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Dynamics and species diversity of communities of lactic acid bacteria and acetic acid bacteria during spontaneous cocoa bean fermentation in vessels

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ABSTRACT

To speed up research on the usefulness and selection of bacterial starter cultures for cocoa bean fermentation, a benchmark cocoa bean fermentation process under natural fermentation conditions was developed successfully. Therefore, spontaneous fermentations of cocoa pulp-bean mass in vessels on a 20 kg scale were tried out in triplicate. The community dynamics and kinetics of these fermentations were studied through a multiphasic approach. Microbiological analysis revealed a limited bacterial species diversity and targeted community dynamics of both lactic acid bacteria (LAB) and acetic acid bacteria (AAB) during fermentation, as was the case during cocoa bean fermentations processes carried out in the field. LAB isolates belonged to two main $(GTG)_5$-PCR clusters, namely *Lactobacillus plantarum* and *Lactobacillus fermentum*, with *Fructobacillus pseudofilculneus* occurring occasionally; one main $(GTG)_5$-PCR cluster, composed of *Acetobacter pasteurianus*, was found among the AAB isolates, besides minor clusters of *Acetobacter ghanensis* and *Acetobacter senegalensis*. 16S rRNA-PCR-DGGE revealed that *L. plantarum* and *L. fermentum* dominated the fermentations from day two until the end and *Acetobacter* was the only AAB species present at the end of the fermentations. Also, species of *Tatumella* and *Pantoea* were detected culture-independently at the beginning of the fermentations. Further, it was shown through metabolite target analyses that similar substrate consumption and metabolite production kinetics occurred in the vessels compared to spontaneous cocoa bean fermentation processes. Current drawbacks of the vessel fermentations encompassed an insufficient mixing of the cocoa pulp-bean mass and retarded yeast growth.

1. Introduction

Raw, freshly harvested cocoa beans have an unpleasant and astringent flavour. Hence, it is absolutely necessary to ferment, dry, and roast raw cocoa beans to obtain the desired organoleptic characteristics (Beckett, 2009; De Vuyst et al., 2009; Thompson et al., 2007). The cocoa bean fermentation process aims at killing the seed embryo, which prevents cocoa beans to germinate, and facilitating removal of the mucilaginous pulp, which surrounds the cocoa beans. In addition, certain aroma precursors (free amino acids, reducing sugars, and peptides) within the cotyledons are formed which contribute, after roasting, to the characteristic chocolate flavour (Afoakwa et al., 2008; Beckett, 2009).

Nowadays, the cocoa tree, *Theobroma cacao* L., is cultivated within a narrow equatorial belt that crosses South and Central America, Africa, and Asia (Wood and Lass, 1985). In the last decade, the microbial diversity of cocoa bean fermentation processes has been studied in detail (Ardhana and Fleet, 2003; Camu et al., 2007, 2008b; Daniel et al., 2009; Jespersen et al., 2005; Kostinek et al., 2008; Lagunes-Gálvez et al., 2007; Nielsen et al., 2005, 2007). These studies have resulted in a better understanding of the microbial succession and activities taking place during fermentation of the cocoa pulp-bean mass (Camu et al., 2007, 2008a,b; Nielsen et al., 2007). The key microorganisms that are crucial for successful cocoa pulp fermentation are yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (Camu et al., 2007; Schwan and Wheals, 2004). Under the initial anaerobic conditions of the tight pulp-bean mass, yeasts produce ethanol from glucose (sucrose). Also, the pectinolytic yeast community is held responsible for liquefying the pulp, allowing the pulp sweatings to drain away out of and air to penetrate into the fermenting pulp-bean mass (Schwan et al., 1995; Schwan and Wheals, 2004). In parallel, LAB convert citric acid and residual carbohydrates in the pulp into mainly lactic acid, acetic acid, and/or mannitol, enabling a slight pH

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increase (Camu et al., 2007). The LAB species diversity is rather limited; only strains of *Lactobacillus fermentum* and *Lactobacillus plantarum* dominate Ghanaian spontaneous cocoa bean heap fermentations (Camu et al., 2007, 2008b). During the aerobic phase, *Acetobacter pasteurianus* is the main AAB species participating in spontaneous cocoa bean fermentation. The AAB oxidize the ethanol produced by the yeasts and the lactic acid produced by the LAB into acetic acid (Camu et al., 2007; Schwan and Wheals, 2004). This volatile short-chain fatty acid is the key metabolite of a cocoa bean fermentation process. Together with ethanol, it diffuses into the beans and, together with the temperature increase due to microbial oxidation of ethanol into acetic acid and overoxidation of the latter into carbon dioxide and water, it causes the death of the seed embryo (Camu et al., 2007; Thompson et al., 2007). In parallel, diffused acetic acid disintegrates cellular membranes inside the cocoa beans. This induces enzymatic transformations of substrates within the cotyledons of the beans that lead to formation of precursors of the characteristic flavour compounds and colour of fully fermented cocoa beans (Thompson et al., 2007).

Today, the cocoa bean fermentation process is still a spontaneous, uncontrolled, on-farm process, without the addition of starter cultures. Although preliminary experiments of the application of defined starter cultures show satisfying results (Buamah et al., 1997; Dzogbefia et al., 1999; Leal et al., 2008; Samah et al., 1992, 1993; Sanchez et al., 1985; Schwan, 1998), they have not been introduced in the field. However, starter cultures may influence the fermentation rate of raw cocoa beans and thus the taste of chocolate made of the corresponding fermented dry beans. Testing starter cultures requires laborious and time-consuming heap and/or box fermentation experiments to be carried out under difficult circumstances in the field or a fermentory in the origin countries.

The present study aimed at developing a benchmark cocoa bean fermentation process to speed up research on the usefulness and selection of bacterial starter cultures. Therefore, spontaneous cocoa bean fermentations were set-up in vessels and analysed multiphasically, encompassing microbiological analysis (culture-dependent and culture-independent), metabolite target analysis, and pilot-scale cocoa liquor production.

2. Materials and methods

2.1. Cocoa bean vessel fermentations

Three individual spontaneous cocoa bean fermentations were carried out in 20 l plastic vessels in a temperature-controlled room (28 °C). The fermentations were carried out simultaneously to exclude as much environmental factors as possible (harvest season, external contamination factors). Mature, freshly harvested, healthy cocoa pods were obtained from the same plantation at the end of the main-crop of 2007 (January to February 2008). Selected pods were broken with washed machetes by workers who first washed their hands, the placenta of the fruits was removed, and the pulp-bean mass was immediately transferred into a clean, decontaminated vessel to obtain a homogeneous mixture of 60 kg of wet beans. After mixing, 60 kg of the pulp-bean mass was transferred and divided into three equal vessels (20 kg/vessel) and lasted to ferment for six days without mixing. The pulp sweatings produced during fermentation were allowed to drain away through a hole in the bottom of the vessels. To simulate the anaerobic phase during the first two days of fermentation, the vessels were closed with a lid to avoid open air contact with the pulp-bean mass. After 48 h of fermentation, the lid was removed to allow air ingress into the pulp-bean mass. After fermentation, the cocoa beans were sun-dried on coverable platforms for approximately seven to ten days, depending on the weather conditions. The three vessel fermentations were monitored online for temperature and pH by inserting a digital pH 340i sensor (WTW GmbH, Weilheim, Germany) in the middle of the fermenting cocoa bean mass. As all three vessel fermentations performed similarly, the courses of one fermentation are shown below.

2.2. Sampling

Samples (±500 g) of the vessel fermentations were taken according to a fixed time schedule, namely at the start of the fermentation (time 0) and after 24, 48, 72, 96, 120, and 144 h of fermentation. Sampling was always done in the middle of the pulp-bean mass. Each sample was aseptically removed and transferred into a sterile plastic bag. After 72, 96, 120, and 144 h of fermentation, 2 kg of wet beans were withdrawn from the fermenting pulp-bean mass, sun-dried, and roasted to check the influence of fermentation time on fermented dry bean quality and sensory properties of cocoa liquors made from the corresponding roasted beans.

2.3. Culture-dependent community dynamics

The culture-dependent analysis was performed immediately after sampling following the protocol of Camu et al. (2007). Briefly, 180 ml of 0.1% (wt/vol) peptone water (Oxoid, Basingstoke, United Kingdom) was added to 20 g of pulp and beans in a sterile stomacher bag that was vigorously shaken for 5 min in a Stomacher 400 (Seward, Worthington, United Kingdom). Samples (1.0 ml) of the homogenate were serially diluted tenfold in 0.1% (wt/vol) peptone water, from which aliquots (0.1 ml) were plated on different selective agar media, which were incubated aerobically at 37 °C in a standard incubator (Jouan, Saint Herblain, France) for enumeration (by recording the number of colony forming units, CFU) of specific groups of microorganisms responsible for the cocoa bean fermentation and isolation of individual colonies: malt extract agar (MEA, Oxoid) plus 100 mg/l of oxytetracycline for yeasts, de Man–Rogosa–Sharpe (MRS, Oxoid) agar plus 200 mg/l of pimaricin for LAB, acetic acid medium (AAM) [1% D-glucose, 0.5% ethanol, 0.3% acetic acid, 1.5% bacteriological peptone, and 0.8% yeast extract; wt/vol] (Lisdiyanti et al., 2003) agar plus 200 mg/l of pimaricin for AAB. Colonies were picked up from a suitable dilution of each sample on MRS and AAM agar media and overnight cultures were stored at −20 °C in the respective broths. After transport to Belgium, colonies were further purified through successive transfers in and plating on the appropriate media, and a catalase test was performed. Potential LAB (catalase-negative) and AAB (catalase-positive) isolates were grown in MRS and mannitol–yeast extract–peptone (MYP) medium [2.5% D-mannitol, 0.5% yeast extract, and 0.3% bacteriological peptone (Oxoid); wt/vol], respectively, and stored at −80 °C.

2.4. Culture-dependent identification of LAB and AAB isolates

LAB and AAB isolates were grouped and presumptively identified through $(GTG)_5$-PCR fingerprinting of genomic DNA, according to the procedures of Gevers et al. (2001) and De Vuyst et al. (2008), respectively, taking into account the following procedures. DNA from bacterial isolates was extracted from cell pellets obtained through centrifugation (21,036 × *g*, 15 min, 4 °C) of overnight cultures (2 ml) of LAB in MRS medium and AAB in MYP medium. For cell lysis of AAB, proteinase K (VWR International, Darmstadt, Germany) was used in an amount of 2.5 mg/ml of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), as described previously (Camu et al., 2007). All PCR amplifications with the single oligonucleotide primer $(GTG)_5$ were performed using a T300 Thermocycler (Biometra GmbH, Goettingen, Germany). Numerical cluster analysis of the $(GTG)_5$-PCR profiles was performed with Bionumerics Version