96 h of Rugoso® 2015 and 120 h of Nicalizo® 2014 as determined by 16S rRNA gene amplicon sequencing (Fig. 2B), reflecting that the heat generated during the fermentations give aerobic sporeformers a competitive advantage. *Bacillus* spp. are generally believed to negatively impact flavour and when they become predominant it is important to interrupt the fermentations to avoid over-processing and off-flavour development (Nielsen et al., 2013).

3.3.2. Regional blends

Both fermentations of the regional blends started with low LAB and AAB counts (~4.8 log CFU/g for O'Payo™; ~3.5 log CFU/g for Nugu™) (Table 1). In the case of O'Payo™, LAB counts rose quickly reaching a peak of 8.21 log CFU/g after 72 h. The peak for LAB during Nugu™ fermentation was reached after 24 h (7.11 log CFU/g) and stayed at this level until 72 h. In both cases LAB counts declined after 96 h but did not drop below 6.0 log CFU/g. The AAB counts increased steadily to a maximum of 7.5–7.9 log CFU/g at 120 h of both fermentations.

Members of Enterobacteriaceae were the predominating bacterial species involved at the onset of the regional blends, too (Fig. 2B). *Weissella* sp. was present during the first 48 h of O'Payo™ fermentation and it appeared during 24–48 h of Nugu™ fermentation (Fig. 2B). Lactobacillaceae appeared earlier in the case of the fermentations of regional blends (24 h) and together with the unusual yeast species composition of O'Payo™, the LAB played an important role during the whole

O'Payo™ process and *L. pentosus* and *L. plantarum*-like were main representatives for this fermentation. *Lactobacillus fermentum* was the main LAB species found during Nugu™ 2015.

Acetic acid bacteria were also present mainly after 48 h (Fig. 2B). In the case of Nugu™, *Acetobacter* spp. appeared early during the fermentation (48 h) and was the only detected group at 120 h.

3.4. Metabolite target analysis

3.4.1. Single-varietals

The fresh pulp of all fine single-varietals was characterized by moderate concentrations of glucose and fructose (15.1–28.1 mg/g and 18.7–60.6 mg/g, respectively). The pulp sugars were consumed simultaneously within 48 h of most fermentations and after 72 h of Chuno® and Johe® 2014. Only traces of sucrose and mannitol were found in the pulp. At the end of the fermentation, pulp sugars were below 4.0 mg/g reflecting completion of the process. The bean sucrose concentration varied within 3.6 and 7.7 mg/g and it was fully depleted into glucose and fructose (approximately 2.0–4.0 mg/g each after 120–144 h of fermentation). No mannitol was found in the beans.

Ethanol in the pulp peaked 24 h after the peak of the yeast population, mostly at 48–72 h of fermentation. The maximum pulp ethanol concentrations were between 13.4 (Johe® 2015) and 23.1 (Chuno® 2015) mg/g. For Chuno® 2014, pulp ethanol concentration peaked only after 96 h. The bean ethanol reached its maximum (6.5–13.5 mg/g) after 72–96 h; For Johe® 2015 the bean ethanol peaked early at 48 h. At the end of all single-varietal fermentations ethanol was ~2.0 mg/g except for the Chuno® trials where it ended at ~5.0–6.8 mg/g. The fresh pulp citric acid was approximately 3.5–6.0 mg/g and in case of Rugoso® 2015 it was at 7.8 mg/g. In all cases, citric acid assimilation started once *Lactobacillus fermentum* dominated the LAB community (48–96 h) dropping below 2 mg/g at the end of the process. Exception is the Rugoso® 2015 fermentation where citric acid remained at high levels until the end, illustrating that the low populations of *L. fermentum* impacted its assimilation. The bean citric acid was ~4.0 mg/g except for Nicalizo® lots which was 6.1–7.2 mg/g and remained stable throughout the process. Lactic acid in the pulp was produced at low levels and did not exceed 4.0–8.3 mg/g; the lactate concentration was higher in the case of Chuno® 2014, where *L. pentosus* and *L. brevis* were involved during the mid-phase of this fermentation. In the bean, lactic acid remained at very low levels (~1.0–2.0 mg/g). Pulp acetic acid peaked after 96 h reaching a maximum of approximately 8.5–11.9 mg/g, while it started diffusing in the beans peaking after 120–144 h at an average maximum of ~6.5 mg/g.

3.4.2. Regional blends

Fresh pulp glucose and fructose concentrations were approximately 20.0 to 30.0 mg/g each also for the O'Payo™ and Nugu™ (Fig. 3A & B). At the end of the fermentation process glucose and fructose were at 2.0 mg/g for O'Payo™ while for Nugu™ they were still at a concentration of 5.2 mg/g and 8.7 mg/g respectively, indicating a slower and maybe slightly incomplete process (Fig. 3A & B). Despite that, bean sucrose (5.0 mg/g) was fully depleted at the end of both processes leaving the reducing sugars at a concentration of 3.0–3.5 mg/g (glucose) and 2.9–4.2 mg/g (fructose) (Fig. 3C & D). No mannitol was produced in the pulp and/or diffused into the beans of the regional blends.

Ethanol was produced by yeasts reaching a maximum of ~18.0 mg/g in the pulp (Fig. 4A & B) and ~12.0 mg/g in the bean (Fig. 4C & D). The fresh pulp of the regional blends had a citric acid concentration of 5.0 mg/g which started being assimilated, presumably by *L. fermentum*, after 24 (Nugu™) and 72 (O'Payo™) h (Fig. 4A & B). Pulp lactic acid concentration was higher in the case of O'Payo™ (7.8 mg/g; 72 h) probably reflecting the presence of more homofermentative LAB while for Nugu™ lactic acid peaked at 96 h (5.7 mg/g) (Fig. 4A & B). Pulp acetic acid was high by the end of the Nugu™ fermentation (15.8 mg/g at 120 h) reflecting the early involvement of AAB. The bean citric acid remained stable throughout the fermentations of the regional blends, too, and lactic acid did not exceed 1.7 mg/g. The acetic acid diffused into the beans in both trials reaching a maximum of 6.0–7.5 mg/g (Fig. 4C & D).

3.5. Bean quality assessment

Making use of the cut-test methodology for bean quality assessment, information about the genetic characteristics of the different bean types could be obtained (Table 2). In detail, the single varietals were found to have high percentages of white (Criollo-like; 4–16%) and lilac (Trinitario-like; 12–28%) beans confirming the fine cocoa genetics of these beans. In contrary, the regional blends showed low percentages of white/lilac beans and did not have a sum higher than 8% of the two categories. The bean size was remarkably varying between 1.20 g and 1.55 g per bean (lowest for regional blends, highest for Nicalizo®). In general, bean size was slightly higher for the fermentation round of December 2014. All lots were free of defects, such as internal mould, infestation or germination. Although the selected post-harvest practices were not focusing just on brown colour development but maximum flavour expression, the colour changes were noted. The Chuno® and Rugoso® fermentations had the highest percentage of fully browned (well-fermented) beans (20–26%) while Nugu™ had the lowest (4%). The percentage of under-fermented (no brown colour development) beans varied between 46% and 82%. Semi-fermented (partial brown colour development) bean percentages were on average 15–30% while Johe® 2014 reached 8% of partially browned cotyledons. The fissuring of the beans was good and together with bean pH decrease of 1.2–1.4 units they added on the conclusion that fermentations were complete.

3.6. Sensory evaluation of liquor samples and overall appreciation

A trained panel of six performed a sensory analysis of liquor samples corresponding to the trials described above (Fig. 5). The general appreciation and the chosen roasting duration for each lot are shown in Fig. 6. The selection of roasting duration was based on the results of cut-test and taste of dry cocoa beans roasted at intervals of 2 min starting with 16′ until 22′ at 120 °C. Almost all lots were highly appreciated scoring between 4.0 and 5.1 for overall quality. The Chuno® lots were equally appreciated (Chuno® 2014: 4.85 ± 0.71; Chuno® 2015: 5.02 ± 0.29) and panelists found them consistent and balanced in terms of flavour despite the small difference in roasting duration (Fig. 6). The main flavours detected for the Chuno® lots were sweet lemon, white flowers, caramel, notes of red berries and spices with tropical fruit acidity. The floral notes were more pronounced for Chuno® 2015 while the citric acidity was slightly more intense for Chuno® 2014. In the case of Rugoso® and Nicalizo®, big differences in overall appreciation were seen between the two rounds. Rugoso® 2015 and Nicalizo® 2014 were the least appreciated scoring below 3.5. These lots got the highest scores for green/grassy notes (2.0 ± 0.63 and 1.75 ± 0.71) and they were characterized as heavy and out of balance with some off-notes which were more intense in the case of Rugoso® 2015. During this fermentation *Bacillus* appeared earlier. In contrary, Rugoso® 2014 was the highest scoring lot for most of the panelists (average score 5.10 ± 0.37). Sweet prunes, woody, herbal notes and vanilla were the main flavours perceived for this lot. Nicalizo® 2015 was a balanced lot and it was characterized by flavours such as intense cocoa, tannic wine, liquorish, hazelnuts, and fragrant (dry herbs) with a soft smoky touch. The Johe® lots expressed similar flavour profiles with very unique characteristics. Delicate floral notes like jasmine and roses, and freshly-brewed green tea gave the special character for Johe® 2015 next to flavours such as chocolate paste, nuts, and soda crackers with citric notes (both lots).

The regional blends had distinct flavour profiles but lacked balance. However, the O'Payo™ 2014 tasted like pilsner beer (yeasty, sparkling

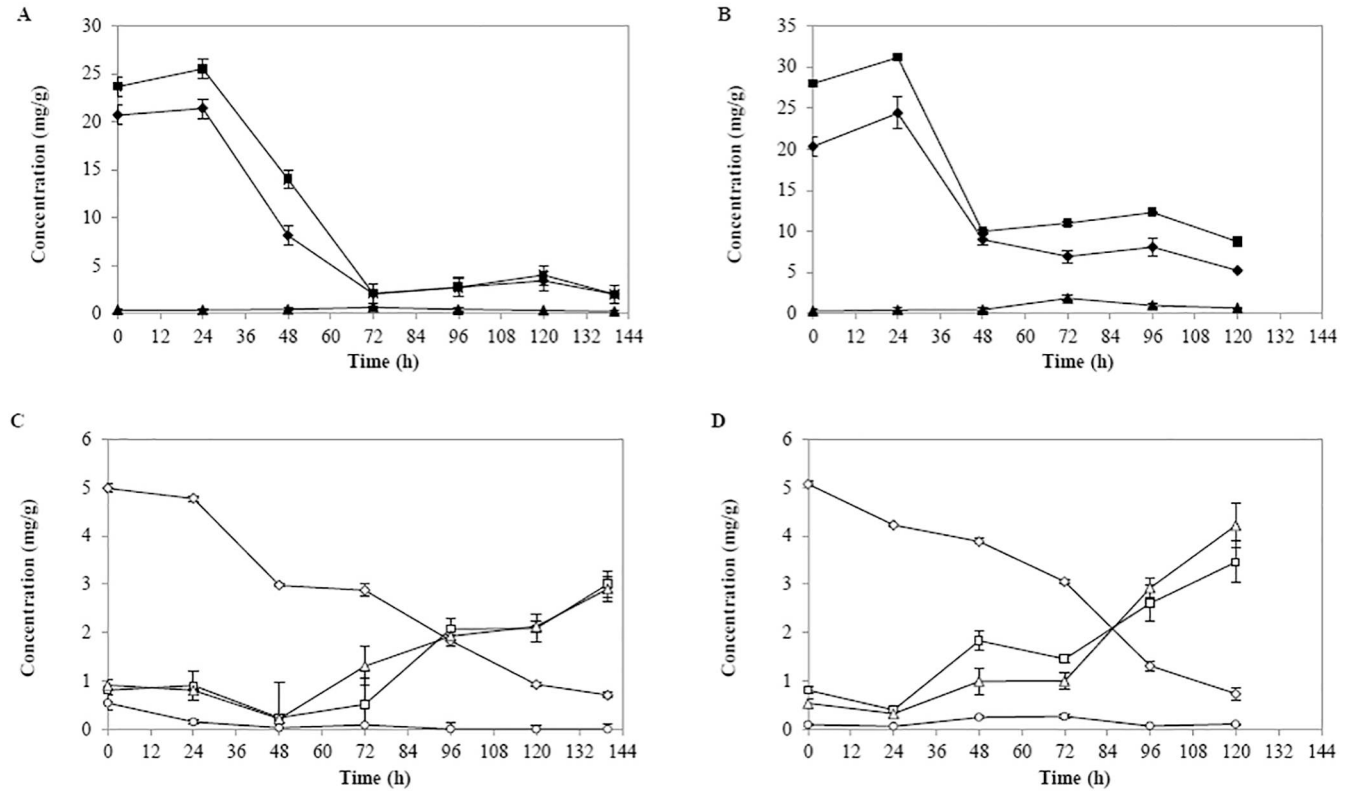

Fig. 3. Course of glucose (♦), fructose (■), and mannitol (▲) in the pulp during O'payo™ 2014 (A) and Nugu™ 2015 (B) box fermentations. Course of sucrose (◊), glucose (□), fructose (Δ), and mannitol (○) in the beans during O'payo™ 2014 (C) and Nugu™ 2015 (D) box fermentations. Bars indicate standard deviations.

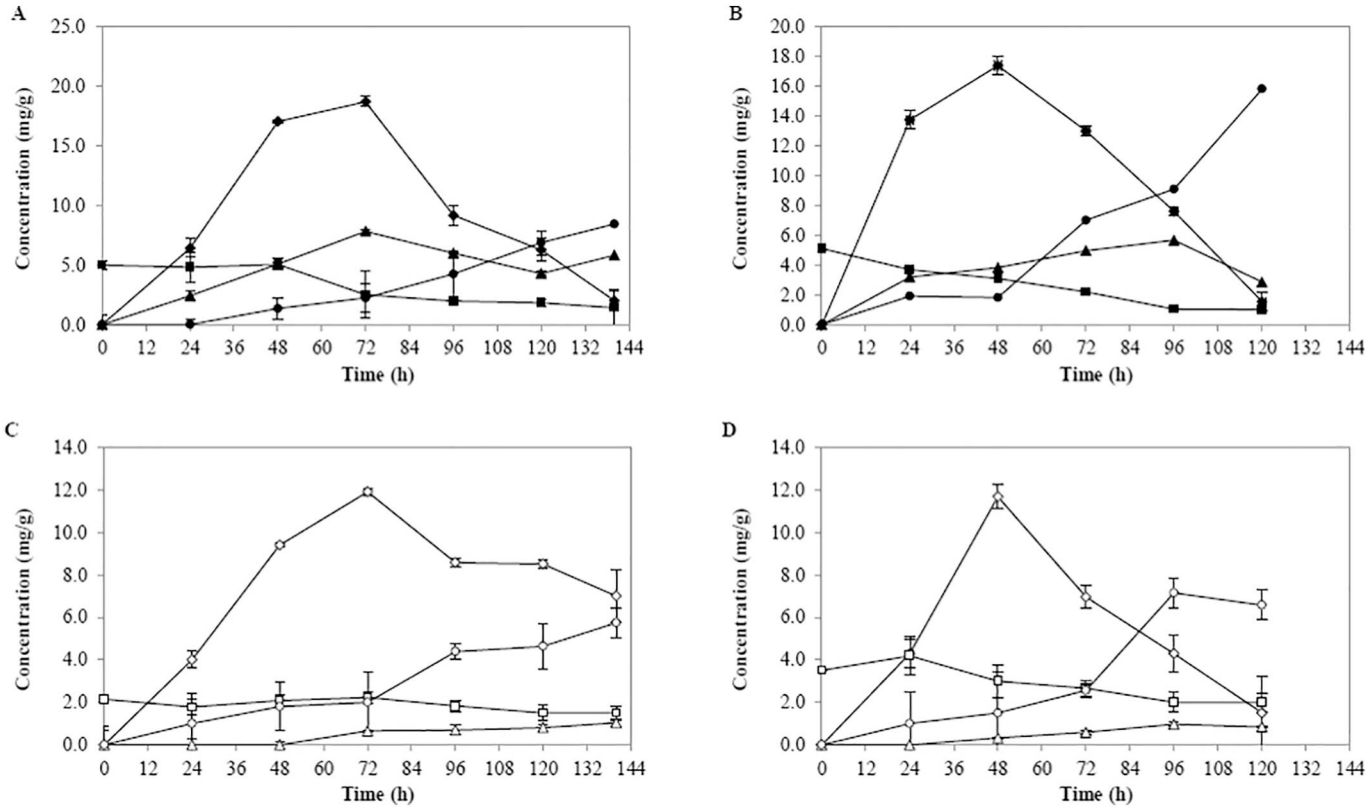

Fig. 4. Course of ethanol (♦), citric acid (■), lactic acid (▲), and acetic acid (●) in the pulp during O'payo™ 2014 (A) and Nugu™ 2015 (B) box fermentations. Course of ethanol (◊), citric acid (□), lactic acid (Δ), and acetic acid (o) in the pulp during O'payo™ 2014 (C) and Nugu™ 2015 (D) box fermentations. Bars indicate standard deviations.

Table 2
Quality assessment (cut-test) of the dry cocoa beans of the Nicaraguan single-varietal and regional blend cocoa bean fermentations.

	Weight of 100 beans (g)	Under-fermented %	Semi-fermented %	Well-fermented %	Over-fermented %	Slaty %	Mouldy %	White beans %	Lilac beans %
Chuno® 2014	148.4	60	14	26	0	0	0	12	20
Rugoso® 2014	142.4	46	30	24	0	0	0	12	18
Nicalizo® 2014	154.9	78	12	10	0	0	0	6	12
Johe® 2014	142.1	82	8	10	0	0	0	6	14
O'Payo™ 2014	146.9	62	24	14	0	0	0	4	2
Chuno® 2015	136.9	50	30	20	0	0	0	14	18
Rugoso® 2015	140.9	60	20	20	0	0	0	16	14
Nicalizo® 2015	140.1	74	14	12	0	0	0	6	28
Johe® 2015	131.2	56	26	16	0	2	0	10	14
Nugu™ 2015	120.3	78	18	4	0	0	0	2	6

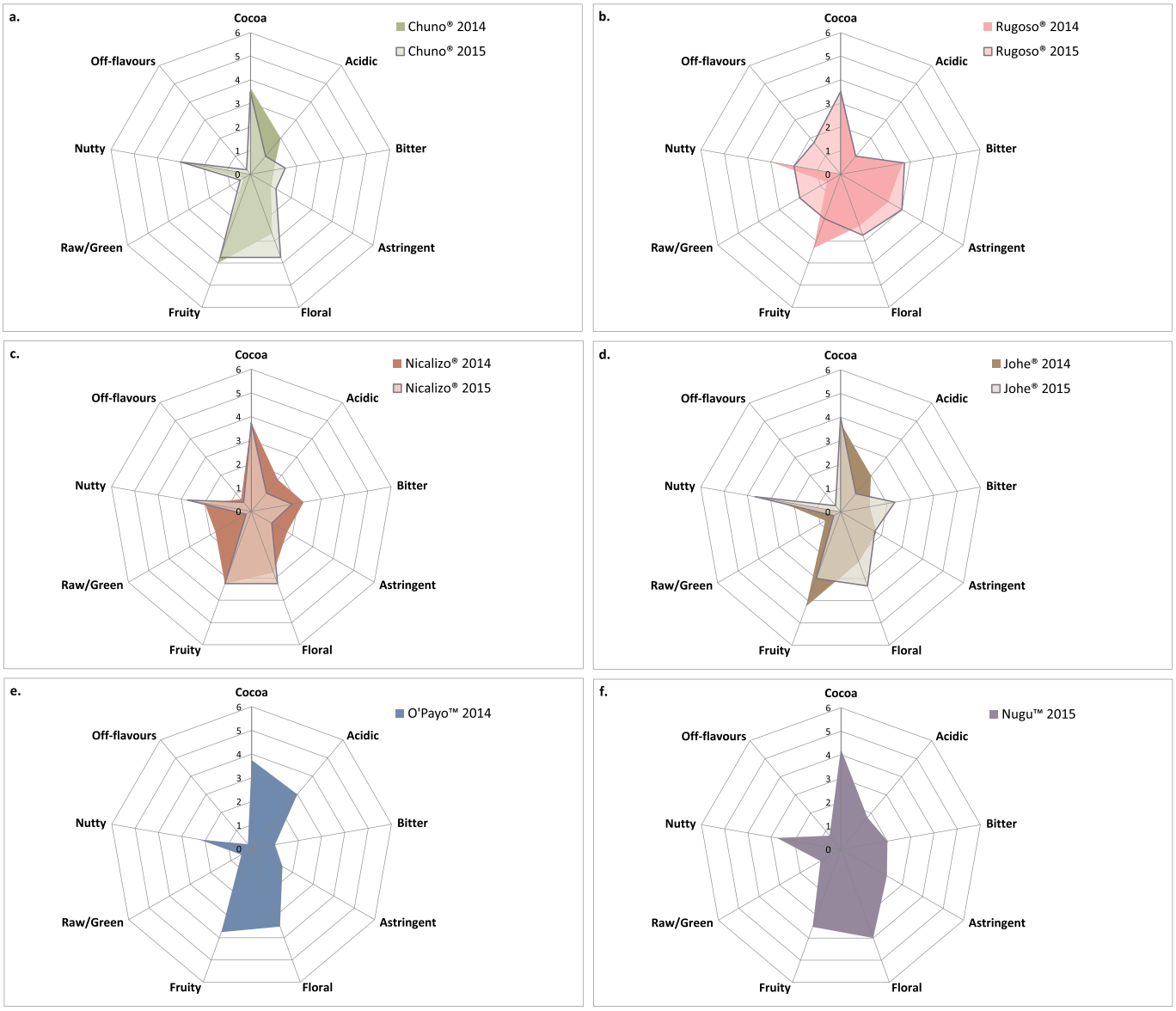

Fig. 5. Flavour profiles of the liquor samples made with fermented dry cocoa beans of the respective cocoa bean fermentations. The flavour intensity of the descriptors increases from the centre to the perimeter. The results were obtained through sensory analysis performed by a trained tasting panel of 6 members. For standard deviations on the individual descriptors please see Supplementary Table 1.

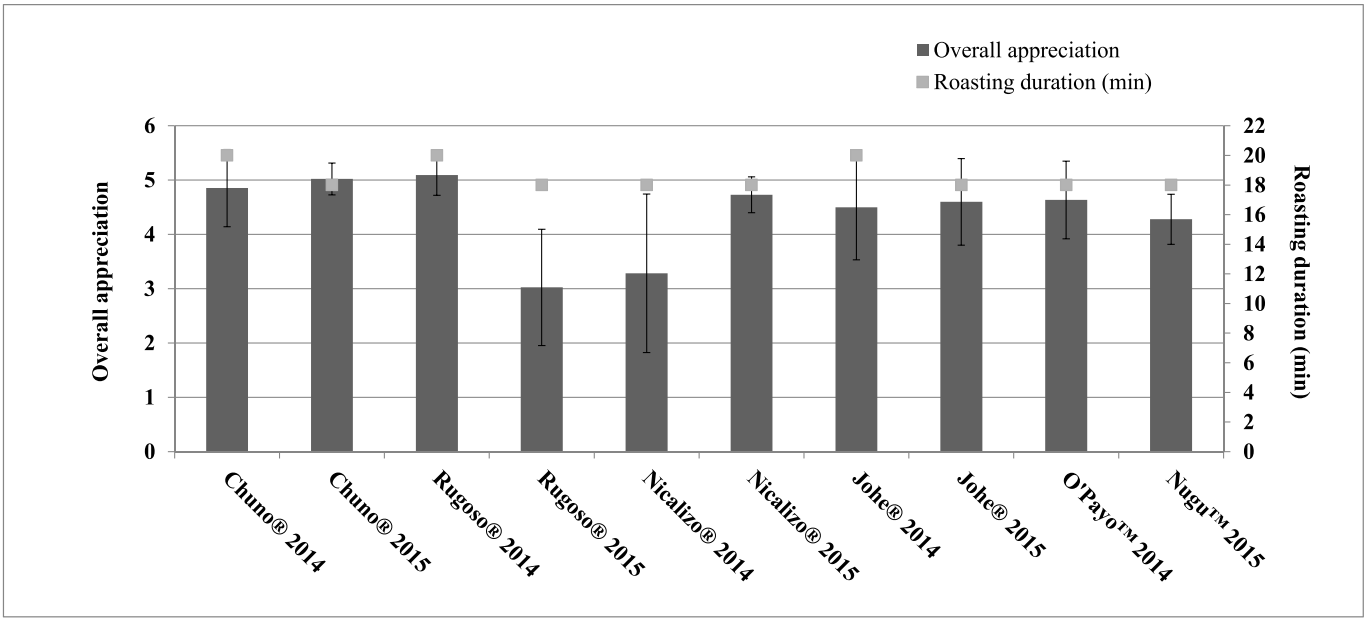

Fig. 6. Average overall appreciation of the liquor samples made with fermented dry cocoa beans of the respective cocoa bean fermentations and selected roasting duration (min). Bars indicate standard deviations.

mouthfeel, and alcoholic) and had a sweet-sour character. There was fruity acidity of kiwi and pineapple as well as citrus and green grapes and sweetness of caramel. The bitter notes were like espresso coffee. Despite the lack of balance of Nugu™ 2015, the lot was appreciated for the strong red fruits and the dominant complex floral notes. Further, no positive correlation between bitterness and/or astringency and under-fermented% beans was seen (results not shown). In contrary, Rugoso® 2014 got a high score for bitterness (2.67) and it had the lowest percentage of under-fermented beans (46%) among the lots of this study.

4. Discussion

The flavour capacity of cocoa beans depends on various factors among which plant genetic variety and post-harvest handling have the biggest impact (Schwan and Fleet, 2014). The microbial fermentation of cocoa allows for the formation of necessary flavour and aroma precursors that will be converted to the final chocolate flavour once cocoa is roasted (Afoakwa et al., 2008). The present study dealt with Nicaraguan fine cocoa bean box fermentations to investigate the impact of cocoa cultivar and/or its origin on the microbial species diversity, metabolism and sensory appreciation of the final product.

The high-throughput rRNA/ITS gene amplicon sequencing analysis proved to be an ideal methodology for a rapid identification of the total microbial composition of the complex cocoa bean fermentations. Even if identification did not reach species level in all cases, it summarized clearly the microbial groups involved in the process in a proportional manner allowing for better explanation of the fermentation metabolism and organoleptic characteristics of the final product.

The conducted experiments confirmed that the microbial species that play a key role for the successful evolution of cocoa bean fermentation were *Hanseniaspora uvarum/opuntiae*, *Lactobacillus fermentum* and *Acetobacter* spp. The Nicaraguan fermentations were however characterized by slow start with lower counts of yeasts and bacteria than observed elsewhere. Low initial counts have been found during Honduran fermentations too (Saerens and Swiegers, 2013). The low counts could reflect more hygienic conditions at collection and transport of wet mass in comparison to the conditions encountered in West Africa (Camu et al., 2007; Nielsen et al., 2007).

Yeasts were predominating during the entire fermentation process. *Saccharomyces cerevisiae* and *Pichia kudriavzevii* were among the key players at mid and end of the process, respectively. The low temperatures for 72 h allow for prolonged yeast activity and eventually production of more aromatic compounds that can enhance the fragrant character of the beans (Meersman et al., 2015; Papalexandratou and Nielsen, 2016). *Pichia kudriavzevii* seemed to add floral character when it dominated the end phase of the fermentations, as seen for Chuno® 2015 and Johe® 2015. This species was among the predominating yeasts of Ecuadorian fermentations, too (Papalexandratou et al., 2011b). Other *Pichia* species such as *Pichia kluyveri* have been found to have very high aroma potential in other fermented products such as wine and have also been utilized as starter culture for cocoa fermentations (Crafack et al., 2013). Also, Pereira et al. (2017) showed that several cocoa-related *P. kudriavzevii* strains may have increased aroma contribution. The genetic cocoa varietal did not seem to have an impact on the microbial diversity. The centralized fermentation processing setup used in the present study reflects an established microbial diversity with little impact from the harvest points. Surprisingly, comparison of the single-variety trials with the origin-blend trials showed that fermentation carried out with organic cocoa from the region of Waslala was dominated by a different yeast species, namely *Kazachstania humilis*. The beer-like flavour that characterized this lot during the tastings might be related to the dominance of this species and the fact that bean ethanol remained at high levels at the end of the process. *Kazachstania humilis* is mainly found in sourdough fermentations and it is an acid tolerant species growing well at pH as low as 3.5 (Guerzoni et al., 2013). It has been reported frequently in cocoa bean fermentations but it does normally not represent the most abundant species, except for Indonesian fermentations (Ardhana and Fleet, 2003; Schwan and Fleet, 2014). In the case of this study, it seemed to have originated from the location where the cacao was harvested from and the micro-environment of this fermentation allowed for a selection of this yeast. *Pichia barkeri* was found at the end of Rugoso® 2015. *Pichia barkeri* is a cactophylic yeast isolated from necrotic tissue of the prickly pear cactus (*Opuntia stricta*) found at the Caribbean or on the cactus *Pilosocereus arrabidae* (Kurtzmann et al., 2011). It is the first time to our knowledge to be found in cocoa bean fermentations.

The low pH of the pulp at the onset of the process seemed to be a substrate stress factor (Nielsen et al., 2014) causing probably a spontaneous selection for citrate and/or low pH tolerant species. Citric acid assimilation did not start before the abundance of *Lactobacillus fermentum*

increased, confirming that this is the main cocoa-related species with this capability (De Vuyst and Weckx, 2016). Bean citric acid levels has previously been shown to remain stable during cocoa bean fermentations around the world (Papalexandratou et al., 2011a; Papalexandratou and De Vuyst, 2011; Papalexandratou et al., 2011b, 2011c, 2013). This could explain the intense citrus and other fresh fruit notes detected during the tasting of the liquor samples of the trials of the present study. Bulk cocoa from West Africa, Brazil, and Malaysia is characterized by higher pulp and bean pH values (~3.9 and 6.5, respectively) (Camu et al., 2007; Nielsen et al., 2007; Papalexandratou et al., 2011a; Papalexandratou and De Vuyst, 2011; Papalexandratou et al., 2011b, 2011c, 2013) than found in the present study. (Voigt and Biehl, 1995; Voigt et al., 1994) suggest that the final bean pH is a key measurable parameter to identify the end of the fermentation process. It is suggested that fermented beans with final pH of 5.5–5.0, reflecting therefore a 1.5 pH-unit drop, would be characterized by good intense cocoa flavour. Taking this theory to the fine cocoa, it can be subsequently assumed that an adequate final bean pH would be approximately 4.3 ± 0.3, depending on the initial pH which is variety-specific. The speed of acidification and bean pH curve is important to the flavour and quality consistency of the final product. According to this theory and in combination with the microbial successions and colour/odour progress, the fermentations of this study were characterized as complete.

The pulp glucose and fructose concentrations were lower than reported in West Africa, Brazil, and Malaysia but similar with some fermentations from Ecuador (Afoakwa et al., 2008; Camu et al., 2007; Nielsen et al., 2007; Papalexandratou et al., 2011a; Papalexandratou and De Vuyst, 2011; Papalexandratou et al., 2011b, 2011c; Schwan and Fleet, 2014) and they were mostly consumed during the first 48 h of all fermentations when Enterobacteriaceae were predominating, occasionally with partial involvement of *Weissella fabalis*. The latter species showed a tendency to appear in fermentations were the pulp fructose concentration was higher than the glucose concentration. *Weissella fabalis* was originally isolated from Brazilian cocoa bean fermentations and it has seen to grow well in the presence of fructose (Papalexandratou and De Vuyst, 2011; Papalexandratou et al., 2011c; Snauwaert et al., 2013). Enterobacteriaceae seem to play a very important role in fermentations of fine cocoa origins, like Nicaragua (current study), Honduras (unpublished data), and Ecuador (Papalexandratou et al., 2011b). The main species found during the first 3 days of fermentation of this study all belonged to the *Tatumella* genera. Enterobacteriaceae have been shown to produce pectinolytic enzymes and assimilate citric acid but the main product of their metabolism is gluconic acid. Gluconic acid has been found in high concentrations in the pulp as well as the beans of Ecuadorian cocoa bean fermentations of Nacional cocoa (Papalexandratou et al., 2011b). The beverage industry suggests that gluconic acid has several positive organoleptic properties (http://www.jungbunzlauer.com); sodium gluconate can reduce bitterness caused by caffeine. Also, it can balance the sourness intensity caused by sharper acids like acetic acid. When gluconic acid is a fermentation product, it can contribute with a long-lasting mild herbal flavour to the taste of the product. Reflecting these properties to cacao, gluconic acid produced by Enterobacteriaceae could diffuse into the bean and add all the above mentioned positive flavour characteristics. The single-variety *Acriollado*-type cocoas of this study received low scores for bitterness, despite the high percentages for under-fermented beans, and were all characterized by fruity acidity. Further, Rugoso®, Nicalizo®, and Johe® lots expressed herbal notes at different intensities.

Comparing tasting results to the total microbial diversity of the lots, a relation was seen between the occurrence of *Bacillus* spp. at the end of the fermentation process and the low sensory appreciation. When *Bacillus* represented 3–8% of the total bacterial community (Rugoso® 2015, Nicalizo® 2014), lots were characterized by off-notes and grassy flavour. (Lopez and Quesnel, 1973) have suggested that C3-C5 chain fatty acids produced by bacilli result in the development of off-flavours and slime on fermenting cocoa beans.

The initial conditions of the Nicaraguan fermentations did not permit dominance of lactic acid bacteria from the onset of the process, which is usually the case for most other origins (De Vuyst and Weckx, 2016). Later involvement of LAB has been seen also in Dominican Republic (Gálvez et al., 2007), although in Nicaragua LAB started getting abundant only after 48–72 h of fermentation. Heterofermentative LAB species, including *Weissella* spp. as well as *L. fermentum* were present revealing the production of lactic acid, ethanol, acetic acid, and CO_2. The latter species can produce high amounts of mannitol out of fructose but it was not seen in the case of these fermentations. During Ecuadorian fine cocoa fermentations, mannitol diffused into the bean and it contributed to the sweet flavour of the dried cocoa beans (Papalexandratou et al., 2011b). The fact that LAB were not involved at the onset of fermentations with low fresh pulp sugar concentrations raises the question if *L. fermentum* was practically restricted only to citrate assimilation, letting yeasts and Enterobacteria consume the available glucose and fructose.

Bean colour development occurs mainly during drying and drying conditions (speed, layer thickness) can affect the intensity of the brown colour. Although based on traditional theory cocoa has to reach > 70% brown coloured “well-fermented” beans through its post-harvest processing, this seems more to be relevant for Forastero varieties and less fine cocoa varieties. The lots of this study were characterized by < 50% fully browned beans. Long processing and high temperatures for long periods will eliminate potential aromatic volatiles produced by yeasts or enzymatic reactions in the bean causing a softening of flavour complexity.

On a side note, it deserves to be mentioned that chocolates produced from fine chocolate makers with beans of some of these lots won gold and silver medals in European and International chocolate competitions (e.g. O'Payo™ 2014: World's Best Organic Chocolate 2016; Silver and Bronze medals for European competition 2016 http://www.internationalchocolateawards.com/2016/07/european-bar-semi-final-competition-2016-winners/).

5. Conclusion

A combination of high throughput amplicon sequencing of yeast and bacterial communities with metabolite target analysis and sensory data allows for an in-depth evaluation and understanding of cocoa post-harvest practices and their impact on final cocoa quality. Unravelling the microbial species diversity of fine cocoa origins brings more information about the impact of fermentation and drying on final flavour and overall cocoa quality. This study showed that the post-harvest treatment of cocoa overrules the impact of the genetic cultivar. Also, even though the main yeast and bacterial species can be found in all cocoa-producing regions, regional microbial differences may be found even within the same country, reflecting the need of appropriate harvest, collection and fermentation practices. Through this study the involvement of *Tatumella* spp. in the cocoa bean fermentation was confirmed and it was illustrated that non-*Saccharomyces* species contribute to fine flavour notes. The metabolism of *Tatumella* species and their contribution to the cocoa bean fermentation still remains an important point of investigation. Further studies are needed to better understand the role of Enterobacteriaceae and supplementary quality methods to the cut-test could help in the future for beans' assessments.

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