International Journal of Food Microbiology 174 (2014) 72–87

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Yeasts are essential for cocoa bean fermentation

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ARTICLE INFO

Article history:
Received 4 October 2013
Received in revised form 17 December 2013
Accepted 17 December 2013
Available online 27 December 2013

Keywords:
Cocoa bean fermentation
Yeasts
Lactic acid bacteria
Acetic acid bacteria
Cocoa quality
Natamycin

ABSTRACT

Cocoa beans (*Theobroma cacao*) are the major raw material for chocolate production and fermentation of the beans is essential for the development of chocolate flavor precursors. In this study, a novel approach was used to determine the role of yeasts in cocoa fermentation and their contribution to chocolate quality. Cocoa bean fermentations were conducted with the addition of 200 ppm Natamycin to inhibit the growth of yeasts, and the resultant microbial ecology and metabolism, bean chemistry and chocolate quality were compared with those of normal (control) fermentations. The yeasts *Hanseniaspora guilliermondii*, *Pichia kudriavzevii* and *Kluyveromyces marxianus*, the lactic acid bacteria *Lactobacillus plantarum* and *Lactobacillus fermentum* and the acetic acid bacteria *Acetobacter pasteurianus* and *Gluconobacter frateurii* were the major species found in the control fermentation. In fermentations with the presence of Natamycin, the same bacterial species grew but yeast growth was inhibited. Physical and chemical analyses showed that beans fermented without yeasts had increased shell content, lower production of ethanol, higher alcohols and esters throughout fermentation and lesser presence of pyrazines in the roasted product. Quality tests revealed that beans fermented without yeasts were purplish-violet in color and not fully brown, and chocolate prepared from these beans tasted more acid and lacked characteristic chocolate flavor. Beans fermented with yeast growth were fully brown in color and gave chocolate with typical characters which were clearly preferred by sensory panels. Our findings demonstrate that yeast growth and activity were essential for cocoa bean fermentation and the development of chocolate characteristics.

1. Introduction

Cocoa beans (*Theobroma cacao*) are the major raw material for chocolate production. Fermentation of the beans is essential for removing the pulp that envelops the beans and developing precursors of chocolate flavor (Fowler, 2009; Thompson et al., 2013). Sugars and polysaccharides of the bean pulp are fermented by microorganisms, producing metabolites and conditions that cause bean death and initiate an array of biochemical reactions within the bean that generate chocolate flavor precursors. These flavors are fully developed on subsequent bean roasting and conching as part of the chocolate making process (Afoakwa et al., 2008; Lima et al., 2011; Schwan and Wheals, 2004). Although chocolate manufacturing is a multibillion dollar industry, estimated to have a global value of approximately US$ 95 billion (Pipitone, 2012), bean fermentation is still an uncontrolled traditional process (Pereira et al., 2013) conducted by a consortium of indigenous species of yeasts, lactic acid bacteria and acetic acid bacteria (Schwan and Wheals, 2004; Lima et al., 2011). To transform the fermentation into a more efficient industrialized process, controlled by the use of defined starter cultures, it is essential to know how each microbial group contributes to the fermentation process and chocolate quality (Saltini et al., 2013).

The microbial ecology of cocoa bean fermentation is complex and involves the successional growth of various species of yeasts, lactic acid bacteria, acetic acid bacteria and, possibly, species of *Bacillus*, other bacteria and filamentous fungi (Ardhana and Fleet, 2003; De Vuyst et al., 2010; Lima et al., 2011; Schwan and Wheals, 2004; Thompson et al., 2013). Within the yeasts, *Hanseniaspora guilliermondii* or *Hanseniaspora opuntiae* generally dominate the early part of fermentation after which *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Pichia membranifaciens*, *Pichia kudriavzevii* and some *Candida* spp. are most often dominant (Ardhana and Fleet, 2003; Daniel et al., 2009; Galvez et al., 2007; Jespersen et al., 2005; Nielsen et al., 2005). Of the lactic acid bacteria, *Lactobacillus plantarum* and *Lactobacillus fermentum* most frequently grow, although contributions from *Pediococcus* and *Leuconostoc* species are sometimes reported (Camu et al., 2008b; Kostinek et al., 2008; Nielsen et al., 2007). For the acetic acid bacteria, *Acetobacter pasteurianus* is most frequently the main contributor, but other species are also involved including *Gluconobacter oxydans*, *Acetobacter tropicalis*, *Acetobacter lovaniensis* and *Acetobacter syzygii* (Ardhana and Fleet, 2003; Camu et al., 2007; Lefeber et al., 2011; Nielsen et al., 2007). It is not fully understood how these microbial groups or individual species determine cocoa bean quality and chocolate character and, indeed, whether or not they are essential to the fermentation process. Roelofsen (1958) has reviewed early studies on the microbiology of cocoa bean fermentation dating from the late 1890s. Based on these early investigations, some of which involved

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0168-1605/$ – see front matter.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.12.014

crude fermentations with inoculated yeasts and bacteria, it was generally concluded that the growth of yeasts was necessary for successful fermentation and the production of cocoa beans with typical chocolate aromas and character. However, analytical evidence to support this conclusion was lacking.

The functional roles of the different groups of microorganisms have been discussed in recent reviews of cocoa bean microbiology by De Vuyst et al. (2010), Lima et al. (2011) and Schwan and Wheals (2004). The yeasts initiate an alcoholic fermentation of the pulp sugars, generating ethanol which, along with acetic acid, enters the bean to kill the embryo and trigger endogenous biochemical reactions that produce the chocolate flavor precursors. The acetic acid is thought to be produced by acetic acid bacteria via oxidation of part of the ethanol produced by the yeasts. The latter reaction generates heat that causes the fermenting bean mass to increase to 45–50 °C, also considered essential for successful fermentation and chocolate flavor development. Some yeasts contribute to pectin degradation in the pulp which facilitates bean aeration and growth of the acetic acid bacteria. The role of lactic acid bacteria and whether or not they are essential to the process is not clear. Their fermentation of pulp sugars to give lactic acid can be detrimental to cocoa bean and chocolate quality, leading to too much acidity. However, their production of this acid and potential to utilize the citric acid of the pulp may contribute to the pH balance of the process. It is believed that the internal pH of cocoa bean (around 7.0 before fermentation) must decrease to between pH 5.0 and 5.5 to allow good activity of endogenous proteases that are essential to the degradation of bean proteins and production of chocolate flavor precursors (Biehl et al., 1985; Hansen et al., 1998). Some early studies have suggested an important role of heterofermentative lactic acid bacteria in producing acetic acid, necessary for killing the beans (Roelofsen, 1958).

To better understand how individual microbial groups and species contribute to cocoa bean fermentation and chocolate character, controlled fermentation studies that integrate microbiological, chemical and sensory analyzes are required. The objective of this paper is to determine the contribution of yeasts, as a group, to cocoa bean fermentation and chocolate character. To investigate their contribution, we conducted controlled cocoa bean fermentations in the presence and absence of Natamycin (Natamax, Danisco, Denmark) which is an approved food additive that inhibits yeast growth (European Food Safety Authority, 2009).

2. Materials and methods

2.1. Cocoa bean fermentation

Cocoa pods (*Trinitario* variety) were harvested from plantations in North Queensland, Australia and transported to the University of New South Wales, Sydney. The pods were stored at 20–25 °C for 7–10 days, then cut with a knife for manual removal of the beans. The mass of beans was uniformly mixed on a tray and deliberately brought into contact with the outer surfaces of extracted pods to provide a source of natural microbial inoculum. The bean mass was divided into two × 5 kg batches which were transferred into two plastic fermentation boxes (17 × 17 × 20 cm) with drilled holes on the sides and the base to facilitate juice drainage and aeration. A solution of Natamycin was sprayed onto the beans in one box, to inhibit yeast growth. The beans were mixed to give a final concentration of 200 ppm of Natamycin throughout the mass. The cocoa beans in the other box were not treated with Natamycin but similarly mixed. The boxes of beans were incubated for fermentation which developed spontaneously due to growth of the indigenous microflora.

For fermentation, the boxes were covered with lids and incubated at 25 °C (0–12 h), 30 °C (12–24 h), 35 °C (24–36 h), 40 °C (36–48 h), 45 °C (48–72 h) and 48 °C (72–144 h) to simulate the temperature evolution that occurs during commercial cocoa fermentations (Schwan and Wheals, 2004; Lima et al., 2011). The fermenting beans were mixed every 48 h. The fermentations were stopped at day 6 when the beans were removed from their boxes and dried as a single layer at 30 °C and RH 70% for 5 days. Samples of beans (100 g total, from locations throughout the fermenting mass) were taken daily for microbiological and chemical analyses. Samples for microbiological analysis were used immediately while those for chemical analysis were stored at −20 °C until examined.

The fermentations were repeated twice, once using cocoa beans harvested in March 2011 and again with beans harvested from another plantation in October 2011. Several preliminary fermentations were conducted to optimize the conditions for fermentation and drying, chocolate production and sensory assessment, and to select the appropriate concentration of Natamycin.

2.2. Microbiological analyses

2.2.1. Enumeration of microbial populations

Cocoa beans (25 g) were aseptically mixed with 225 ml of 0.1% peptone water in a Stomacher bag and manually shaken for 5 min to give a uniform suspension of the pulp material. One ml of the suspension was serially diluted in 0.1% peptone water and 0.1 ml samples from each of three consecutive dilutions were spread inoculated onto duplicate plates of different agar media. The enumeration of yeasts was done on plates of Malt Extract Agar (MEA) (Oxoid) containing 100 mg/l of oxytetracycline and Dichloran Rose Bengal Chloramphenicol Agar (DRBC) with incubation at 25 °C for 3–4 days. Lactic acid bacteria were enumerated on de Man Rogosa Sharpe (MRS) Agar (Oxoid) containing 100 mg/l of cycloheximide at 30 °C for 3–4 days. Acetic acid bacteria were enumerated on Wallerstein Laboratories Nutrient Agar (WLNA) (Oxoid) and Glucose–Yeast Extract Agar (GYEA) (5% glucose, 0.5% yeast extract and 1.5% agar) containing 100 mg/l of cycloheximide at 30 °C for 4–5 days. Plates for culture of yeasts and acetic acid bacteria were incubated under aerobic conditions while a candle-jar was used for the incubation of lactic acid bacteria.

After incubation, counts of the yeast and bacterial groups were determined as well as those of individual yeast and bacterial species following observations of their colonial and cellular morphologies and subsequent identification. At least three representatives of each colony type were isolated from each sampling time, and purified for identification. Population data reported are the means of duplicate analyses.

2.2.2. Identification of yeast and bacterial species

Cellular morphologies of the isolates were determined by phase contrast microscopic observation. For the bacteria, Gram stains and catalase tests were conducted. Yeasts and bacteria were identified to species by a combination of rDNA sequencing and restriction fragment length polymorphism (RFLP) analysis.

2.2.2.1. DNA extraction and amplification by Polymerase Chain Reaction (PCR). Extraction of DNA from cells of yeasts (grown in Malt Extract Broth) and bacteria (grown in MRS broth) followed the protocol of Cocolin et al. (2002).

The 5.8S-Internally Transcribed Spacer (5.8S-ITS) rDNA region of yeast isolates was amplified by PCR using the primers ITS1 (5′-TCCG TAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described by Esteve-Zarzoso et al. (1999).

The 16S rDNA of bacterial isolates was amplified by PCR using the primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1495R (5′-CTAC GGCTACCTTGTTACGA-3′) for lactic acid bacteria (Yu et al., 2009) and the primers 16Sd (5′-GCTGGCGGCATGCTTAAC ACAT-3′) and 16Sr (5′-GGAGGTGATCCAGCCGCAGGT-3′) for acetic acid bacteria (Ruiz et al., 2000). The primers were purchased from SigmaAldrich (Sydney, NSW, Australia) and the nucleotides and enzyme from New England Biolabs (Ipswich, MA, USA).