



The microbial ecology of cocoa bean fermentations in Indonesia

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Abstract

Cocoa beans are the principal raw material of chocolate manufacture. The beans are subject to a microbial fermentation as the first stage in chocolate production. The microbial ecology of bean fermentation (Forastero and Trinitario cultivars) was investigated at three commercial fermentaries in East Java, Indonesia by determining the populations of individual species at 12-h intervals throughout the process. The first 2–3 days of fermentation were characterised by the successional growth of various species of filamentous fungi, yeasts, lactic acid bacteria and acetic acid bacteria. The principal species found were *Penicillium citrinum*, an unidentified basidiomycete, *Kloeckera apis*, *Saccharomyces cerevisiae*, *Candida tropicalis*, *Lactobacillus cellobiosus*, *Lactobacillus plantarum* and *Acetobacter pasteurianus*. The later stages of fermentation were dominated by the presence of *Bacillus* species, mostly *Bacillus pumilus* and *Bacillus licheniformis*. Glucose, fructose, sucrose and citric acid of the bean pulp were utilised during fermentation, with the production of ethanol, acetic acid and lactic acid that diffused into the beans. The filamentous fungi were notable for their production of polygalacturonase activity and probably contributed to the degradation of bean pulp.

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1. Introduction

Cocoa beans are the principal raw material of chocolate manufacture. The beans originate as seeds in fruit pods of the tree, *Theobroma cacao*, which is cultivated in plantations in tropical regions throughout the world. The beans are embedded in a mass of mucilaginous pulp within the pod. The beans and associated pulp are removed from the pod and subject to microbial fermentation as the first stage in preparation for chocolate production. These fermentations

are generally conducted as traditional, indigenous processes, the details of which have been well reviewed (Roelofsen, 1958; Lehrian and Patterson, 1983; Lopez and Dimick, 1995; Thompson et al., 2001; Schwan and Wheals, in press). Microbial action during fermentation solubilises the pulp material surrounding the beans, and produces a range of metabolic end-products (e.g. alcohols, organic acids) which diffuse into the beans to cause their death. These changes induce an array of biochemical reactions within the beans and generate the chemical precursors of chocolate flavour, aroma and colour (Lehrian and Patterson, 1983; Jones and Jones, 1984; Hansen et al., 1998; Hashim et al., 1998; Thompson et al., 2001).

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The importance of bean fermentation in contributing to chocolate quality has been recognised for over 100 years, and numerous studies have been conducted in different countries to determine the microbial species associated with this process (Schwan et al., 1995; Schwan and Wheals, in press). The studies can be summarised as follows. The bean pulp is rich in fermentable sugars such as glucose, fructose and sucrose, and has a low pH of 3.0–3.5, mainly due to the presence of citric acid. These conditions select for the initial growth of yeasts and lactic acid bacteria. The yeasts conduct an alcoholic fermentation and involve the sequential growth of *Kloeckera* and its teleomorphic form *Hanseniaspora*, *Saccharomyces*, *Candida*, *Pichia* and *Kluyveromyces* species. The lactic acid bacteria also ferment pulp sugars and utilise citric acid, and involve the growth of *Lactobacillus*, *Leuconostoc* and *Lactococcus* species. Acetic acid bacteria (*Acetobacter* and *Gluconobacter* spp.) eventually grow, oxidising ethanol, initially produced by the yeasts, to acetic acid. Finally, various species of *Bacillus* develop when the pH of the bean mass becomes less acid and its temperature increases to 40–50 °C due to heat generated by the total process. Various fungal species have been isolated from fermenting beans, but their role in the process is not clear (Thompson et al., 2001; Schwan and Wheals, in press).

Although significant progress has been made in defining the diversity of microbial species associated with the fermentation, quantitative information on the growth profiles of individual species throughout the process is lacking. These quantitative data, combined with appropriate chemical and sensory analyses of bean and chocolate quality, are necessary to determine which microbial species are most important to the fermentation. With this knowledge, it should be possible to optimise the fermentation process, and if necessary, select appropriate species for use as starter cultures (Schwan, 1998).

Indonesia produces about 15% of the world's cocoa beans, and ranks third in terms of international production statistics (Anonymous, 2000). Early studies on the microbiology of beans fermented at several locations in Java identified the contributions of yeasts, lactic acid bacteria, acetic acid bacteria and aerobic, spore-forming bacteria, but the growth profiles of individual species within these groups were not

reported. The contributions of filamentous fungi were not considered to be significant (Steinmann, 1928; Roelofsen and Giesberger, 1947; Roelofsen, 1958). This paper reports a detailed study of the growth of species of yeasts, filamentous fungi, lactic acid bacteria, acetic acid bacteria, *Bacillus* and other bacteria during the commercial fermentation of cocoa beans at three locations in Java, Indonesia.

2. Materials and methods

2.1. Cocoa bean fermentations

Cocoa beans were fermented as commercial operations at three estates (A, B, C) in East Java. The beans were harvested from plantations by traditional methods and used in fermentations within 6 h of harvest. Typically, cocoa pods were harvested from the tree and opened within 30 min for removal of the beans. The pods were cut with a machete by plantation workers and the beans plus surrounding pulp were scooped out by hand and placed in piles on a plastic mat. The beans were collected from the mat, transferred into plastic or hessian sacks and transported to the fermentary. Beans of the Forastero cultivar were used at estate A and beans of the Trinitario cultivar were used at estates B and C. At each fermentary, the beans (1000 kg approximately) were placed in wooden boxes (150–200 cm long × 100 cm wide × 75–100 cm deep) where natural fermentation developed. After 12–16 h, the beans were mixed by transferring them to another box. Such transfers were repeated every 20–24 h until fermentation was complete, which was 6 days for estate A and 4 days for estates B and C. The wooden boxes were not cleaned between operations, and contained holes drilled in the base and sides to allow drainage of liquid (sweatings) generated by the fermentation. Fermentations at each estate were monitored over a period of 8–9 weeks. The data reported are representative of a typical fermentation.

2.2. Microbiological analyses

Samples (250 g) of beans were aseptically scooped from the centre of the fermenting mass at intervals of 12 h. These were mixed with 250 ml of 0.1% peptone

water (Oxoid, Melbourne) in a Stomacher bag and vigorously shaken and massaged for 3 min to give a uniform homogenate. Samples (1.0 ml) of the homogenate were serially diluted in 0.1% peptone water from which aliquots (0.1 ml) were spread-inoculated in duplicate over the surface of plates of agar media for the isolation and enumeration of specific organisms. Media used for specific microbial groups were: Tryptone Yeast Extract Agar (Gibco, Melbourne) containing 100 mg/l of cycloheximide, for bacteria; MRS agar (Oxoid) with 10% tomato juice and 100 mg/l of cycloheximide, pH 5.5, for lactic acid bacteria; Glucose Yeast Extract Calcium Carbonate Agar with 100 mg/l of cycloheximide for acetic acid bacteria (Drysdale and Fleet, 1988); Malt Extract Agar (Oxoid) containing 100 mg/l of oxytetracycline; and Dichloran Rose Bengal Chloramphenicol Agar (Oxoid) for yeasts and filamentous fungi. Inoculated agar plates were incubated aerobically at 28 °C for 1–4 days after which colonies were counted. The morphological properties of the different colony types were recorded and counts made for each type. Three representative samples of each colony type were isolated and purified by restreaking, and maintained for identification. Isolation, enumeration and preliminary screening of strains for Gram reaction, cell morphology and catalase activity (Harrigan and McCance, 1976) were done on location at each fermentary.

Detailed characterisation and identification of isolates were done at The University of New South Wales, Sydney. Bacteria were identified using the range of morphological, biochemical and physiological tests outlined in Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) supplemented with the use of API test strips (API System, La Balme, Les Grottes, France). Lactic acid bacteria were identified according to cell and colony morphology, Gram, catalase and oxidase reactions, gas production from glucose, growth at pH 3.0–5.5, growth at 15–47 °C and fermentation of carbohydrate substrates in API 50CHL galleries (Krieg and Holt, 1984). *Bacillus* species were identified according to cell and colony morphology, endospore shape and location, Gram, catalase and oxidase reactions, aerobic growth, nitrite reduction, utilisation of citrate and reactions in API 50CHB and API 20E galleries (Krieg and Holt, 1984). Tests for the identification of *Acetobacter* spp. have been described by Drysdale and Fleet (1988), and in

addition to cell morphology, colony morphology and Gram and catalase reactions, included growth on ethanol and acetate, oxidation of ethanol and calcium lactate, ketogenesis of glycerol, production of 5-ketogluconic acid from glucose and formation of water-soluble brown pigments. Yeasts were identified according to the morphological, physiological, fermentation and assimilation properties described in Kreger-van Rij (1984) and Kurtzman and Fell (1998). Fungal isolates were identified by microscopic observations of their asexual and sexual morphologies on Malt Extract Agar according to Pitt and Hocking (1997) and Samson and van Reenan-Hoekstra (1988). The identities of fungal isolates were confirmed by J.I. Pitt and A.D. Hocking (Food Science Australia, CSIRO, North Ryde, Sydney) and the Central Bureau Voor Schimmel Cultures, Af Baarn, The Netherlands.

Pectinolytic activity was determined according to Speck (1984). The basal medium containing pectin (Sigma, USA) was prepared and adjusted to either pH 7.2 ± 0.2 for testing pectate lyase or to pH 5.0–6.0 for testing polygalacturonase activity. The plates were inoculated and incubated at 25 °C for fungi, at 28 °C for yeasts and at 30 °C for bacteria. Pectinolytic activity was determined, after growth of the organisms (2–6 days), by flooding the plate with 7 N hydrochloric acid. Pectinolytic colonies were surrounded by a clear zone against an otherwise opaque medium. Cellulolytic activity was determined according to Speck (1984). The basal medium containing cellulose (Whatman, UK) was prepared and adjusted to pH 7.2 ± 0.2 . After inoculation and incubation, cellulolytic activity was determined by flooding the plate with 7 N hydrochloric acid, and noting clear zones surrounding colonies. Hydrolyses of gelatin (Oxoid), casein (Difco, Detroit) and skim milk (Difco) were conducted to test for proteolytic activity of the isolates. The media were prepared following the procedures of Harrigan and McCance (1976). The plates were inoculated with the isolates, incubated at 25–30 °C for 5–7 days and then flooded with acidic mercuric chloride. Proteolytic colonies were surrounded by a clear zone. Amylolytic activity was examined on starch (Oxoid) agar plates (Harrigan and McCance, 1976). Plates were inoculated with the isolates, incubated at 25–30 °C for 5–7 days and then flooded with Gram iodine solution. Colonies sur-

rounded by a clear zone against a blue-coloured background indicated the hydrolysis of starch. Lipolytic/esterase activity was tested on tributyrin agar (Oxoid). Growth at different temperatures was done in tubes of either Malt Extract Broth (Oxoid), MRS Broth (Oxoid) or Trypticase Soy Broth (Oxoid) for yeasts, lactic acid bacteria and other bacteria, respectively. Growth was indicated by turbidity development and microscopic examination after 7 days. Growth in the presence of different concentrations (2–10%) of ethanol (v/v) was determined in the same media (plus ethanol) after incubation at 25 °C for 7 days. Growth of filamentous fungi at different temperatures was done on plates of Malt Extract Agar.

2.3. Chemical analyses

Bean samples (250 g) were physically separated from pulp by shaking with an equal volume of distilled water and decanting. Samples (20 g) of each fraction (pulp/bean) were mixed with 60 ml of distilled water and blended in an omnimixer (Bamix M100, Mettlen, Switzerland) for 4 min. The homogenate was centrifuged at $10,000 \times g$ at 10 °C for 15 min and the supernatant retained. The sediment was washed twice with 20 ml of distilled water and the washings combined with the first supernatant to provide the extract for analysis. The extracts were clarified by filtration through 0.45- μm membranes (Oxoid N47/45G), followed by ultrafiltration through an Amicon YMS membrane (Amicon, Lexington, USA). The filtrate was then passed through a Sep-pak C18 cartridge (Waters Associates, Milford, USA) and stored at –20 °C until analysis. The concentrations of monosaccharide and disaccharide carbohydrates were determined by high performance liquid chromatography (HPLC) through a Silica Radial Pak Column (Waters Associates 8SI, 10 μm) and elution with acetonitrile–water (85:15) as described elsewhere (Davis et al., 1986). Organic acids were determined by HPLC through an Aminex Ion Exclusion HPX-87 column (Bio-Rad, Richmond, CA) and elution with 0.08% H_3PO_4 (Davis et al., 1986). Ethanol concentrations were measured with enzymatic kits (Boehringer, Mannheim, Germany). The pH was measured on extracts before clarification by filtration.

3. Results

3.1. Physical and chemical changes during cocoa bean fermentation

Freshly harvested beans were enveloped in a white-cream mucilaginous pulp. This pulp represented about half of the dry weight of Forastero beans and about one-third of the dry weight of Trinitario beans. The pulp was solubilised during fermentation, giving rise to a liquid material (sweetings) that drained away through holes in the fermentation boxes. The temperature of the bean-pulp mass gradually increased throughout fermentation, from initial values of 20–25 °C (depending on the estate) to values of 48–50 °C, when the process was terminated. The pH of the pulp before fermentation was 3.7–3.9 and increased to 4.8–4.9 by the end of fermentation, while that of the bean decreased from 6.3–6.5 to 5.0–5.1 (Table 1). Pulp sugars (fructose, glucose and sucrose) were utilised during fermentation and lead to significant increases in the concentrations of ethanol, lactic acid and acetic acid. The ethanol concentration was greatest (5–6%) at 24–36 h, after which it declined (Table 1). Pulp citric acid decreased by about 55%. Sucrose was the main sugar of the beans, and decreased to nondetectable levels. The concentra-

Table 1
Changes in the chemical composition of the pulp and bean fractions during cocoa bean fermentation

Component	Concentration							
	Before fermentation				After fermentation			
	A ^a		B ^b		A ^a		B ^b	
Pulp	Bean	Pulp	Bean	Pulp	Bean	Pulp	Bean	
Fructose (mg/g)	62	1.0	42	0.8	11	0.4	9	0.3
Glucose (mg/g)	41	0.7	24	0.6	7	0.1	5	0.1
Sucrose (mg/g)	32	19	21	18	0	0	0	0
Ethanol (% w/w) ^c	0.5	0.2	0.3	0.2	0.1	0.4	1.6	1.6
Citric acid (mg/g)	24	9.0	21	7.4	11	4.0	9	3.5
Lactic acid (mg/g)	0.3	0.1	0.3	0.1	6	2.0	5	1.8
Acetic acid (mg/g)	0.4	1.0	0.4	0.7	12	25	10	15
pH	3.7	6.3	4.8	6.5	3.9	5.1	4.9	5.0

^a Estate A (Forastero beans); before, 0 h; after, 120 h.

^b Estate B (Trinitario beans); before, 0 h; after, 72 h.

^c Concentrations of ethanol after 24 h for estate A were 6.5% (pulp) and 2.8% (bean), and for estate B were 5.2% (pulp) and 0.6% (bean).

tions of ethanol, lactic acid and acetic acid in the beans increased during fermentation, most notably for acetic acid. The citric acid content of the beans, while less than that of the pulp, decreased by 50–60%.

3.2. Filamentous fungi

Filamentous fungi (10^2 – 10^3 cfu/g) were detected in the beans at the beginning of fermentation, and grew to 10^6 – 10^7 cfu/g by 24–36 h, after which they died off to nondetectable populations (<100 cfu/g) (Fig. 1). *Penicillium citrinum* and an unidentified basidiomycete were the dominant species at

each estate, followed by contributions from *Aspergillus versicolor* and *Aspergillus wentii*. The unidentified basidiomycete grew as a large white colony on Malt Extract Agar at 25 °C (colony diameter 50–60 mm in 3 days). No fruiting bodies were produced, even after mating trials with different isolates. *Penicillium purpurogenum* and another species closely related to *Penicillium ochrochloron* were also present during the fermentations at estate B. All the fungal isolates grew at 37 °C but not at 40 °C, except *A. versicolor* which grew at 40 °C but not at 45 °C (results not shown). None of the isolates exhibited pectate lyase or cellulase activities. Strong polygalacturonase reactions were found

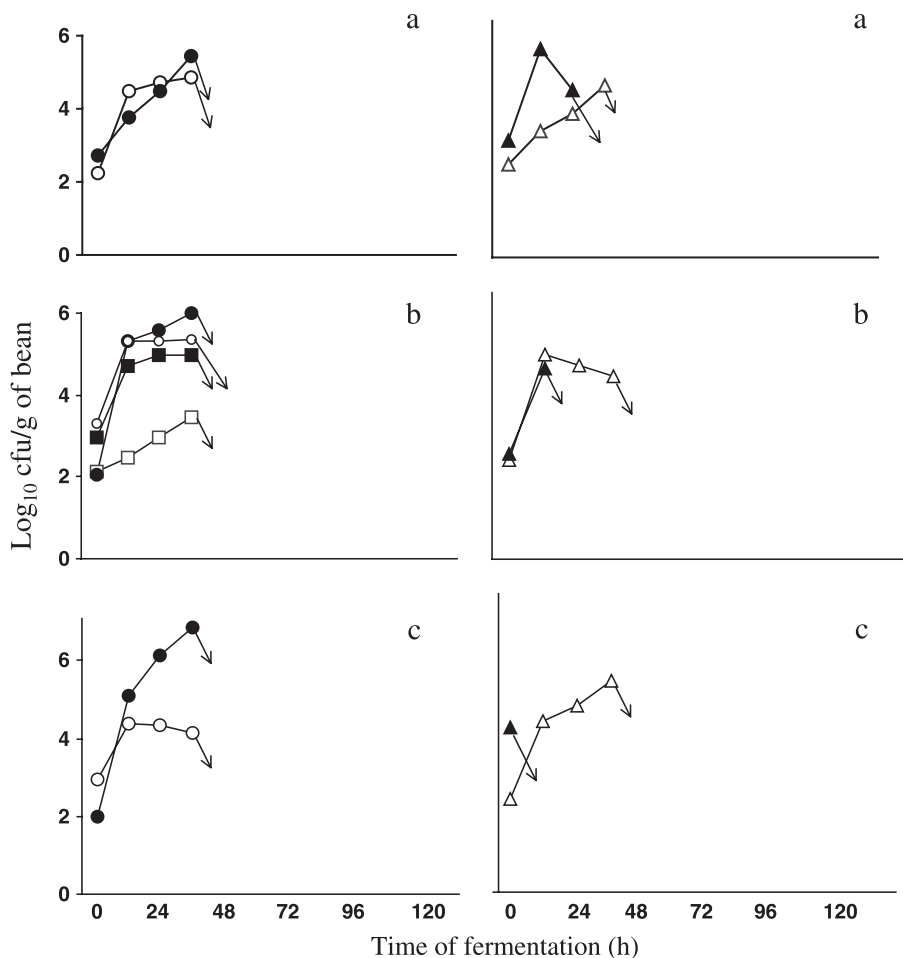


Fig. 1. Growth of unidentified basidiomycete (O), *P. citrinum* (●), *P. purpurogenum* (□), *P. ochrochloron* (■), *A. versicolor* (Δ) and *A. wentii* (▲) during coca bean fermentation at (a) estate A, (b) estate B and (c) estate C. Arrows indicate counts below 50 cfu/g of bean.

in strains of the unidentified basidiomycete and *P. citrinum*, and weaker reactions occurred with *A. wentii* and *A. versicolor*. All the isolates gave extracellular amylolytic reactions (except those of the basidiomycete) and proteolytic reactions. None were lipolytic, except for a weak reaction by *A. versicolor*.

3.3. Yeasts

Yeasts (10^4 – 10^5 cfu/g) were present in the bean mass at the commencement of fermentation, and grew to maximum populations of 10^7 – 10^8 cfu/g during the subsequent 24–36 h. Thereafter, the yeast population declined (Fig. 2). For each estate, *Kloeckera apis* was the dominant species during the first 24–36 h, after which it declined to nondetectable levels. It accounted for 70–90% of the yeast population during this early phase. *Kloeckera javanica* and *Kloeckera africana* also contributed to the fermentations at estate B (data not shown). After, *K. apis*, *Saccharomyces cerevisiae* and *Candida tropicalis* were the most significant yeasts in the fermentation at all three estates (Fig. 2). These species grew to 10^6 – 10^7 cfu/g during the first 24–36 h before declining to lower populations that persisted until fermentation was terminated. Because of this survival, they were the predominant yeasts during the later stages (48–120 h) of fermentation. Other species of *Candida*, especially *Candida pelliculosa* and *Candida humicola* (not shown), were detected during the early stages of fermentation, but contributed less than 5% of the yeast flora. *Rhodotorula rubra* (estate A) and *Rhodotorula glutinis* (estate B) were also isolated (10^5 – 10^6 cfu/g) but not after 36 h.

Only isolates of *S. cerevisiae* and *C. tropicalis* gave significant growth at the higher temperature of 40 °C, with a small proportion of these strains demonstrating weak growth at 47–50 °C. Isolates of *K. apis* grew at 37 °C and some gave weak growth at 40 °C. Isolates of *K. javanica* and *K. africana* did not grow at 37 °C. All isolates of *S. cerevisiae* and *Candida* species grew well in the presence of 7% ethanol and weakly at 10% ethanol. Isolates of *Kloeckera* species grew at 5% ethanol, but not at higher concentrations. None of the yeast isolates gave positive reactions for pectate lyase, polygalacturonase, cellulase, amylase or protease. Most gave positive reactions on tributyrin agar.

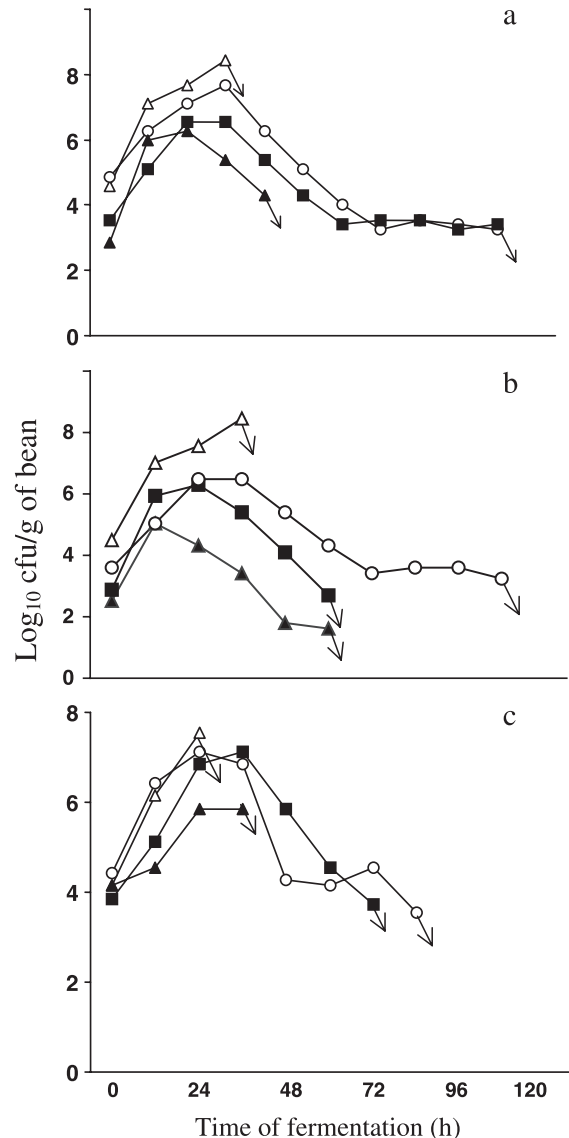


Fig. 2. Growth of *S. cerevisiae* (○), *K. apiculata* (△), *C. tropicalis* (■) and *C. pelliculosa* (▲) during cocoa bean fermentation at (a) estate A, (b) estate B and (c) estate C. Arrow indicates counts below 50 cfu/g of bean.

3.4. Lactic acid bacteria

Lactic acid bacteria grew during fermentation to maximum populations of 10^8 – 10^9 cfu/g at 36 h (Fig. 3). *Lactobacillus cellobiosus* was the most predominant species at each estate and accounted for 60–

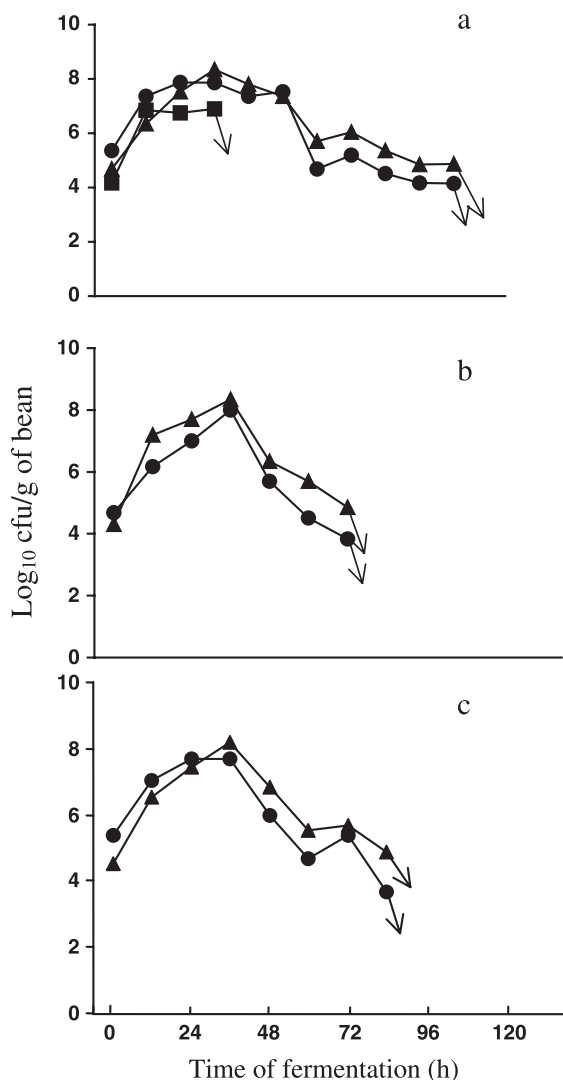


Fig. 3. Growth of *L. plantarum* (●), *L. hilgardii* (■) and *L. cellobiosus* (▲) during cocoa bean fermentations at (a) estate A, (b) estate B and (c) estate C. Arrow indicates counts below 50 cfu/g of bean.

85% of the lactic acid bacteria by 36–48 h. *Lactobacillus plantarum* was also significant, especially during the first 24 h of fermentation at estates A and C. *Lactobacillus hilgardii* grew during fermentations at estate A, but had died off by 48 h. At its maximum population (36 h), it constituted less than 2% of the lactic acid bacteria. None of the isolates of lactic acid bacteria grew at 50 °C, but those of *L.*

cellobiosus grew well at 45–47 °C and those of *L. plantarum* grew at 40–45 °C. *L. hilgardii* strains did not grow at 45 °C and gave weak growth at 40 °C. Isolates of all three species grew in the presence of 7% ethanol, but only those of *L. plantarum* and *L. hilgardii* grew at 10% ethanol. None of the isolates gave positive reactions for pectate lyase, polygalacturonase, cellulase, amylase, esterase/lipase and protease.

3.5. Acetic acid bacteria

Both *Acetobacter pasteurianus* and *Acetobacter aceti* contributed to fermentations during the first 24 h at estate A and the first 12 h at estates B and C, reaching maximum populations of 10^5 – 10^6 cfu/g before declining sharply (Fig. 4). *A. pasteurianus* was the more dominant species and survived longer throughout the fermentations. None of the isolates gave significant growth at temperatures above 35 °C. Isolates of *A. pasteurianus* were tolerant of 10% ethanol, whereas those of *A. aceti* did not grow at ethanol concentrations above 5–7%. None of the acetic acid bacteria gave positive pectolytic, cellulase, amylase, protease or lipase reactions.

3.6. Bacillus species

Bacillus species grew throughout fermentation and became the dominant microflora (10^7 – 10^8 cfu/g) after 48–72 h, when other species had died off (Fig. 5). They remained at this high population until the fermentations were considered complete. Four species, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus cereus* were significant in the fermentations conducted at estate A, with *B. pumilus* showing final dominance. Fermentations at estate B had a dominance of *B. licheniformis* and *B. pumilus*, and a minor contribution from *Bacillus sphaericus*. *Bacillus coagulans* dominated during fermentations at estate C, along with a lesser presence of *B. pumilus* (Fig. 5). Most of the *Bacillus* isolates grew at 50 °C (except *B. cereus* and *B. sphaericus*), and gave significant reactions for protease, amylase (except *B. pumilus* and *B. sphaericus*) and lipase (except *B. cereus* and *B. sphaericus*). None of the isolates gave pectolytic or cellulolytic reactions.

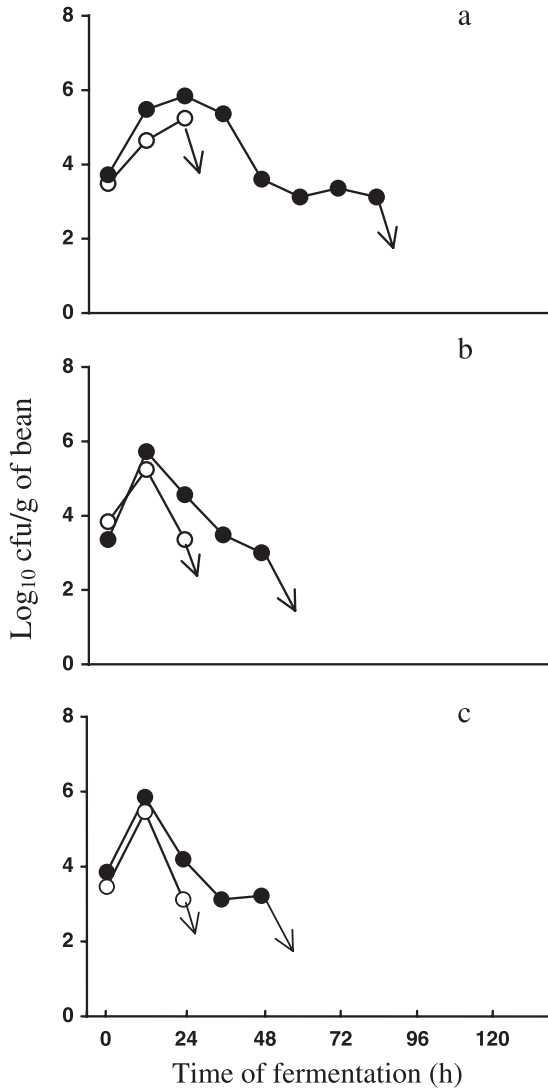


Fig. 4. Growth of *A. pasteurianus* (●) and *A. aceti* (○) during cocoa bean fermentations at (a) estate A, (b) estate B and (c) estate C. Arrow indicates counts below 50 cfu/g of bean.

3.7. Other bacterial species

A range of other bacterial species grew throughout the fermentations, but their populations did not exceed 10^5 – 10^6 cfu/g. Prominent among these were: *Staphylococcus capitis*, *Staphylococcus aureus* and *Micrococcus kristinae* (estate A); *S. capitis*, *Staphylococcus hominii*, *M. kristinae* and *Pseudomonas cepacea* (estate B); *S. aureus*, *M. kristinae* and *P.*

cepacea (estate C). Generally, these species grew during the first 24–36 h and subsequently died off. None of these isolates were pectolytic, cellulolytic or amylolytic but, some strains of the staphylococci and

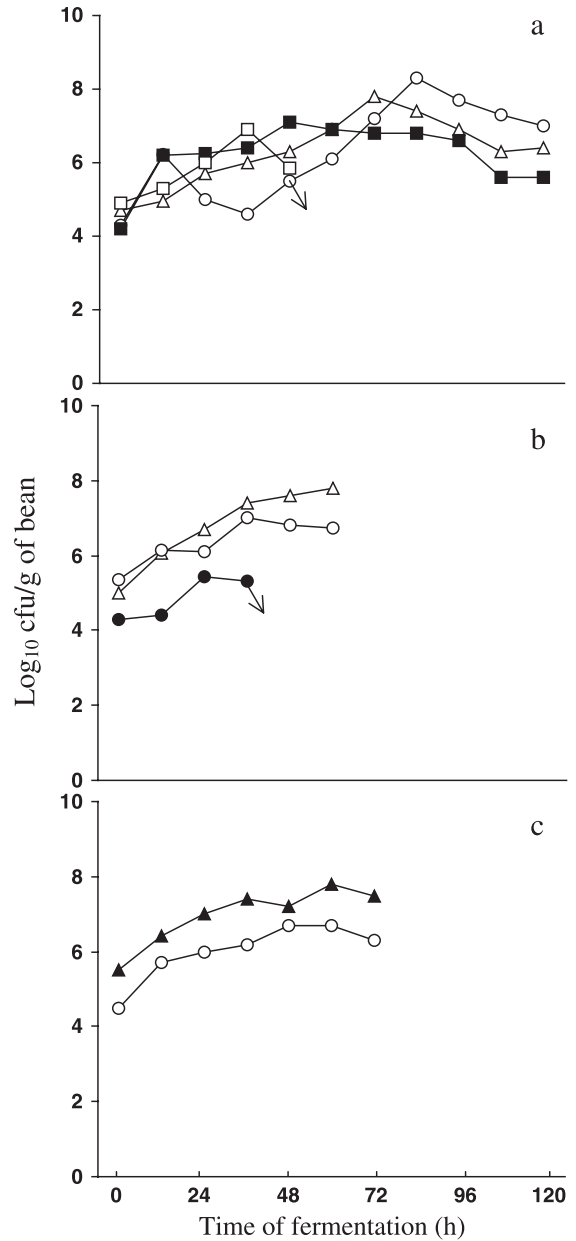


Fig. 5. Growth of *B. pumilus* (○), *B. licheniformis* (△), *B. cereus* (□), *B. sphaericus* (●), *B. coagulans* (▲) and *B. subtilis* (■) during cocoa bean fermentation at (a) estate A, (b) estate B and (c) estate C. Arrows indicate counts below 50 cfu/g of bean.

P. cepacea, exhibited lipolytic and proteolytic activities.

4. Discussion

Cocoa beans have been cultivated and fermented on a commercial basis in Indonesia since the late 1800s (Soenaryo, 1985). The process continues to be conducted in a traditional manner as we have described, and also reported by Roelofsen (1958). The fermentation is characterised by microbial metabolism of pulp sugars and production of significant amounts of ethanol, lactic acid and acetic acid (Table 1, Lehrian and Patterson, 1983; Jones and Jones, 1984; Lopez and Dimick, 1995). The concentration of ethanol in the pulp peaked at about 1–2 days, and then declined as it is oxidatively metabolised to acetic acid. At harvest, the bean pulp was acidic (pH 3.7–3.9) due to the presence of about 2–2.5% (w/w) of citric acid. Citric acid was utilised during fermentation, causing the pulp to increase to about pH 5.0 by the end of fermentation (Table 1). These transformations in the pulp lead to an accumulation of ethanol and acids (especially acetic acid) within the bean. The concentrations of free amino acids in the pulp and beans increased about fivefold during fermentation (unpublished data), suggesting proteolytic activity. The intense metabolic activity that occurred during fermentation caused the temperature of the bean mass to increase to about 50 °C by the end of the process. Overall, the physical and chemical changes that we observed in the bean mass were similar to those reported by others (Lehrian and Patterson, 1983; Lopez and Dimick, 1995). They provided an ecosystem that selected for the successional growth of various species of filamentous fungi, yeasts, lactic acid bacteria, acetic acid bacteria and *Bacillus*.

Our data suggest that filamentous fungi have a greater role in the microbial ecology of cocoa bean fermentation than previously thought (Roelofsen, 1958, Schwan and Wheals, in press). The total populations (10^5 – 10^7 cfu/g) produced by the fungi were quantitatively significant and would contribute to biochemical transformations in the pulp and beans. Moreover, these populations are likely to be an underestimation of the actual contribution because of the difficulty of quantifying fungal presence by agar plate

count methods (Pitt and Hocking, 1997). Fungal contribution to the fermentation was restricted to the first 36 h. The increasing temperature and ethanol concentration of the bean mass would be restricting factors. None of the species isolated from the bean mass could grow or survive above 40 °C.

The main species associated with the fermentation (Fig. 1) were different from those isolated by previous workers from dried cocoa beans (Bunting, 1928; Dade, 1928; Ciferri, 1931) or beans during fermentation (Maravalhas, 1966). In those studies, *Aspergillus fumigatus*, *Aspergillus glaucus*, species of *Mucor* and other species of *Penicillium* were reported. However, Maravalhas (1966) did report the isolation of *P. citrinum* from the fermenting beans. It seems that fungal species, which develop on dried beans after fermentation, are different from those associated with the fermentation (Maravalhas, 1966; Niles, 1981). The unidentified basidiomycete is a novel finding. Molecular and physiological characterisation of this isolate is required, as it could represent a new species.

The two prominent fungi of fermentation, *P. citrinum* and the unidentified basidiomycete were strongly pectolytic, and probably play a key role in degradation of pectin of the pulp. As noted by many authors (Lehrian and Patterson, 1983, Sanchez et al., 1984; Ravelomanana et al., 1985; Schwan et al., 1997), degradation of bean pulp is a major requirement of cocoa bean fermentation. In the past, the degradation of this pulp has been attributed to the pectolytic activity of certain yeast species, but none of the yeasts isolated in our study were pectolytic. Several of the fungal species (*A. wentii*, *A. versicolor* and *P. purpurogenum*) were strongly amylolytic. The pulp contains little or no starch, but any amylases produced by these species may be significant in degrading starch which is present in the beans (Lopez and Dimick, 1995). Similarly, the proteolytic enzymes produced by *A. wentii* and *P. citrinum* could affect the protein composition of the beans, as well as degrade any protein in the pulp. Lipolytic enzymes produced by fungi could affect the lipid composition of the beans. Lipid is the main constituent of the bean, comprising approximately 50% of its weight (Lehrian and Patterson, 1983). Hansen (1975a,b) has mentioned that fungal species may degrade bean lipids to increase the concentration of free fatty acids, which then impact on chocolate flavour. Moreover, changes

in these lipids could affect the melting point of cocoa butter derived from the beans. Future studies should aim to determine whether these fungal enzymes are produced and are active under the conditions of fermentation, as it seems they would be significant in contributing to the final quality of the cocoa beans. The impact of other fungal activities such as utilisation of pulp sugars and acids, and generation of metabolites upon bean and chocolate quality need investigation.

Yeasts were prominent in the early stages (12–48 h) of cocoa bean fermentation at all three estates. These findings agree with previous studies conducted in Indonesia (Steinmann, 1928; Roelofsen and Giesberger, 1947; Roelofsen, 1958) and other countries (Rombouts, 1952; de Camargo et al., 1963; Ostovar and Keeney, 1973; Gauthier et al., 1977; Passos et al., 1984; Schwan et al., 1995). Maximum yeast populations of 10^7 – 10^8 cfu/g will be quantitatively significant in the context of their biochemical activity. It is generally accepted that they conduct a strong alcoholic fermentation, thereby creating conditions (e.g. production of ethanol and its conversion to acetic acid) that contribute to death of the beans. Bean death initiates an array of endogenous biochemical changes that are essential for the development of characteristic chocolate flavour (Lehrian and Patterson, 1983; Lopez and Dimick, 1995; Hansen et al., 1998). Other functions of yeasts include degradation of pulp through the production of pectolytic enzymes and decreasing pulp and bean acidity through the utilisation of citric acid (Schwan and Wheals, *in press*). The secondary products of yeast metabolism (e.g. organic acid, aldehydes, ketones, higher alcohols, esters) and glycosidase production are likely to be significant and should impact on bean and chocolate quality. However, these potentially important influences have been overlooked in previous literature, and require investigation.

Reports on the species of yeasts associated with cocoa bean fermentations date from 1899 have been critically reviewed by Knapp (1937), Rombouts (1952), Schwan et al. (1995) and Schwan and Wheals (*in press*). The diversity of species isolated from the fermentations examined in our studies was less than that reported for fermentations conducted in Brazil (de Camargo et al., 1963; Schwan et al., 1995), Malaysia and Ghana (Carr et al., 1979; Carr and Davies, 1980), the Ivory Coast (Gauthier et al., 1977; Ravelomanana

et al., 1985; Sanchez et al., 1985) and the West Indies (Rombouts, 1952). Species of *Pichia*, *Hansenula*, *Kluyveromyces*, *Lodderomyces* and *Schizosaccharomyces*, for example, were not found. Earlier studies of fermentations in Indonesia also revealed the occurrence of *Pichia* and *Schizosaccharomyces* species (Roelofsen, 1958). Nevertheless, the isolation and prevalence of various species of *Kloeckera* and *Candida* as well as *S. cerevisiae* (Fig. 2) are consistent with other studies. The early dominance and disappearance of *Kloeckera* species followed by the growth and dominance of *S. cerevisiae* and various *Candida* species is a trend reported in most cocoa bean fermentations (Schwan et al., 1995; Schwan and Wheals, *in press*) and is analogous to the yeast ecology of wine fermentations (Fleet, 2001). This succession is explained by the more rapid growth of *Kloeckera* species at lower pH values, but their early death due to weaker tolerance of the increasing concentrations of ethanol produced by *S. cerevisiae* and *Candida* species. Tolerance to temperatures in the range of 35–50 °C is likely to be another property of cocoa bean fermentations that will impact on the successional growth of yeast species and strains. Unlike other authors, we found *K. apis* rather than *Kloeckera apiculata* to be the principal *Kloeckera* species and this is probably due to its greater tolerance of higher temperatures (Kurtzman and Fell, 1998). It is noteworthy that some of our isolates of *S. cerevisiae* and *C. tropicalis* exhibited distinct, but weak growth at 50 °C. Probably, the main difference between our study and those of others was the absence of pectolytic strains of yeasts, such as those of *S. cerevisiae* var. *chevalieri*, *Kluyveromyces marxianus* and *Candida norvegensis* (Sanchez et al., 1984; Ravelomanana et al., 1986; Schwan et al., 1997; Buamah et al., 1997). As explained already, filamentous fungi probably played a key role in the degradation of pulp pectin during the fermentations we examined.

Early studies, reviewed by Knapp (1937) and Roelofsen (1958), mentioned the growth of lactic acid bacteria during cocoa bean fermentations, but at that time, there was little understanding of the species present or their significance. Subsequent studies by Ostovar and Keeney (1973) in Trinidad, Carr et al. (1979) and Carr and Davies (1980) in Malaysia and Ghana, and Passos et al. (1984) in Brazil showed that these bacteria grew to 10^6 – 10^8

cfu/g during the first 36–48 h of fermentation, thereafter decreasing to lower populations. A diversity of species within the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* were isolated from the different locations (Thompson et al., 2001; Schwan and Wheals, in press), with *L. plantarum* being the most consistent representative. It is now accepted that lactic acid bacteria have a predominant role in the microbial ecology of bean fermentation. They metabolise pulp sugars by homofermentative and heterofermentative mechanisms, producing significant amounts of lactic acid that impacts on the acidity and quality of the bean. Moreover, some species may metabolise pulp citric acid (Thompson et al., 2001; Schwan and Wheals, in press). We have confirmed the prominent growth of lactic acid bacteria during the first 2 days of fermentation (Fig. 3), but in contrast to other studies, we did not encounter a broad diversity of species. In the fermentations of Trinitario beans (estates B and C), only two species, *L. plantarum* and *L. cellobiosus* were found. These two species plus *L. hilgardii* were also recovered from fermentation of the Forastero beans (estate A). *L. plantarum* seems to be a species that is common to most cocoa bean fermentations (Thompson et al., 2001; Schwan and Wheals, in press). *L. cellobiosus* has not been reported in other studies of cocoa bean fermentation. Most of our isolates of these species grew at 45–47 °C and this may account for their prevalence in the fermentations.

The characteristic vinegar-like aroma of cocoa bean fermentations lead early investigators to conclude and demonstrate that acetic acid bacteria were significant contributors to the process (Bainbridge and Davies, 1912; Knapp, 1937; Roelofsen and Giesberger, 1947). Various isolates, now classified as *A. aceti*, *A. pasteurianus* and *Gluconobacter oxydans*, were identified (Ostovar and Keeney, 1973; Carr et al., 1979). They grow during the early stages of fermentation in association with or soon after the growth of yeasts, reaching populations of 10^6 – 10^9 cfu/g according to the extent of bean mixing and aeration. They oxidise the ethanol, produced by yeasts, to acetic acid, which contributes to the acidity and death of the beans. Acetic acid bacteria can also metabolise sugars and organic acids to produce various aldehydic, ketogenic and other volatile products

(Drysdale and Fleet, 1988) that could impact on the sensory quality of beans.

We have confirmed the contribution of acetic acid bacteria to bean fermentations (Fig. 4), but unlike other reports (Rombouts, 1952; Carr et al., 1979), we did not find the higher populations of 10^7 – 10^9 cfu/g, and also, we noted an earlier (12–36 h) rather than later association of these bacteria with the process. Generally, our data were more consistent with those reported by Ostovar and Keeney (1973). Acetic acid bacteria are aerobic in their metabolism and are not particularly tolerant to high temperatures. Consequently, the degree of bean mixing and aeration, the location (surface/middle of bean mass) from which samples were taken for analysis, and the ambient temperature and rate of temperature increase could account for these varying observations. However, it should be noted that acetic acid bacteria can survive quite well and possibly grow under nonaerobic conditions, as has been found in winery environments (Drysdale and Fleet, 1988).

The association of aerobic spore-forming bacteria (*Bacillus* spp.) with cocoa bean fermentations has been recognised for some time (Preyer, 1913; Bainbridge and Davies, 1912; Rombouts, 1952). They become the predominating microorganisms (10^7 – 10^8 cfu/g) in the later stages of fermentation when conditions are less acidic and more aerobic. Also, they are tolerant of the higher temperatures, 45–50 °C at that stage. Numerous species have been identified, with *B. subtilis*, *Bacillus stearothermophilus*, *B. cereus*, *B. licheniformis*, *B. coagulans* and *B. pumilus* being among the most prevalent (Ostovar and Keeney, 1973; Carr et al., 1979; Carr and Davies, 1980; Schwan et al., 1986). Our data (Fig. 5) are consistent with these observations, and show the growth profiles of individual species. The profile of species varied with the estate, with *B. pumilus* being the only species common to all estates. It has been suggested that *Bacillus* species could impact on bean and chocolate quality through the production of organic acids and flavourants such as pyrazines and 2,3-butanediol (Schwan and Wheals, in press). Some of our isolates gave strong protease, amylase and lipase reactions. Presumably, such enzymes could diffuse into the beans and impact upon their overall composition.

Bacterial species other than those belonging to the groups of lactic acid bacteria, acetic acid bacteria and

Bacillus can be isolated from cocoa bean fermentations but, generally, their populations are less than 10^6 cfu/g, and they constitute less than 5–10% of the total bacterial flora (Bainbridge and Davies, 1912; Rombouts, 1952; Barrile et al., 1971; Ostovar and Keeney, 1973). They become more prevalent if the fermentations are extended beyond 6–8 days, and have the potential to cause putrefactive spoilage. Various species of coliforms, micrococci, *Flavobacterium*, *Propionibacterium* and *Zymomonas* have been reported. The occurrence and significance of *Zymomonas mobilis* needs more consideration because it can conduct an alcoholic fermentation (de Camargo et al., 1963; Ostovar and Keeney, 1973). Minor bacterial species, principally those of *Staphylococcus* and *Micrococcus* were consistently isolated from the fermentation at estates A, B and C, and emphasise the importance of monitoring the hygiene and conduct of the fermentation to ensure that they do not become predominant and spoil the beans.

Studies on the microbial ecology of cocoa bean fermentations are complicated by several factors. Many fermentaries are located in remote regions of developing countries, and have minimal facilities for scientific analyses. In our case, rudimentary analytical capability needed to be established on each site. Collection of samples from the bean mass is another variable, since the beans are periodically mixed and transferred from one box to another on a daily basis. In preliminary analyses at each fermentary, we examined samples taken from the top, middle and bottom locations of the fermentation boxes and noted similar populations and colony types on enumeration media. Consequently, we examined samples from only one location, namely, the middle of the fermenting mass. The maturity of the cocoa beans at harvest is a further variable that could influence microbial ecology. While beans at the correct stage of maturity were generally harvested and fermented, we did note, on occasions, that immature or overmature beans were sometimes included in the fermenting mass. At each fermentary, we conducted controlled, experimental fermentation (5 kg bean mass) with either immature (1–2 weeks before maturity), mature or overmature (1 week post maturity) beans (data not reported). Generally, we found similar growth profiles as reported in Figs. 1–5, suggesting no obvious impact of bean maturity on the ecology. Moreover, these experiments con-

firmed the overall ecological data found in commercial fermentaries.

Using conventional microbiological procedures, we have demonstrated the complex microbial ecology of cocoa bean fermentations. Additional studies using molecular analyses such as denaturing gradient gel electrophoresis (DGGE) (Fleet, 1999) may reveal even greater complexity. Nevertheless, we have identified several species, which were consistently dominant throughout the fermentations. These are *P. citrinum*, an unidentified basidiomycete, *S. cerevisiae*, *C. tropicalis*, *K. apis*, *L. cellobiosus*, *L. plantarum*, *A. pasteurianus*, *B. pumilus*, *B. licheniformis* and *B. coagulans*. Further research is needed to determine if and how each of these species are essential to the fermentation and the development of cocoa bean and chocolate quality.

References

- Anonymous, 2000. The World Cocoa Market Outlook. LMC International. www.treecrops.org/crops/cocoaoutlook.pdf.
- Bainbridge, J.S., Davies, S.H., 1912. The essential oil of cocoa. *J. Chem. Soc.* 101, 2209–2220.
- Barrile, J.C., Ostovar, K., Keeney, P.G., 1971. Microflora of cocoa beans before and after roasting at 150°C. *J. Milk Food Technol.* 34, 369–371.
- Buamah, R., Dzogbefia, V.P., Oldham, J.H., 1997. Pure yeast culture fermentation of cocoa (*Theobroma cacao* L.): effect on yield of sweatings and cocoa bean quality. *World J. Microbiol. Biotech.* 13, 457–462.
- Bunting, R.H., 1928. Fungi occurring in cacao beans. *Dept. Agric. Gold Coast Year Book Bull.* 16, 44–62.
- Carr, J.G., Davies, P.A., 1980. Cocoa fermentation in Ghana and Malaysia: Part 2. Further microbiological methods and results. A report available at Long Ashton Research Station, Long Ashton, Bristol, England.
- Carr, J.G., Davies, P.A., Dougan, J., 1979. Cocoa fermentation in Ghana and Malaysia. A report available at Long Ashton Research Station, Long Ashton, Bristol, England.
- Ciferri, R., 1931. Studies on cocoa. *J. Dep. Agric. P. R.* 15, 223–286.
- Dade, H.A., 1928. Internal moulding of prepared cocoa. *Dept. Agric. Gold Coast Year Book Bull.* 16, 74–100.
- Davis, C.R., Wibowo, D., Lee, T.H., Fleet, G.H., 1986. Growth and metabolism of lactic acid bacteria during and after malolactic fermentation of wines at different pH. *Appl. Environ. Microbiol.* 51, 539–545.
- De Camargo, R., Leme, J., Martinelli Filho, A., 1963. General observations on the microflora of fermenting cocoa beans (*Theobroma cacao*) in Bahia (Brazil). *Food Technol.* 17, 116–118.
- Drysdale, G.S., Fleet, G.H., 1988. Acetic acid bacteria in wine-making: a review. *Am. J. Enol. Vitic.* 39, 143–154.

- Fleet, G.H., 1999. Microorganisms in food ecosystems. *Int. J. Food Microbiol.* 50, 101–117.
- Fleet, G.H., 2001. Wine. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), *Food Microbiology Fundamentals and Frontiers*. ASM Press, Washington, DC, pp. 747–772.
- Gauthier, B., Guiraud, J., Vincent, J.C., Parvais, J.P., Galzy, P., 1977. Remarques sur la flore de levures de la fermentation traditionnelle du cacao en Cote d'Ivoire. *Rev. Ferment. Ind. Aliment.* 32, 160–163.
- Hansen, A.P., 1975a. Microbiological activity and its effect on cocoa beans. *Manuf. Confect.* 55, 35–39.
- Hansen, A.P., 1975b. Understanding the microbiological deterioration of cacao. *Candy Snack Ind.* 140, 44–47.
- Hansen, G.E., Olmo, M.D., Burns, C., 1998. Enzyme activities in cocoa beans during fermentation. *J. Sci. Food Agric.* 77, 273–281.
- Harrigan, W.F., McCance, M.E., 1976. *Laboratory Methods in Food and Dairy Microbiology*. Academic Press, London.
- Hashim, P., Selamat, J., Muhammad, S.K.S., Ali, A., 1998. Changes in free amino acid peptide-N, sugar and pyrazine concentration during cocoa fermentation. *J. Sci. Food Agric.* 78, 535–542.
- Jones, K.L., Jones, S.E., 1984. Fermentations involved in the production of cocoa, coffee and tea. *Prog. Ind. Microbiol.* 19, 411–433.
- Knapp, A.W., 1937. *Cacao Fermentation*. John Bale, Sons and Curnow, London.
- Krieg, N.R., Holt, J.G. (Eds.), 1984. *Bergey's Manual of Systematic Bacteriology*, vol. 1. The Williams and Wilkins, London.
- Kreger-van-Rij, N.J.W., 1984. *The Yeasts—A Taxonomic Study*, 3rd ed. Elsevier, Amsterdam.
- Kurtzman, C., Fell, J., 1998. *The Yeasts—A Taxonomic Study*, 4th ed. Elsevier, Amsterdam.
- Lehrian, D.W., Patterson, G.R., 1983. Cocoa fermentation. In: Reed, G. (Ed.), *Food and Feed Production with Microorganisms*. Biotechnology, vol. 5. Verlag Chemie, Weinheim, pp. 529–575.
- Lopez, A.S., Dimick, P.S., 1995. Cocoa fermentation. In: Reed, G., Nagodawithana, T.W. (Eds.), *Enzymes, Biomass, Food and Feed*, 2nd ed. Biotechnology, vol. 9. VCH, Weinheim, pp. 561–577.
- Maravalhas, M., 1966. Mycological deterioration of cocoa beans during fermentation and storage in Bahia. *Rev. Int. Choc.* 21, 375–378.
- Niles, E.V., 1981. Microflora of imported cocoa beans. *J. Stored Prod. Res.* 17, 147–150.
- Ostovar, K., Keeney, P.G., 1973. Isolation and characterization of microorganisms involved in the fermentation of Trinidad's cacao beans. *J. Food Sci.* 38, 611–617.
- Passos, F.M.L., Silva, D.O., Lopez, A., Ferreira, C., Guimaraes, W.V., 1984. Characterization and distribution of lactic acid bacteria from traditional cocoa bean fermentation in Bahia. *J. Food Sci.* 49, 205–208.
- Pitt, J.I., Hocking, A.D., 1997. *Fungi and food spoilage*, 2nd ed. Blackie Academic & Professional, London.
- Preyer, A., 1913. Chapter I. In: Smith, H.H. (Ed.), *The Fermentation of Cacao*. John Bale, Sons & Danielson, London, pp. 1–31.
- Ravelomanana, R., Guiraud, J.P., Vincent, J.C., Galzy, P., 1985. The yeast flora of cocoa bean fermentation in the Ivory Coast. *MIRCEN J.* 1, 319–326.
- Ravelomanana, R., Guiraud, J.P., Galzy, P., 1986. Isolation of a pectin-utilising yeast from cocoa beans. *Syst. Appl. Microbiol.* 8, 230–233.
- Roelofsen, P.A., 1958. Fermentation, drying and storage of cacao beans. *Adv. Food Res.* 8, 225–296.
- Roelofsen, P.A., Giesberger, G., 1947. Onderzoekingen over cacao-bereiding. *Arch. Koffiecult. Ned.-Indie* 16, 119–159.
- Rombouts, J.E., 1952. Observations on the microflora of fermenting cacao beans in Trinidad. *Proc. Soc. Appl. Bacteriol.* 15, 103–111.
- Samson, R.A., van Reenan-Hoekstra, E.S., 1988. *Introduction To Foodborne Fungi*, 3rd ed. Centraalbureau voor Schimmelcultures, Delft.
- Sanchez, J., Guiraud, J.P., Galzy, P., 1984. A study of the polygalacturonase activity of several yeast strains isolated from cocoa. *Appl. Microbiol. Biotechnol.* 20, 262–267.
- Sanchez, J., Daquenet, G., Guiraud, J.P., Vincent, J.C., Galzy, P., 1985. A study of the yeast flora and the effect of pure culture seedling during the fermentation process of cocoa beans. *Lebensm.-Wiss. Technol.* 18, 69–75.
- Schwan, R.F., 1998. Cocoa fermentations conducted with a defined microbial cocktail inoculum. *Appl. Environ. Microbiol.* 64, 1477–1483.
- Schwan, R.F., Wheals, A., 2002. Understanding the microbiology of cocoa bean fermentations in order to improve quality. *Crit. Rev. Food Sci. Nutr.* (in press).
- Schwan, R.F., Vanetti, M.C.D., Silva, D.O., Lopez, A., De Moraes, A., 1986. A research note—characterization and distribution of aerobic, spore-forming bacteria from cacao fermentations in Bahia. *J. Food Sci.* 51, 1583–1584.
- Schwan, R.F., Rose, A.H., Board, R.G., 1995. Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. *J. Appl. Bacteriol.* 79, 96S–107S (symposium supplement).
- Schwan, R.F., Cooper, R.M., Wheals, A.E., 1997. Endo-polygalacturonase secretion by *Kluyveromyces marxianus* and other pulp-degrading yeasts. *Enzyme Microb. Technol.* 21, 234–244.
- Soenaryo, 1985. The prospect of Indonesia cacao to the year 2000. Trade and Investment Centre, Ministry of Agriculture of the Republic of Indonesia.
- Speck, M.L., 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd ed. American Public Health Association, Washington.
- Steinmann, A., 1928. Onderzoekingen over cacao-fermentatie: II. De biologie der cacao-gisten. *Arch. Cacao Andere Klainne Cult. Ned.-Indie* 3, 71–80.
- Thompson, S.S., Miller, K.B., Lopez, A.S., 2001. Cocoa and coffee. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), *Food Microbiology Fundamentals and Frontiers*. ASM Press, Washington, DC, pp. 721–736.