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The environmental and intrinsic yeast diversity of Cuban cocoa bean heap fermentations

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ABSTRACT

The environmental yeast diversity of spontaneous cocoa bean fermentations in east Cuba was investigated. Seven fermentations, 25 equipment- and handling-related samples, and 115 environmental samples, such as flowers, leaf and cocoa pod surfaces, as well as drosophilid insects, were analysed. The basic fermentation parameters temperature and pH were recorded during five fermentations for at least six days. A total of 435 yeast isolates were identified by a combination of PCR-fingerprinting of genomic DNA with the M13 primer and sequence analysis of DNA from representative isolates, using the internal transcribed spacer region, the D1/D2 region of the large subunit rRNA gene, and an actin gene-encoding fragment, as required. Among 65 yeast species detected, *Pichia manshurica* and *Hanseniaspora opuntiae* were the most frequently isolated species, obtained from five and four fermentations, followed in frequency by *Pichia kudriavzevii* from two fermentations. *Saccharomyces cerevisiae* was isolated only occasionally. Cocoa fermentation yeast species were also present on processing equipment. The repeated isolation of a preliminarily as *Yamadazyma* sp. classified species, a group of strains similar to *Saccharomycopsis crataegensis* from fermentations and equipment, and the isolation of fifteen other potentially novel yeast species in low numbers provides material for further studies. Environmental samples showed higher yeast diversity compared to the fermentations, included the most frequent fermentation species, whereas the most frequently isolated environmental species were *Candida carpophila*, *Candida conglobata*, and *Candida quercitrusa*. Potential selective advantages of the most frequently isolated species were only partly explained by the physiological traits tested. For instance, tolerance to higher ethanol concentrations was more frequent in strains of *Pichia* spp. and *S. cerevisiae* compared to *Hanseniaspora* spp.; the ability to also assimilate ethanol might have conferred a selective advantage to *Pichia* spp. In contrast, high glucose tolerance was common among strains of *Hanseniaspora* spp., *Torulaspora delbrueckii*, and *Candida tropicalis*, among which only *Hanseniaspora* spp. were frequently isolated.

1. Introduction

Cocoa trees (*Theobroma cacao* L.) are assumed to have been introduced into Cuba by the Spanish after the colonization of Mexico in the mid-16th century (Hartmann and Larramendi, 2011). With a production of 1425 t of cocoa beans in 2013 (Food and Agriculture Organisation of the United Nations), Cuba is a small-scale cocoa-producing country and strives to offer high-quality fermented dry cocoa beans to the world market. Although cocoa growing was widespread throughout Cuba from the 17th until the 19th century, the current production is centred in Baracoa, surrounded by tropical rainforest-covered mountains, and located in the easternmost province of Cuba, Guantánamo. More than 70% of the national cocoa production takes place in this region (Oficina Nacional de Estadísticas e Información, 2012). Cocoa cultivation and harvest are performed by individual farmers with familial transmission of the techniques (Márquez Rivero and Aguirre Gómez, 2008). Most cocoa plantations contain a variety of crops and other

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plants, such as banana, coconut, and the endemic royal palm *Roystonea regia*. The farmers deliver the largest part of the harvest to government-owned cooperatives that centralise the fermentation and drying processes. Small domestic-scale fermentations are carried out by farmers only rarely. Traditionally, they use a *yagua*, a container made from a dry leaf of the Cuban royal palm to collect the cocoa pulp-bean mass at the harvest sites and to carry out fermentations of 10–20 kg close to their farm houses. Empirically, smaller fermentations are said to result in better quality of fermented cocoa beans. Detailed practices, such as turning, leaf covers, and exposure to sun, differ among farms.

Cocoa pulp contains mostly fructose and glucose in concentrations of 40–80 mg/g, which are fermented to alcohol largely by yeasts and metabolised by bacteria to lactic acid, acetic acid, and mannitol (Camu et al., 2007, 2008; Ho et al., 2014; Papalexandratou et al., 2011b). The initial low pH of the cocoa pulp-bean mass of 3.3 to 4.0, mainly due to 5–40 mg/g of citric acid (Ho et al., 2014 and references therein), favours growth of acid-tolerant yeast strains of the genera *Hanseniaspora* and *Pichia* in the first phase of the fermentation process (Daniel et al., 2009). The pH then rises due to the metabolic conversion of citric acid by lactic acid bacteria (Camu et al., 2007, 2008; Ho et al., 2014). The draining of pulp compounds caused by pectin degradation by the yeasts facilitates oxygen access, allowing acetic acid bacteria to aerobically oxidize ethanol into acetic acid in an exothermic reaction that causes a temperature increase from ambient temperature (25–32 °C) to up to 50 °C (Camu et al., 2007, 2008; Schwan and Wheals, 2004). Increasing temperatures together with rising ethanol concentrations of up to 20 mg/g in spontaneous fermentations (Camu et al., 2007, 2008) limit the yeast activity, depending on the yeast species. From major cocoa bean fermentation species isolated in Ghana, *Saccharomyces cerevisiae* strains were ethanol-tolerant but not thermotolerant, while certain *Pichia kudriavzevii* strains combined ethanol, temperature, and acid tolerance (Daniel et al., 2009). The high temperatures and various metabolites, including organic acids produced during cocoa bean fermentation, loosen the seed coat, kill the germ, reduce bitter taste compounds, and provide precursors for aroma formation during the subsequent roasting process. The study of bacterial starter cultures without yeasts shows highly variable fermentation results (Lefeber et al., 2011). Cocoa bean fermentations inoculated with *S. cerevisiae*, *Pichia kluyveri*, and *Kluyveromyces marxianus* demonstrate the differential influence of these yeast species on the flavour profile of the resulting chocolates compared to spontaneous fermentation (Crafack et al., 2013; Lefeber et al., 2011). By comparing yeast-free fermentations with common spontaneous cocoa bean fermentations, the essential role of yeasts for flavour development has been shown and a likely link to the lack of ethanol in such fermentations has been established (Ho et al., 2014).

The spontaneous nature of cocoa bean fermentations may be the source of microbial variation, and hence of variable end-product quality. More than one hundred yeast species have been isolated from cocoa bean fermentations and the processing equipment involved (Daniel et al., 2009 and references therein; Ho et al., 2014; Meersman et al., 2013; Nielsen et al., 2010; Papalexandratou et al., 2011a, 2013). The most frequently encountered species are *S. cerevisiae*, *P. kudriavzevii*, and *Hanseniaspora guilliermondii* (12, 10, and 8 out of 15 reports, respectively), although other species such as *Pichia membranifaciens*, *P. kluyveri*, *Candida tropicalis*, *Hanseniaspora opuntiae*, *Pichia manshurica*, *Torulaspora delbrueckii*, and *Meyerozyma guilliermondii* have been found frequently (7 to 4 reports).

Whereas the yeast diversity of cocoa bean fermentations, including equipment and cocoa handling-related samples has been studied, neither the habitat in which cocoa bean fermentation yeasts reside between the harvest and fermentation seasons nor the vectors that inoculate spontaneous cocoa bean fermentations have yet been explored. The lack of reports on the isolation of yeasts from the environments of cocoa bean fermentations, other than tools and cocoa pods, lead to the present study of the yeast diversity in habitats that may harbour yeasts that could potentially initiate cocoa bean fermentations. This study documents cocoa bean fermentation data from east Cuba, from where such data were missing.

2. Materials and methods

2.1. Harvest and fermentation

Field experiments were carried out in 2013 and 2014. Selected cocoa pods were cut from the trees using a 2 to 3 m long stick with an attached knife. The pods were piled up on the ground close to the harvest site. On the delivery day, pods were opened with a knife and the cocoa pulp-bean mass scooped out from the pods by hand, sometimes protected by a glove, into a *yagua*. Fabric bags were filled with 40 to 50 kg of cocoa pulp-bean mass each from the *yagua* and transported by horse to either a collection point or the cooperative. The duration of the transport and waiting period in bags varied between 8 h to two days. At the cooperative, cocoa pulp-bean mass from different farms was used to form long fermentation heaps (several m) of about 70 cm height on a concrete platform exposed to the sun (further referred to as industrial-scale fermentations). The heaps were covered by a black plastic covering. From day 3 onwards, the heaps were turned daily in the morning by manual shovelling. Fermentation was considered as completed when the inner colour of the beans was reddish, which usually required seven days. Also, two small fermentations, further referred to as domestic-scale fermentations (not turned), performed in *yaguas* close to the respective harvest sites were sampled.

2.2. Sampling sites

The sampling sites and their specifications are listed in Table 1 and visualised in the Supplementary Fig. 1. Cocoa-related sites located within a radius of 3.5 km were contrasted with rural sites without cocoa trees or cocoa bean fermentation activities. These rural sites were located within a radius of about 40 km.

2.3. Fermentation sampling

In the case of the industrial-scale fermentations, the cocoa pulp-bean mass was transported on the harvest day (2013 field experiment) or within three days after the harvest (2014 field experiment) to the cooperative and was set up into large fermentation heaps together with cocoa pulp-bean mass from other plantations. The domestic-scale fermentation heap was formed directly after harvest. Four industrial-scale fermentation heaps as well as one small domestic-scale fermentation (Table 2) were followed over their entire duration by three to four samples on day 1 and 2, two samples on day 3, and then daily samples until yeast growth ceased (between 80 and166 h) or until the beans were spread out for drying. Two fermentations were only partially sampled.

2.4. Sampling methods

Sampling of cocoa pulp-bean mass was started 10–20 min after opening the cocoa pods. Initial samples were taken from the *yagua*, followed by samples from the fabric bags used by the farmers to transport the cocoa pulp-bean mass, and finally from the assembled fermentation heaps to which the content of the transport bags had been added. One sample of 50 to 100 g from about 20–30 cm below the surface was collected in sterile plastic sample bags per sampling site and time point. The samples were transported to the laboratory on ice and processed on the same day or kept refrigerated for one night. Flowers and tree exudates were either collected in sterile tubes and later submerged in saline (0.85% NaCl, m/v) or liquid was taken from the flowers by 10 μL microcapillaries and transferred into tubes containing saline. Insects were either enclosed in tubes and later submerged in saline or enclosed