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An untargeted metabolomic assessment of cocoa beans during fermentation

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ABSTRACT

Fermentation is a critical step in the processing of high quality cocoa; however, the biochemistry behind is still not well understood at a molecular level. In this research, using a non-targeted approach, the main metabolomic changes that occur throughout the fermentation process were explored. Genetically undefined cocoa varieties from Trinidad and Tobago (n = 3), Costa Rica (n = 1) and one clone IMC-67 (n = 3) were subjected to spontaneous fermentation using farm-based and pilot plant controlled conditions. Samples were collected daily, and acetone/water/acetic acid (70/29.5/0.5%) extracts were obtained and measured using a UPLC-ESI$^+$-Q-Tof-MS system. Analysis of pre-processed data, applying a PLS-DA model, highlighted significant differences between the three fermentation periods, observed at 0–2, 3–4 and 5–6 days, respectively. Among the most discriminating metabolites, sucrose, flavanols such as (epi) catechin, procyanidin dimers and trimers, anthocyanins and oligopeptides ranging in size from 3 to 12 amino acids were tentatively identified. In general, flavanols and sugars tended to decrease throughout fermentation, whereas most oligopeptides increased to maximal levels after 3 to 4 days of fermentation and decreased thereafter. These results shed new insight into cocoa fermentation optimization strategies and may also contribute to the development of novel alternatives for cocoa processing based on biochemical and functional values.

1. Introduction

Traditionally, cocoa beans are subjected to fermentation and drying on farms prior to their acquisition by the chocolate industry. Fermentation is recognized as a key step, which determines final cocoa quality (Biehl & Ziegleder, 2003b; Kongor et al., 2016).

During fermentation, microbial development triggers a series of changes as pulp degradation, diffusion of metabolites across the testa and diverse *postmortem* reactions that take place inside the bean. All of these phenomena result in the development of color, flavor and aroma precursors (Biehl & Ziegleder, 2003b; Buyukpamukcu et al., 2001; Nigam & Singh, 2014; Saltini, Akkerman, & Frosch, 2013). After fermentation, cocoa beans are dried. This step contributes to cocoa quality, by easing the loss of volatile organic acids, and oxidizing polyphenols. The overall result of this two steps has a profound impact in the color and flavor potential of the beans (Kongor et al., 2016).

The pulp surrounding the cocoa nibs contains approximately 11% of sugars, mainly hexoses, and has a pH of 3.5–3.8 due to the high concentration of organic acids, such as citric acid (Biehl & Ziegleder, 2003a; Dand, 2011). These conditions boost the spontaneous development of microorganisms that ferment the pulp. A sequential development of yeasts (e.g., *Kloeckera*, *Saccharomyces*, *Candida*, *Pichia and Kuyveromyces spp.*), lactic acid bacteria (e.g., *Lactobacillus*, *Leuconostoc and Lactococcus*), and acetic acid bacteria has been described (e.g., *Acetobacter and Gluconobacter spp.*) (Ardhana & Fleet, 2003; Mozzi, Ortiz, Bleckwedel, De Vuyst, & Pescuma, 2013).

Generation of microbial metabolites such as ethanol, lactic and acetic acid, combined with an increase in the cocoa mass temperature due to exothermic reactions, leads to the death of the embryo within the seed. Consequently, post mortem changes take place, for example cell membrane disruption, followed by the release of the cell's storage components. This facilitates the occurrence of biochemical reactions mainly catalyzed by glycosidases, invertases, polyphenol oxidases, peroxidases and proteases (Afoakwa, Quao, Takrama, Budu, & Saalia, 2012; Biehl & Ziegleder, 2003a).

In cocoa beans, proteins are the second most abundant macronutrients and constitute approximately 10–15% (dry weight) of the bean (Marseglia et al., 2014). The most abundant proteins in cocoa are albumins and vicilin-like globulins, which undergo changes during fermentation, mediated by enzymes, such as cysteine endoprotease, leucine-*p*-nitroanilide cleaving seryl exopeptidase, aspartic endoprotease, and carboxypeptidase (sery-exopeptidase) (Biehl & Ziegleder, 2003a). Meanwhile albumin degradation is limited during fermentation,

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vicilin-type (7S) globulin is highly broken down, in derivatives such as hydrophobic oligopeptides which then turn into hydrophilic oligopeptides and hydrophobic free amino acids (Kongor et al., 2016). Both play an important role in the development of cocoa flavor, as contributors in Maillard reactions during the roasting step (Amin, Jinap, & Jamilah, 1997; Biehl & Ziegleder, 2003a; Kongor et al., 2016; Marseglia et al., 2014; J. Voigt, Heinrichs, Voigt, & Biehl, 1994). Several oligopeptides have been identified in fermented cocoa beans with sequences that allows identification of the source proteins vicilin or albumin (Buyukpamukcu et al., 2001; Marseglia et al., 2014). Some oligopeptides have been putatively classified as aroma-related peptides as for example FILPH and IFVPH (Voigt, Janek, Textoris-Taube, Niewienda, & Wöstemeyer, 2016). However, the dynamic of peptides formation in the course of fermentation is not well known.

From a health functional perspective, certain peptides may also improve cocoa quality, as specific peptide classes have been reported to have antioxidant and angiotensin-converting enzyme inhibitory activities (Sarmadi, Ismail, & Hamid, 2011).

On the other hand, cocoa beans are rich in metabolites such as polyphenols (Biehl & Ziegleder, 2003a) mainly flavonoids, which account nearly 13% of the dried unfermented cocoa beans mass (Kim, Lee, & Lee, 2011). Proanthocyanidins, catechins and anthocyanins represent 58–65%, 29–38% and 1.7–4%, of the total polyphenols found in cocoa, respectively (Biehl & Ziegleder, 2003a). Important metabolites include epicatechin, catechin, procyanidin oligomers and some glycosides such as cyanidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside (Kim et al., 2011; Niemenak, Rohsius, Elwers, Omokolo Ndoumou, & Lieberei, 2006; Ortega et al., 2008). Flavonoids have been shown to decrease during fermentation, caused by lixiviation, oxidation, hydrolysis and polymerization to form complex tannins (Afoakwa et al., 2012; Nazaruddin, Seng, Hassan, & Said, 2006).

Flavonoids also influence cocoa quality, as they impart astringent flavors and drive a wide range of colors, from violet to dark brown. In addition, the bioactivity of the cocoa flavonoids include antioxidant, anti-inflammatory, immune modulating, and antimicrobial activities (Hii, Law, Suzannah, Misnawi, & Cloke, 2010; Rusconi & Conti, 2010).

From a global perspective, the biochemical changes that occur during cocoa fermentation have been hypothesized; yet, the comprehension of the dynamics at a molecular level still has some gaps to be fulfilled. This study attempts to contribute in this direction, by trying to identify the main metabolites (<1000 Da) of a cocoa extract, that enhance the differences between three fermentation stages.

2. Materials and methods

2.1. Samples

Three varieties of cocoa were used in this study. Two genetically undefined varieties were provided from the Cocoa Research Centre of The University of The West Indies, Trinidad and Tobago (n = 3) and from a local producer from Upala, Costa Rica (n = 1). The clone IMC-67 was provided by CATIE (The Tropical Agricultural Research and Higher Education Center), Costa Rica (n = 3).

2.2. Chemicals

Hexanes and acetone ACS grade were acquired from Avantor Performance Materials. The following standards were employed for UPLC–MS analysis: citric acid (99.5% ACS reagent, Sigma-Aldrich, St. Louis, Missouri, USA), malic acid (99%, Sigma-Aldrich), L-tryphtophan (≥98%, Sigma-Aldrich) and L-phenylalanine (≥98%, Sigma-Aldrich), sucrose (≥98%, Sigma-Aldrich), and (+)-catechin (98%, Sigma-Aldrich). Standards were prepared in Milli-Q water. Acetonitrile (Avantor Performance Materials, Center Valley, Pennsylvania, USA) and methanol (LiChrosolv®, Merck Millipore, Billerica, Massachusetts, USA) were acquired with HPLC grade. Formic acid was added to the solvents (98%, Fluka, Sigma-Aldrich) at a 0.1 % (v/v) concentration.

2.3. Fermentation trials

Cocoa pods were visually selected and those with physical damage or disease symptoms were dismissed. Selected pods were manually opened and the beans were placed in boxes following spontaneous fermentation. Internal temperature was monitored throughout the fermentation process in the central point, and mixing of the cocoa mass was done manually at 48 and 96 h. The mix of cocoa varieties from Upala (Northern Costa Rica), were fermented at a farm in a wooden box (1 * 1 * 0.5 m) that was divided into four equal dimensions, with approximately 100 kg of cocoa raw beans stored *per* unit. Cocoa from Trinidad and Tobago was fermented at a farm using the outlined box method, with a load of 32 kg. The clone IMC-67 was fermented in polystyrene boxes (23 * 22 * 17 cm) with an average of 40 kg of cocoa raw beans *per* box, that were placed inside a cabin with controlled temperature (30 °C). Nibs were manually sampled, daily, and in the center of the fermentation mass. Sampling mass ranged in between 10 and 100 g depending on the size of the batch; and preserved using flash freezing in liquid nitrogen and subsequently stored at −18 °C. Afterwards, samples from Costa Rica (clon and mix of varieties) were dehydrated at the same time and for a week, with a combination of solar and artificial forced-air tray drying at less than 30 °C. The end of the drying step was determined by evaluating the following criteria: color, odor, hardness and degree of cracking of shell under manual pressure, to obtain approximated moisture below 7% (w/w).

2.4. Cocoa extracts

Dried samples were de-shelled manually and grinded in a blade grinder. Next, the samples were subjected to the procedure outlined in (Ortega et al., 2010) with some modifications. An exact mass (m) of approximately 1–3 g of the grinded cocoa material was weighed and followed defatting, extraction and dilution steps. The volume of the solvents and the dilution factors employed were determined in relation to the initial grinded cocoa mass (m), to reduce variance related to sample mass. Grinded material was defatted with 8.3 * m hexane volume (mL) for 20 min in an orbital shaker at 250 rpm and followed by centrifugation at 5000 rpm for 15 min. This defatting procedure was repeated three more times. Afterwards, defatted cocoa material was extracted with a 5 * m volume (mL) of acetone-water-acetic acid (70:29.5:0.5), vortexed for 3 min and centrifuged at the same conditions mentioned previously. This extraction procedure was repeated three more times. Combined supernatants were filtered through glass-microfibre discs (Boeco, Germany) and acetone was evaporated at maximum temperature of 40 °C and under vacuum. Concentrated extracts were diluted with Milly-Q water 21 * m times. Extracts were filtered through a 0.45 μm regenerated cellulose filter (Minisart-RC 25) prior to analysis.

2.5. Instrumental analysis

Samples were analyzed using an UPLC-ESI-QTof-MS system (Acquity, Waters, Milford, Massachusetts, USA). Chromatographic separation was performed using a 1.7 μm CSH C_{18} column of 2.1 * 100 mm (Acquity, Waters) coupled to a 1.7 μm CSH C_{18}VanGuard pre-column in an ACQUITY Ultra Performance LC™ system equipped with an auto sampler and PDA detector (Waters). Gradient elution was carried out using water (solvent A) and acetonitrile (solvent B), both adjusted with 0.1% formic acid. The elution started at 0% of B and continued as follows (min, B %): (7, 10); (22, 95); (22.1, 0) and ended with a re-equilibration step (26, 0).The flow rate was fixed at 0.4 mL/min with a total run time of 26 min. Column temperature was fixed at 30 °C. Sample volumes of 6 μL were injected and their temperatures were controlled at 5 °C before injection.