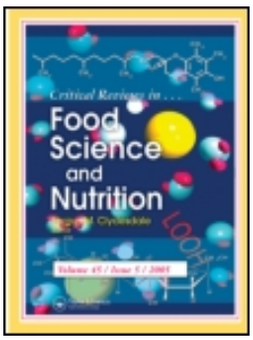

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Theobroma cacao L., "The Food of the Gods": Quality Determinants of Commercial Cocoa Beans, with Particular Reference to the Impact of Fermentation

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The quality of commercial cocoa beans, the principal raw material for chocolate production, relies on the combination of factors that include the type of planting material, the agricultural practices, and the post-harvest processing. Among these, the fermentation of the cocoa beans is still the most relevant since it is the process whereby the precursors of the cocoa flavor arise. The formation of these precursors depends on the activity of different microbial groups on the beans pulp. A comparison of fermentations in different countries showed that a well-defined microbial succession does not always take place and that the role of Bacillus spp. *in this process remains unclear. Considering the overriding importance of the fermentation to achieve high quality commercial cocoa beans, we discuss the need of addressing the impact of the farming system, the ripeness state of the pods, and the role of microbial interactions on the fermentation in future research. In addition, the problem of high acidification cocoa beans, aspects dealing with the volatile fraction of the flavor, and the cocoa butter properties, all were identified as critical aspects that need further investigation. The standardization of the microbiological methods and the application of metagenomic approaches would magnify the knowledge in this domain.*

Keywords Post-harvest, organic production, microbial interactions, cocoa flavor, cocoa butter

ABBREVIATIONS

AA = Acetic acid
AAB = Acetic acid bacteria
B = Basionym
BCCCA = The Biscuit Cake Chocolate and Confectionary Alliance
CA = Citric acid
CBS = Centraalbureau voor Schimmelcultures
CMAA = Cocoa Merchants' Association of America
DGGE = Denaturing Gradient Gel Electrophoresis
DNA = Deoxyribonucleic acid
E = Ethanol
EU = European Union
F = Fructose
FAO = Food and Agriculture Organization
FCC = Federation of Cocoa Commerce
FDA = Food and Drug Administration
FFA = Free Fatty Acid
G = Glucose
ICCO = International Cocoa Organization
ISO = International Organization for Standardization
LA = Lactic acid
LAB = Lacic acid bacteria
M = Mannitol
PCR = Polymerase chain reaction
rRNA = Ribosomal ribonucleic acid
S = Sucrose

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SA = Succinic acid
SOO = Saturated fatty acid, Oleic acid, Oleic acid
SOS = Saturated fatty acid, Oleic acid, saturated fatty acid
UNCTAD = United Nations Conference on Trade and Development

INTRODUCTION

From the twenty-two species that constitute the genus *Theobroma* (family *Sterculiaceae*), *Theobroma cacao* L., is commercially the most important, due to the value of its seeds (Bartley, 2005; Wood, 1975). The seeds, commonly known as cocoa beans, are the principal raw material for chocolate production. Not only is it not possible to make chocolate without cocoa beans, but also the distinctive flavor of chocolate is due to the presence of these beans. Other products derived from cocoa beans are cocoa powder, widely used in the food industry, and cocoa butter that in addition to its confectionary use also has cosmetic and pharmaceutical applications. However, before cocoa beans can be traded and processed into final industrial products they have to undergo post-harvest processing on farms and plantations comprising the steps of pod opening and beans removal from the pod, beans fermentation, and drying. In this sequence, the fermentation constitutes an essential critical step for the development of flavor quality attributes of the commercial cocoa beans (Rohan, 1964). This is due to the fact that during the fermentation, biochemical transformations are induced within the beans leading to the formation of important precursors of the cocoa flavor, some of its highly volatile compounds, as well as causing browning, and reduction of bitterness and astringency of the beans (Almeida, 1999; Cros and Jeanjean, 1998). The full cocoa flavor is developed later upon roasting, through complex reactions, mainly of the Maillard type (Afoakwa et al., 2008).

Two important milestones achieved in the scientific area of cocoa beans fermentation consisted of the understanding of the basic mechanisms leading to the formation of cocoa flavor precursors (see for instance, the work by Forsyth and Quesnel (1963)); and the conduction of the first controlled fermentation using a defined microbial inoculum as starter culture (Schwan, 1998). This contributed to a significant improvement of cocoa post-harvest processing. However, we have identified relevant aspects linked to the fermentation whose comprehension could bring important breakthroughs for the future of cocoa fermentation science and practice. Therefore, this article will focus on the impact of the fermentation on the quality of commercial cocoa beans, although it is a well known fact that this results from a combination of factors ranging from the planting material to the drying method (Figure 1) (Almeida, 1999; Clapperton et al., 1994; Jeanjean, 1995; Kattenberg and Kemmink, 1993). After an introduction on cocoa botany, production, and methods of post-harvest processing, the current state of knowledge on the microbiota of cocoa beans fermentation, and the mechanisms leading to the formation of the cocoa flavor precursors are presented. Next, we have discussed the research needs in the field of cocoa fermentation. These deal with the influence of the farming system and pod ripeness on the fermentation, and the subsequent way through which the fermentation affects beans acidification, the volatile fraction of the flavor, and the cocoa butter properties. Finally, the quality requirements of cocoa beans have been addressed and the conclusions arrived at have been presented in this paper.

BOTANY, PRODUCTION AND POST-HARVEST PROCESSING

The cocoa tree is a perennial tree, 8 to 15 m in height, which under a more intensive cultivation is limited to 2.5 to 3 m by pruning, for better phytosanitary control (Fowler, 1999; Wood, 1975). Its natural habitat is the lower storey of the evergreen rain forest in the Amazon basin and other tropical areas of South and Central America (Fowler, 1999; Lass, 1999). Although there is still some controversy concerning the origin and domestication of wild populations of cocoa, recent investigations suggest their center of origin is in South America (Motamayor et al., 2002).

It was the Swedish botanist Linnaeus who named the genus "Theobroma," from the Greek "Theos" (meaning God) and "broma" (meaning food), prompted by the recognized legendary Mayan and Aztec popular belief of the deific origin of the cocoa tree (Cook, 1982). After Hernando Cortés' encounter with the Aztecs in actual Mexico city in 1520, cocoa drinks were introduced in the Spanish court (Coe and Coe, 1996). Their popularity would later lead to the spread of cocoa tree rootstocks to other European colonies bearing their growth (Lass, 1999; Thompson et al., 2007).

The fruit of the cocoa tree is botanically an indehiscent drupe, usually called pod (Lass, 1999; Wood, 1975). These are oval in shape, measure between 12 and 30 cm, and contain 30 to 40 beans embedded in a mucilaginous pulp, which comprises approximately 40% of the bean fresh weight (Schwan and Wheals, 2004). The pulp is characterized by a sugar content around 9 to 13% (w/w), high acidity, conferred by the presence of diverse organic acids, but mainly citric acid, and a protein content in the range of 0.4 to 0.6% (w/w) (Table 1, where the amount of fat, minerals, and vitamins is also presented).

Each cocoa bean consists of two cotyledons ("nibs") and a small embryo, all enclosed in a skin ("shell"). The cotyledons comprise of two types of cells—storage or parenchyma cells, containing fat globules, protein bodies and starch granules, and bigger pigmented cells, containing polyphenols and methylxanthines (Biehl et al., 1977; Del Boca, 1962; Ferrão, 2002). In the cotyledons, the fat is the most important nutrient, representing about half the weight of the dry seed (Table 2). The methylxanthines, mainly theobromine and caffeine may occur at an average level of 1.5% (w/w) in the dried nibs, imparting a very bitter taste to the cocoa beans (Table 2). The total amount of polyphenols in dried fresh cocoa beans may vary between 12

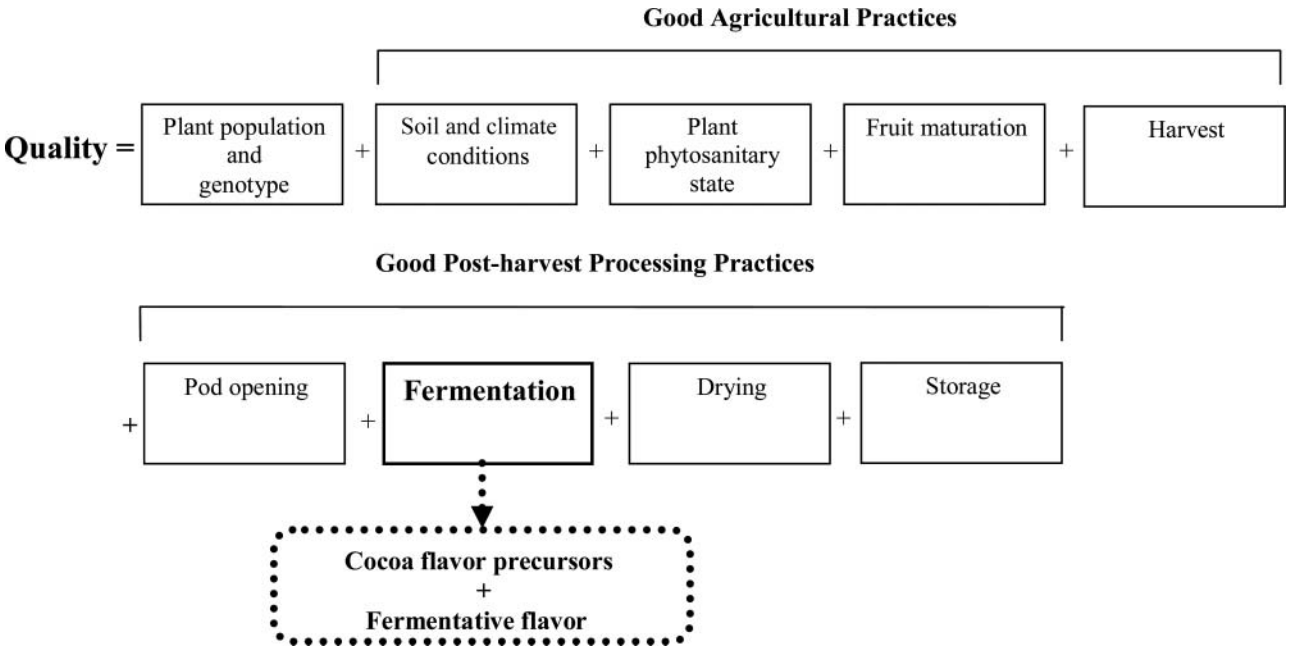

Figure 1 Stages that contribute to the quality of commercial cocoa beans, where the fermentation has a core importance.

and 20% (w/w) and these are responsible for its high astringency, contributing to their bitterness as well (Forsyth and Quesnel, 1957; Kim and Keeney, 1984). Three main groups of polyphenols are present: anthocyanins, flavan-3-ol (catechins), and proanthocyanidins, corresponding to approximately 4, 37, and 58%, respectively (Kim and Keeney, 1984; Wollgast and Anklam, 2000). Within the catechins group, the (-)-epicatechin is the predominant fraction amounting to 35% of the total polyphenol content (Kim and Keeney, 1984).

Notwithstanding the existence of morphological and genotypical differences among cocoa populations, the most prominent classification system is based on flavor quality attributes of the cocoa seeds. There are three main botanical populations: Forastero, Criollo, and Trinitario (Bartley, 2005; Cheesman, 1944; Motamayor et al., 2002). Forastero populations, probably native to the Amazon basin, supply over 95% of the world's cocoa (ICCO, 2007a). The seeds of this population are flat, astringent, and purple in color (more rarely ivory or pale) due to the presence of anthocyanins. Forastero cocoa trees are very productive and are considered to be moderately resistant to pests and diseases (Bartley, 2005; Ferrão, 2002). Nowadays, most populations around the world use locally adapted Forastero varieties augmented by specifc genotypes (Sounigo et al., 2005). These hybrids are then selected based on their biological resistance and yield (Jonfia-Essien et al., 2008). The commercial beans called "bulk cocoa in trade" are used for the manufacture of chocolate milk, cocoa butter, and cocoa powder (Fowler, 1999; Fowler, 1994; Thompson et al., 2007). Criollo is the original cultivated population, indigenous to Northern, South, and Central America. The beans are white to ivory or have a very pale purple color, due to an anthocyanin inhibitor gene (Ferrão, 2002; Fowler, 1999). The low yields and their susceptibility

Table 1 Composition of cocoa pulp (g.100 g^{-1} fresh weight pulp)

				Indonesia[b]		
	Ivory Coast[a]	Nigeria[a]	Malaysia[a]	F1	F2	Ghana[c]
Water	82.60	82.50	85.90	—	—	—
Glucose and fructose	6.80	11.13	10.25	10.3	6.6	10.77
Sucrose	4.35	1.92	1.35	3.2	2.1	0
Plant and cell wall polymers	2.81	—	1.48	—	—	—
Citrate	1.31	0.79	0.29	2.4	2.1	0.93
Protein/peptides	0.57	0.51	0.43	—	—	—
Free amino acids	0.15	0.11	0.21			
Fat	0.45	0.75	0.35	—	—	—
Metals	0.24	0.22	—	—	—	—
Vitamins (composite sample)	0.05	0.05	—	—	—	—

[a](Pettipher, 1986b); [b](Ardhana and Fleet, 2003); [c](Camu et al., 2007).
F1, F2- Fermentary 1 and 2; - Not determined.

Table 2 Average chemical composition of roasted nibs* (g.100 g^{-1}).

Constitutents	Roasted nibs	
Water[a]	3.0	3.7
Fat	54.0	54.0
Protein	12.5	
Starch	6.0	6.0
Fiber	2.5	2.5
Ash	3.0	2.8
Theobromine	1.3	1.3
Caffeine	0.2	0.1
Others	—	
References	(Valiente et al., 1994)	(Minifie, 1980)

[a]Varies according to the degree of drying and roasting.
*Nibs consist of shelled and ground cotyledons of commercial cocoa beans.
—Not determined.

to many diseases make them rare to cultivate. Nowadays, its cultivation is limited to Central America and a few regions in Asia (Ferrão, 2002; Fowler, 1999; Thompson et al., 2007). The Trinitario type originated in Trinidad and covers all the products of natural hybridization and recombination of the Criollo and Forastero populations (Ferrão, 2002; Fowler, 1999). The beans are variable in color, although rarely white, and the trees show a susceptibility to pests and diseases intermediate to Forastero and Trinitario populations (Bartley, 2005; Ferrão, 2002; Fowler, 1999). Both the Trinitario and the Criollo varieties produce the "fine" or "flavor" cocoas, whose share in the total world production is below 5% (ICCO, 2007a). These cocoas are used to make high quality dark chocolate (Fowler, 1999). Nonetheless, some special Forastero types produce "fine" cocoa due to its distinctive aromatic nature. This includes for instance the Nacional type grown in Ecuador, or some cocoa from São Tomé (Fowler, 1994; UNCTAD, 2001).

In the producing countries, the post-harvest processing of cocoa beans starts by breaking the fruits during a period of 3 to 4 days to 2 weeks after the harvesting (Lass, 1999; Thompson et al., 2007). Next, the wet beans are removed manually or, on some large estates in West African countries, in Mexico, and in Brazil, by means of mechanical systems (Ferrão, 2002). When care is given to the processing, cocoa pods that are diseased or broken are separated to a different pile or buried in the soil, and the defective beans are separated from the main fermenting mass (Ferrão, 2002). Subsequently, the wet beans are fermented or cured. The method for cocoa beans fermentation consists of piling a certain quantity of fresh beans and allowing naturally occurring microorganisms to develop (Fowler, 1999). The growth of these microorganisms is supported by the sugars and other minor components present in the cocoa beans pulp (Ardhana and Fleet, 2003; Nielsen et al., 2007b).

Different methods of cocoa beans fermentation are used worldwide but wooden boxes, heaps, baskets, or drying platforms are used the most (Cook, 1982; Thompson et al., 2007; Wood, 1975). The fermentation time may take between two to three days in the case of Criollo populations and five to ten days in the case of Forastero and Trinitario populations (Afoakwa et al., 2008; Cook, 1982; Rohan, 1964; Thompson et al., 2007; Wood, 1975). During this period the beans can be aerated and mixed ("turning" operation) by transfer to another box or heap. When the fermentation is completed, the moisture content of the beans has to be reduced to levels between 6–8% and stored at a relative humidity of 65 to 70% in order to avoid mold growth (Cook, 1982; Fowler, 1999; Schwan and Wheals, 2004). The drying process is conducted either by sun drying during a minimum of 7 days to 3 weeks, or, alternatively, in mechanical driers (Cook, 1982; Thompson et al., 2007). Finally, the beans are cleaned, selected, calibrated, and bagged in 60 kg jute sacks or they are transported to the manufacturing plants in bulk.

The world cocoa beans production in 2006/2007 was 3.4 million tonnes. Ivory Coast is the world's largest producer (38% of the world production in the 2006/2007 season), followed by Ghana, Indonesia, Nigeria, Cameroon, Brazil, and Malaysia. Together these countries contributed to an output of 86% of the total production in 2006/2007 season. Ivory Coast and Ghana alone supplied 56% of the global fraction (ICCO, 2008). However, in the producing countries, the economic importance of cocoa differs considerably. While in countries like Indonesia or Malaysia it faces competition from other crops such as palm oil, coffee, or rubber (Fowler, 1999), elsewhere it is a main source of foreign exchange earnings, as in the case of Ivory Coast and Ghana (FAO, 2007).

It is estimated that about 90 to 95% of all cocoa in the world is produced by small holder farmers, with a typical size of a farm being around 3 hectares (ICCO, 2008).

Grindings of cocoa beans are expected to continue to rise caused by consumer demand for chocolate with higher cocoa content, as the perceived health-beneficial effects from polyphenol consumption gain greater attention (Arlorioa et al., 2008; Scalbert et al., 2005). At the same time, consumer concerns about social, ethical, environmental safety, and economic sustainability of the cocoa chain will probably shape the pattern of cocoa production (ICCO, 2008).

BIOCHEMICAL TRANSFORMATIONS DURING THE FERMENTATION

Although the term "cocoa beans fermentation" is widely used, it does not truly designate the fermentation of the cocoa beans themselves, but the totality of the activity of microorganisms in the pulp surrounding the beans and the subsequent transformations in the cotyledons (Ferrão, 2002). It can be divided into external and internal fermentations. The external fermentation refers to the microbial activity in the pulp and to the metabolites thus formed, while the internal fermentation refers to enzymatic and other chemical reactions inside the cotyledons (Ferrão, 2002).

The focus of this next section of this article is on Forastero cocoa, the most cultivated population worldwide.

The Microbiology of Cocoa Beans Fermentation

Microbial Groups

The results from investigations on the microbiology of cocoa beans fermentation have come from several producing regions but mainly from Trinidad, Ivory Coast, Brazil, Ghana, Indonesia, the Dominican Republic, and Nigeria. Table 3 summarizes thirteen fermentations, investigated in five of these countries. These five countries were selected for the studies performed on different microbial groups, as well as physical and chemical analyses of the cocoa beans pulp had to be performed (Table 4). In these countries, the taxonomic studies on the microbial isolates revealed that different types of microorganisms are present during cocoa beans fermentation. These microorganisms belong mainly to the group of yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB), and spore-forming bacteria of the genus *Bacillus* and related genera. A number of other bacterial species and molds were also found in some fermentations.

These data illustrate the diversity of the fermentation methods and show how different practices even in a single country can be adopted. In 6 out of the 13 presented fermentations, wooden boxes of variable capacities and depths were used, while the seven described Ghanaian fermentations took place in heaps of different sizes or in trays. Turning of the cocoa beans mass was carried out in 9 out of the 13 fermentations and the fermentation duration varied between 96 and 168 h.

The microbial activity in cocoa beans pulp is considered as a well-defined microbial succession led by yeasts that will dominate the total microbial population during the first hours, after which their level is surpassed by those of LAB, that in turn decline after 48 h of fermentation in detriment of the vigorous development of AAB. Towards the end of the fermentation, *Bacillus* spp. would predominate over all the other microbial groups (Forsyth and Quesnel, 1963; Schwan and Wheals, 2004; Thompson et al., 2007). While for some fermentations in Table 3, a microbial progression by way of a clear succession takes place, for others this sucession was somehow dimmed by the dominance of a certain group, particularly LAB. This novel aspect on the microbial ecology of cocoa beans fermentation has not been reported in earlier investigations. In the former situation fall the fermentations of Trinidad (1-2A), Brazil (3A), Indonesia (4-5A), the Dominican Republic (6A), and Ghana (9A). The remaining fermentations epitomize the latter situation, all reported in recent studies in Ghana.

A common pattern of microbial development in the fermentations (Table 3) consisted of the dominant colonization of the cocoa pulp by yeasts at the start of the process. However, in 11 out of 13 of the fermentations, LAB were also present at the onset of the process. In fact, in 7 out of the 11 fermentations the LAB number was at the same level or significantly surpassed those of the yeasts (considering a difference of 0.5 log cfu/g as significant). This was the case in the fermentations in Trinidad (1-2DE), Indonesia (4-5DE), and Ghana (11-13DE). Maximum levels of yeasts were reached between 12 and 96 h (for example, in Ghana 7D and Ghana 8D), whereas for LAB, peak levels were generally observed at later stages, the earliest maximum at 18 h (Ghana 12-13E) and the latest at 96 h (Ghana 8E). Towards the end of the process, both levels of yeasts and LAB decreased and they did not persist until the end for some of the fermentations. This was the case, for instance, of Indonesia fermentation 4-5DE.

Another general pattern of these fermentations is the early appearance of AAB. In 9 out of the 13 fermentations, they were detected in samples at the start of the process, in some fermentations at levels of the same order of magnitude as yeasts and LAB (e.g., Ghana 12-13DEF), while in others they constituted a fraction of the population of 10% or lower (e.g., Ghana 7DEF and Brazil 3DEF). This shows that AAB can also have an important role to play from the start of the fermentation, in addition to yeasts and LAB. However, as opposed to yeasts and LAB, the activity of AAB was chiefly arrested ahead of the former and this seemed to be independent of the oxygen tension in the cocoa beans mass, since, for example, in Trinidad (2F) and Indonesia (5F) fermentations, aeration was practiced but AAB were not detected after 24 and 48 h, respectively. In contrast, a striking exception occurred in the Ghana fermentation (13F), where while the yeasts were not detected at the samples taken at 144 h, AAB were detected at the end of the fermentation and at approximately the same levels as LAB.

The occurrence of *Bacillus* spp. during cocoa beans fermentation is less predictable in comparison to the other microbial groups. In the fermentations in the Dominican Republic (6G) and Ghana (11-13G), no reference has been made to this group and it is not clear from the publications whether the authors did not investigate their presence or whether *Bacillus* spp. simply did not occur. Only in Ghana (10G) the authors clearly reported the absence of *Bacillus* spp. during tray fermentation, while in Ghana (11G) the authors stated that in addition to yeasts, LAB and AAB, no other major group participated in the process. The fermentations conducted in Trinidad (1-2G) and Ghana (7-9G) were characterized by a late appearance of *Bacillus* spp., typically between 60 and 96 h. On the contrary, in the Indonesia fermentations (4-5G), *Bacillus* spp. were detected at the start of the process at approximately the same level as yeasts and LAB, becoming the dominant group throughout the fermentation. In Ghana fermentations (7-8G), the pattern of occurrence of *Bacillus* spp. was intermediate between that of Indonesia (4-5G) and Trinidad (1-2G) fermentation since this group was detected in samples taken from 48 h. In Brazil fermentation (3G), spore-formers rather than total viable counts were determined. Interestingly, the fact that spore counts remained constant until 72 h correlates well with the results of fermentations of Trinidad (1-2G), Indonesia (5-6G), and Ghana (7-9G), which might indicate unfavorable conditions for the development of this group of microorganisms. With regard to the fate of *Bacillus* spp. towards the end of the fermentation, this constitutes a microbial group that persists until the end.

A range of microbial groups other than yeasts, LAB, AAB, and *Bacillus* spp. have been isolated and identified in

Table 3 Characteristics of cocoa beans fermentation conducted in different countries

	A Country; Culivar; Ref.	B Fermentation Method; Sampling	C Cocoa mass Aeration	D Yeasts	E LAB	F AAB	G *Bacillus* spp.	H Other microorganisms
1	Trinidad; Mention to pinkish colored beans- possibly a Forastero cultivar (Ostovar and Keeney, 1973)	Centeno Estate 168 h; 4 m^3 wooden boxes (1 m depth) covered by plantain leave; Sampling at 45 cm from surface	Beans transfer after 72 and 120 h	Initial level 4.8 log cfu/g (0 h); maximum level of 5.7 log cfu/g (48 h), followed by a decrease to 4.9 log cfu/g (96 h); not detected in samples after 96 h	Initial level 4.9 log cfu/g (0 h); maximum level of log 6.1 cfu/g (48 h), followed by a decrease to 4.6 log cfu/g (168 h)	Detected at levels of 4.8 log cfu/g (48 h); maximum level of 5.1 log cfu/g (72 h); not detected in samples taken from 96 h	Detected at levels of 5.2 log cfu/g (96 h); maximum level of 5.2 log cfu/g (96 h) and decrease to 5.1 log cfu/g (168 h)	*Micrococcus colpogenes* and *Nocardia* were restricted to 0 h, together at levels of 4.4 log cfu/g. *Zymomonas mobilis* was detected at an average level of 5.7 log cfu/g between 24-48 h and at 4.4 log cfu/g at 72 h; not detected after 72 h
2	Trinidad; Mention to pinkish colored beans- possibly a Forastero cultivar (Ostovar and Keeney, 1973)	San Louis Estate 168 h; 18 m^3 wooden boxes (2 m depth) covered by plantain leaves; Sampling at 45 cm from surface	Beans transfer after 72 and 120 h	Initial level 5.5 log cfu/g (0 h); maximum level of 6.5 log cfu/g (24 h), followed by a decrease to 4.3 log cfu/g (168 h)	Initial level of 5.1 log cfu/g (0 h); maximum level of 5.8 log cfu/g (72 h), followed by a decrease to 4.6 log cfu/g (144 h); not detected in the sample taken at 168 h	Initial level of 4.8 log cfu/g (0 h); maximum level of log 5.8 cfu/g (24 h); not detected in samples taken after 24 h	Detected at levels of 5.5 log cfu/g (96 h); maximum level of 5.86 log cfu/g (120 h), followed by a decrease to 5.56 log cfu/g (168 h)	*Micrococcus flavus*, *Cellulomonas cellasea* and *Brevibacterium ammoniagenes* were present together at levels of 4.9 log cfu/g (0 h); not detected from 24 h
3	Brazil; Forastero *Comum* hybrids (Schwan, 1998)	168 h; wooden boxes (1 m depth), covered by jute sacs; 200 kg cocoa beans; Sampling at 45 cm from surface	Beans transfer daily	Initial level 7.0 log cfu/g (0 h); maximum level of log 8.3 cfu/g (36 h), followed by a decrease to 1.8 log cfu/g (120 h) and then an increase to 4.1 log cfu/g (168 h)	Initial level 6.0 log cfu/g (0 h); maximum level of 6.5 log cfu/g (36 h), followed by a decrease to 0.5 log cfu/g (84 h) and then an increase to 2.0 log cfu/g (168 h)	Initial level 3.8 log cfu/g (0 h); maximum level of 7.0 log cfu/g (72 h), followed by a decrease to undetectable levels from samples taken at 132 h onwards	Spore-formers levels were initially 3.8 log cfu/g (0–96 h); maximum level of 7.0 log cfu/g (150–168 h)	Not mentioned
4	Indonesia; Forastero (Ardhana and Fleet, 2003)	Estate A 144 h; wooden boxes, 1.125-2 m^3; 0.75-1 m depth; Sampling at 37.5-50 cm from surface	Beans transfer after 12-16 h and then every 20-24 h	Initial level ~ 5.0 log cfu/g (0 h); maximum level of log 8.5 cfu/g (36h), followed by a sharp decrease to ~3.5 log cfu/g after 72 h until 120 h; not detected after 120 h	Initial level of 5.5 log cfu/g (0 h); maximum level of log 8.0 cfu/g (48 h), followed by a decrease to 5.0 log cfu/g (96-108 h); not detected after 108 h	Initial level of 3.8 log cfu/g (0 h); maximum level of 6.0 log cfu/g (24 h), followed by a decrease to 3.0 log cfu/g (84 h); not detected after 84h	Initial level of 5.0 log cfu/g (0 h); maximum level of 8.0 log cfu/g (84 h), followed by a decrease to 7.0 log cfu/g (120 h) (no data provided after 120 h)	Molds were present at initial level between 2-3.0 log cfu/g (0 h); maximum level of 5.6 log cfu/g (12 h); not detected after 36 h

5	Indonesia; Trinitario (Ardhana and Fleet, 2003)	Estate B 96 h; fermentation, 1.125–2 m^3 wooden boxes (0.75–1 m depth); sampling at 37.5–50 cm from surface	Beans transfer after 12–16 h and then every 20–24 h	Initial level of 4.5 log cfu/g (0 h); maximum level of log 8.5 cfu/g (36 h), followed by a decrease to 3.0 log cfu/g after 72 h until 120 h; not detected after 120 h	Initial level of 4.8 log cfu/g (0 h); maximum level of log 8.1 cfu/g (48 h), followed by a decrease to ~ 5.0 log cfu/g (72 h); not detected after 72 h	Initial level of ~ 3.9 log cfu/g (0 h); maximum level of 5.8 log cfu/g (12 h), followed by a decrease to 3.0 log cfu/g (48 h); not detected after 48 h	Initial level of 5.30 log cfu/g (0 h); maximum level of 7.8 log cfu/g (60 h) (no more information provided after 60 h)	Molds were present at initial level between 2.0-3.3 log cfu/g (0 h); maximum level of 6.0 log cfu/g (36 h); not detected after 36 h
6	The Dominican Republic; Trinitario and Trinitario hybrid trees (Gálvez et al., 2007)	144 h; wooden boxes, 0.216 m^3 (0.6 m depth); 100 kg cocoa beans); Composite sample from different zones	Beans transfer after 24, 48, and 96 h	Initial level of 5.8 log cfu/g (0 h); maximum level of 6.2 log cfu/g (24 h), followed by a decrease to 5.8 log cfu/g (96 h); no data provided after 96 h	Initial level of 3.8 log cfu/g (12 h); maximum level of 7.2 log cfu/g (48 h), followed by a decrease to 6.4 log cfu/g (144 h)	Initial level of 5.4 log cfu/g (0 h); maximum level of 7.4 log cfu/g (48 h), followed by a decrease to 5.9 log cfu/g (144 h)	Not mentioned	Not mentioned
7	Ghana; Mixed hybrids (Nielsen et al., 2007b)	96 h; heap fermentation; ~50 kg cocoa beans covered by plantain leaves; Sampling at 15 cm from surface	Not performed	Initial level of 7.8 log cfu/g (0 h); maximum level of 8.0 log cfu/g (12 h), followed by a decrease to 4.3 log cfu/g (96 h);	Initial level of 6.3 log cfu/g (0 h); maximum level of 9.2 log cfu/g (48 h), followed by a decrease to 6.4 log cfu/g (84 h); not detected at 96 h	Initial level of 6.2 log cfu/g (12 h); maximum level of 7.9 log cfu/g (36 h), followed by a decrease to 6.6 log cfu/g (84 h); not detected at 96 h	Detected at levels of 4.1 log cfu/g (48 h); maximum level of 5.8 log cfu/g (84–96 h)	Not mentioned
8	Ghana; Mixed hybrids (Nielsen et al., 2007b)	144 h; heap fermentation; 500–750 kg cocoa beans covered by plantain leaves; Sampling at 15 cm from surface	Beans transfer after 48 and 96 h	Initial level of 7 log cfu/g (0 h); maximum level of 7.3 log cfu/g (96 h), followed by a decrease to 5.6 log cfu/g (144 h)	Initial level of 6.0 log cfu/g (0 h); maximum level of 9.2-9.3 log cfu/g (84–96 h), followed by a decrease to 9.0 log cfu/g (144 h)	Initial level of 5.6 log cfu/g (0 h); maximum level of 7.8 log cfu/g (48 h), followed by a decrease to 6.0 log cfu/g (144 h)	Detected at levels of 5.5 log cfu/g (48 h); maximum level of 9.1 log cfu/g (96 h) followed by a decrease to levels of 7.8 log cfu/g (144 h)	Not mentioned
9	Ghana; Mixed hybrids (Nielsen et al., 2007b)	144 h; heap fermentation; 500–750 kg cocoa beans covered by plantain leaves; Sampling at center of the fermenting mass	Beans transfer after 48 and 96 h	Initial level of 7.2 log cfu/g (0 h); maximum level of 7.5 log cfu/g (12 h), followed by a decrease to 4.3 log cfu/g (144 h)	Initial level of 5.5 log cfu/g (0 h); maximum level of 9.4 log cfu/g (48 h), followed by a decrease to 5.6 log cfu/g (144 h)	Initial level of 5.6 log cfu/g (24 h); maximum level of 7.1 log cfu/g (60 h), followed by a decrease to 3.7 log cfu/g (120 h), not detected in samples taken from 132 h	Detected at levels of 7.8 log cfu/g (60 h); maximum level of 7.8 log cfu/g (60 h); followed by a decrease to 6.8 log cfu/g (144 h)	Not mentioned

(Continued on next page)

Table 3 Characteristics of cocoa beans fermentation conducted in different countries *(Continued)*

	A Country; Culivar; Ref.	C Fermentation Method; Sampling	B Cocoa mass Aeration	D Yeasts	E LAB	F AAB	G *Bacillus* spp.	H Other microorganisms
10	Ghana; Mixed hybrids (Nielsen et al., 2007b)	96 h; tray fermentation; 0.108 m^3 (0.1 m depth); 100 kg; Sampling at center from the top tray	Not performed	Initial level of 7.5 Log cfu/g (0 h); maximum level of 7.6 log cfu/g (12 h), followed by a decrease to 4.3 log cfu/g (96 h)	Initial level of 7.2 log cfu/g (0 h); maximum level of 9.7 log cfu/g (24 h), followed by a decrease to 8.6 log cfu/g (96 h)	Detected at levels of 7.0 log cfu/g (24 h); maximum level of 7.7 log cfu/g (36 h); followed by a decrease to 4.0 log cfu/g (84 h); not detected at 96 h	Not found	Not mentioned
11	Ghana; Mixed hybrids (Criollo and Forastero) (Camu et al., 2007)	144 h; heap fermentation; 250–1000 kg cocoa beans covered by plantain leaves; Sampling at 30 cm from surface in different points	Not performed	Initial level of 6.3 log cfu/g (0 h); maximum level of 7.8 log cfu/g (12 h), followed by a decrease to 4.0 log cfu/g (144 h)	Initial level of 7.0 log cfu/g (0 h); maximum level of ~ 8.8 log cfu/g (42 h), followed by a decrease to 6.4 log cfu/g (144 h)*	Initial level of 4.0 log cfu/g (0 h); maximum level of ~ 7.0 log cfu/g (66 h), followed by a decrease to 5.0 log cfu/g (144 h)	Not mentioned	
12	Ghana; Mixed hybrids (Criollo and Forastero) (Camu et al., 2008b)	144 h; heap fermentation (heap 12); ~150 kg cocoa beans covered by plantain leaves; Sampling at 30 cm from surface in different points	Beans transfer after 48 and 96 h	Initial level of ~ 6.0 log cfu/g (0 h); maximum level of 7.5 log cfu/g (18 h), followed by a decrease to 4.0 log cfu/g (120 h); not detected at 144 h	Initial level of ~ 6.0 log cfu/g (0 h); maximum level of ~ 8.9 log cfu/g (18 h), followed by a decrease to 6.5 log cfu/g (144 h)	Initial level of 5.5 log cfu/g (0 h); maximum level of 7.8 log cfu/g (24 h), followed by a decrease to ~6.6 log cfu/g (144 h)**	Not mentioned	For both 12-13A reference is made to *Pantoea sp.*and other spp. that constituted between 0.9–8% and 39–72% of the total population, respectively, depending on the type of medium used
13	Ghana; Mixed hybrids (Criollo and Forastero) (Camu et al., 2008b)	144 h; heap fermentation (heap 13); ~150 kg cocoa beans covered by plantain leaves; sampling at 30cm from surface in different points (placenta removed from beans)	Not performed	Initial level of 6.3 log cfu/g (0 h); maximum level of 7.2 log cfu/g (18 h), followed by a decrease to 4.0 log cfu/g (120 h); not detected at 144 h	Initial level of 6.6 log cfu/g (0 h); maximum level of 7.3 log cfu/g (18 h), followed by a decrease to 6.3 log cfu/g (144 h)	Initial level of 6.5 log cfu/g (0 h); varying between 6.5 log cfu/g and values below 7.5 log cfu/g until before 96 h; maximum level of 7.5 log cfu/g (96 h), followed by a decrease to 5.8 log cfu/g (144 h)**	Not mentioned	

Abbreviations: LAB- Lactic Acid Bacteria; AAB- Acetic Acid Bacteria.

*The data retrieved from this article for LAB refers to growth in MRS medium (de Man-Rogosa-Sharpe); **The data retrieved from this article for AAB refers to growth in DMS medium (Deoxycholate-mannitol-sorbitol agar).

Note: This table is the result of data conversion from graphs from the original articles exception for the work by Nielsen et al. (2007b). The microbiological levels were rounded to the decimals.

Table 4 Pulp conditions during cocoa beans fermentation

	A Country/Ref.	I T (°C)	J pH	K Sugars (mg g^{-1})	L Pulp metabolites (mg g^{-1})
1	Trinidad- Centeno Estate (Ostovar and Keeney, 1973)	T_{0h}: 28.5, T_{48h}: 42.5, T_{72h}: 56 = T_{Max},T_f: 45	pH_{0h}: 3.3, pH_{48h}: 4.05, pH_{72h}: 4.15 pH_{Max}: 4.6 (156 h), pH_f: 4.5		
2	Trinidad- San Louis Estate (Ostovar and Keeney, 1973)	T_{0h}: 36, T_{48h}: 39, T_{72h}: 49 = T_{Max}, T_f: 42	pH_{0h}: 3.5, pH_{48h}: 4.0, pH_{72h}: 4.5 pH_{Max}: ~ 4.53 (156 h), pH_f: 4.4		
3	Brazil (Schwan, 1998)	T_{0h}: 26, T_{48h}: 36.5, T_{72h}: 46, T_{Max}: 52.5 (96 h), T_f: 45	pH_{0h}: 3.5, pH_{48h}: 4.0, pH_{72h}: 4.25 pH_{Max} = pH_f: 5.9		E_i: 0; E_{Max}: 7.4 (24 h); E_f: 0 LA_i: 0; LA_{Max}: 1.9 (48 h); LA_f: 0.7 AA_i: 0; AA_{Max}: 5.5 (72 h); AA_f: 0.75
4	Indonesia- Estate A (Ardhana and Fleet, 2003)	Indonesia 4-5A T_{0h}: 20-25°C, T_f: 48-50°C	pH_{0h}: 3.7; pH_{120h}: 3.9	F_{0h}: 62; F_{120h}: 11 G_{0h}: 41; G_{120h}: 7 S_{0h}: 32; S_{120h}: 0	CA_i: 24; CA_{120h}: 11 E_i: 0.5; E_{24h}: 65; E_{120h}: 0.1 LA_i: 0.3; LA_{120h}: 6.0 AA_i: 0.4; AA_{120h}: 12
5	Indonesia- Estate B (Ardhana and Fleet, 2003)		pH_{0h}: 4.8; pH_{72h}: 4.9	F_{0h}: 42; F_{72h}: 9 G_{0h}: 24; G_{72h}: 5 S_{0h}: 21; S_{72h}: 0	CA_i: 21; CA_{72h}: 9 E_i: 0.23; E_{24h}: 52; E_{72h}: 1.6 LA_i: 0.3; LA_{72h}: 5.0 AA_i: 0.4; AA_{72h}: 10
6	The Dominican Republic (Gálvez et al., 2007)	T_{0h}: 24, T_{48h}: 44, T_{72h}: 49, T_{Max}: 50 (78 h), T_f: 47	pH_{0h}: 4, pH_{48h}: 4.4, pH_{72h}: 4.3; pH_{Max} = pH_f: ~ 4.5 (96 h)	F_{0h}: 21; F_f: 3.75 G_{0h}: 50; G_f: 0.6	CA_i: 6; CA_{72h}: 0.75 E_i: 0; E_{Max}: 2.2 (24 h); E_{72h}: 0 LA_i: 0; LA_{Max}: 0.76 (48 h); LA_f: 0 AA_i: 0; AA_{Max}: 5.18 (72 h); AA_f: 0.75
7	Ghana- Small heap (Nielsen et al., 2007b)	T_{0h}: 28.5, T_{48h}: 46, T_{Max}: 47 (60 h), T_{72h}: 45, T_f: 44.5	pH_{0h}: 3.94, pH_{48h}: 4.06, pH_{72h}: 4.12 pH_{Max}: 4.29 (84 h), pH_f: 4.22	Ghana 7-10A G_{0h}: 54-66 F_{0h}: 63-74 S_{0h}: 3	Ghana 7-10A CA_i: 6-7; CA~0 (12h) E_{Max}: 20 (after 24–36 h) ; E_f: 0 LA_i, < 2; LA_{Max}: 10 (after 24–48 h) AA_i < 2; AA_{Max}: 20 (after 60-72 h); AA_f: 7-10 Lower production of AA in the center of the large heap
8	Ghana- Big heap (Nielsen et al., 2007b)	T_{0h}: 28, T_{48h}: 43, T_{72h} = T_{Max}: 48 (60 h), T_f: 44	pH_{0h}: 4.10, pH_{48h}: 3.98, pH_{72h}: 4.21 pH_{Max} = 4.58 (132 h), pH_f: 4.55	80% of sugars consumed in 24 h	
9	Ghana- Big heap (Nielsen et al., 2007b)	T_{0h}: 28, T_{48h}: 33.5, T_{72h}: 44, T_{Max}: 46 (84 h), T_f: 44	pH_{0h}: 3.95, pH_{Max}: 4.69, pH_{48h}: 4.21, pH_{72h}: 4.12 pH_f: 4.41		
10	Ghana- Tray (Nielsen et al., 2007b)	T_{0h}: 28.5, T_{48h}: 45, T_{Max}: 46 (60, 72, 84 h), T_f: 45.5	pH_{0h}: 4.12, pH_{48h}: 3.98, pH_{72h}: 4.20 pH_{Max} = 4.29 (84 h), pH_f: 4.26		
11	Ghana (Camu et al., 2007)	T_{0h}: 27.5, T_{48h}: 40, T_{72h} = T_{Max} = T_f: 45	pH_{0h}: 3.4, pH_{48h}: 3.7, pH_{72h}: ~ 3.8, pH_{Max} = pH_f: 4.3	F_{0h}: 57; F_f: 5 G_{0h}: 54; G_f: 5	CA_i: 9.2; C_f: 2.5 (144 h) E_i: 0; E_{Max}: 22.5 (54 h); E_f: 7 LA_i: 0; LA_{Max}: 8.6 (60 h); LA_f: 8.2 AA_i: 0; AA_{Max} = AA_f: 6 (60 h) M_i: 0; M_{Max} = M_f: 15 (48 h)
12	Ghana- Heap 12 (Camu et al., 2008b)	T_{0h}: 26.3, T_{Max} = 45.9*	pH_{0h}: 3.5; pH_f: 4.4	F_{0h}: 44.5; F_f: 8 G_{0h}: 42; G_f: 2.5	E_i: 0; E_{Max}: 18 (36 h); E_f: 0 AA_i: 0; A_{Max}: 12 (120 h); A_f: 9.5
13	Ghana- Heap 13 (Camu et al., 2008b)	T_{0h}: 27.0, T_{Max} = 42.8*	pH_{0h}: 3.7; pH_f: 4.2	F_{0h}: 52; F_f: 17 G_{0h}: 50; G_f: 10.5	E_i: 0; E_{Max}: 20 (30 h); E_f: 2.5 AA_i: 0; AA_{Max}: 11.5 (54 h); AA_f: 10

Abbreviations: F- Fructose, G- Glucose, S- Sucrose, CA- Citric acid, E-Ethanol, LA- Lactic acid, AA- Acetic acid, M- Mannitol.
Índices: i- Initial, Max- Maximum, f- Final; * The time was not indicated.
Note: This table is the result of data conversion from graphs in the original articles, exception for the work by Ardhana and Fleet (2003).and Nielsen et al. (2007b).

cocoa beans fermentations. Regarding the Trinidad fermentation (1-2H), a report has been made about the diversity of Gram-positive bacteria like *Micrococcus* spp., *Nocardia*, *Cellulomonas cellasea*, *Brevibacterium ammoniagenes*, and the Gram-negative bacterium *Zymomonas mobilis.* In both Trinidad Estates, Gram-positive bacteria were isolated just at the start of the fermentation at levels that were of the same order of magnitude as of yeasts, LAB and AAB (Table 3: 1-2H). In Centeno Estate (1H), the bacterium *Zymomonas mobilis* was detected at 24 h, constituting 50% of the total microflora, after which its level decreased, and then it was not detected after 72 h. In San Louis Estate, reference was also made to *Zymomonas mobilis*, but deeper in the cocoa beans mass (at 90 cm), where it was present in samples taken at 24 and 48 h, constituting, respectively, 18 and 24% of the total population (Ostovar and Keeney, 1973). In Indonesia fermentation (4-5H) molds were observed at initial levels between 2.0–3.2 log cfu/g and their level increased to 5.6–6.0 log cfu/g at 12 h and 36 h, respectively in Estates A and B. Nevertheless, in both Estates, after a time span of 36 h, this group was not detected. In addition, in both Estates *Micrococcus kristinae*, several *Staphylococcus* spp. (S. *capitis*, S. *aureus* and S. *hominii*) together with *Pseudomonas cepacea* (Estate B) were detected at levels between 5–6 log cfu/g, but their growth was restricted to the first 24–36 h (Ardhana and Fleet, 2003).

The divergent microbial dynamics observed during the reported fermentations of the cocoa beans (Table 3), indicates that more research is required on the properties and ecophysiology of the microbial species.

Microbial Properties and Functional Roles

At the onset of the fermentation, the pulp has ambient temperature, low oxygen availability due to the tightly packed structure of the cocoa beans mass, low pH, and high sugar levels (Table 4). With respect to pH, levels as low as 3.3–3.5 have been reported in the Trinidad (1-2J), Brazil (3J), and Ghana fermentations (11-12J). Higher values for the pulp pH were observed in Indonesia (5J) and in Ghana (8 and 10J), where the pH varied between 4.1 and 4.8. In most cases, the sucrose levels were very low indicating the complete conversion of sucrose into glucose and fructose (Table 4: 6-13K).

Yeasts and LAB are the microbial groups that are physiologically better adapted to thrive under the conditions of cocoa beans fermentation, since both groups display a remarkable tolerance to low pH values (Axelsson, 2004; Deak, 2006). Aditionally, yeasts can withstand high sugar concentrations (Barnett et al., 2000).

The metabolic activity of yeasts in the cocoa beans pulp leads to the production of ethanol, carbon dioxide, acids, and volatile compounds, with a concomitant increase of the temperature (Table 4 and Table 5). With respect to ethanol, different maximum concentrations have been detected during the fermentation of cocoa beans. In the case of Ghana fermentations (Table 4: 7-10L), concentrations of around 20 $mg.g^{-1}$ were detected after 24–36 h, with the same level of concentrations being detected in Ghana (11L), after 54 h. On the contrary, in Brazil (3L) and the Dominican Republic (6L), the peaks corresponded to a concentration of 7.4 and 2.2 $mg.g^{-1}$, respectively, and both these peaks registered at 24 h.

Some yeasts are able to metabolize citric acid (CA) (Jespersen, 2003), which contributes to an increase in the pH value of the pulp, as reported for instance in fermentations in Trinidad (Table 4: 1-2J) and Brazil (3J). Jespersen (2003) reported that while *Pichia kluyveri* had the ability to assimilate CA, *Pichia fermentans* strains exhibited a weak or variable assimilation. Moreover, yeasts have the ability to produce pectinolytic enzymes, including polygalacturonase or pectin methylesterease, but no pectin lyase (Gauthier et al., 1977). Their secretion reduces the pulp viscosity, increasing the oxygen availability and provides a source of supplementary carbohydrates (Gálvez et al., 2007; Schwan et al., 1995; 1997). The ability to produce these enzymes is not only extremely variable among yeasts (Ardhana and Fleet, 2003; Schwan et al., 1997), but also not exclusive of this group (Table 5).

Products of the metabolism of LAB include LA, E, AA, and other organic acids, as well as glycerol, mannitol, carbon dioxide and volatiles (Table 5). Lactic acid can be produced by a homofermentative metabolism, with a yield higher than 85%, or heterofementative metabolism, with a yield of only 50% (Axelsson, 2004). The production of the acids causes a drop in pH of the pulp, which can be detected around 24 to 48 h (Nielsen et al., 2007a; Ostovar and Keeney, 1973). However, the fact that yeasts can assimilate LA, in conjunction with the fermentative ability of LAB to use CA, partly explains why the overall effect of acids production may not produce such a pronounced drop in pH during the first two days of fermentation (Camu et al., 2007; Ostovar and Keeney, 1973). Another reason for this is the fact that LA and AA have lower acid-dissociation constants than CA. As observed for ethanol, LA levels varied considerably between the fermentations (Table 4). In the Dominican Republic fermentation (Table 4: 6L) a relatively low maximum value of LA (0.76 $mg.g^{-1}$) was reported, when compared to the concentrations between 8.6 and 10 $mg.g^{-1}$ detected in Ghana fermentations (7-13L). Final levels of LA varied between concentrations close to zero, in Brazil (3L) and the Dominican Republic (6L), while in Indonesia (4-5L) and Ghana (11L), concentration between 5.0–8.2 $mg.g^{-1}$ were present. This contradicts previous works that mention that mainly AA is present in excess at the end of the fermentation (Schwan, 1998).

The activity of yeasts and LAB reduce the sugars to residual levels, with usually a slightly higher level of fructose being measured than glucose, probably due to the preferential use of glucose by microorganisms (Gancedo, 1998) (Table 4: K).

The increase access of air in the cocoa beans mass resulting from the enzymatic collapse of the pulp stimulates the development of AAB. Due to the low levels of glucose and fructose caused by their fast metabolization by yeasts and LAB (Nielsen et al., 2007b), the metabolism of AAB is shifted towards the

Table 5 Functional role of microbial groups during cocoa beans fermentation as reported

		YEASTS	LAB	AAB	*Bacillus* spp.	MOLDS
Oxygen Requirements		Facultative anaerobic or aerobic	Aerotolerant anaerobic	Obligate aerobic	Aerobic or facultative anaerobic	Aerobic
Assimilation	Ethanol	+		+		+
	Mannitol	−		+		+
	Acetate			+		
	Citrate	+				
	Lactate	+		+		
Fermentation	Ethanol	+				
	Mannitol	−	+			
	Citrate		+			
Production of Acids	Acetate	+	+	+	+	*
	Citrate		+			
	Lactate		+	+	+	
	Oxalate	+				
	Phosphorate	+				
	Succinate	+		+		
	Malate	+				
Production of Volatiles	Alcohols					
	- Ethanol	+	+			
	- Methanol	+				
	- Mannitol		+			
	- Butanediol	+			+(?)	
	Aldehydes	+				
	Ketones	+	+			
	Fatty acids				+	
	Esters	+				
	Pyrazines				+	
Excretion of Enzymes	Cellulases	+				+
	Pectinases	+			+	+
	Proteases				+	+
	Amylases				+	+
	Lipases				+	+**
	References	(Deak, 2006; Gálvez et al., 2007; Gauthier et al., 1977; Jespersen et al., 2005; Schwan et al., 1997; Schwan and Wheals, 2004)	(Deak, 2006; Gálvez et al., 2007; Gauthier et al., 1977; Jespersen et al., 2005; Schwan et al., 1997; Schwan and Wheals, 2004)	(Axelsson, 2004; Camu et al., 2007; Nielsen et al., 2007a; Passos et al., 1984)	(Bartowsky and Henschke, 2008; Camu et al., 2007; Cleenwerck and De Vos, 2008; Schwan and Wheals, 2004)	(Ardhana and Fleet, 2003; Lopez and Quesnel, 1971; Ouattara et al., 2008; Schwan and Wheals, 2004; Zak and Keeney, 1976)

Abbreviations: LAB- Lactic Acid Bacteria; AAB- Acetic Acid Bacteria.
*A discrimination of acids was not made (Ribeiro et al., 1986). **Weak reaction (Ardhana and Fleet, 2003); (?) Role not understood yet.

utilization of ethanol as a main carbon source. AAB can oxidize ethanol to AA and species from the genus *Acetobacter* have the additional capacity to oxidize AA to carbon dioxide and water (a property also characteristic of *Gluconoacetobacter* species) (Bartowsky and Henschke, 2008) (Table 5). They are able to perform this activity both at neutral and pH levels until 4.5. The oxidation of ethanol can explain its negligible levels in the samples at the end of the fermentations (Table 4: L). The levels of AA may be higher at the end of the fermentation when turning of the cocoa beans is not performed by direct impact on the activity of AAB or due to a reduced evaporation (Table 4: 11-13L). During the fermentations the maximum concentrations of AA were higher than those of LA, although towards the end they tended to be present at comparable levels (Table 4). Maximum concentrations of AA varied between 5.5 $mg.g^{-1}$ in Brazil (3 L) to 20 $mg.g^{-1}$ in Ghana fermentations (7-10 L). The acetic acid fermentation is an extremely exothermic process enhancing the rise in temperature initiated by the activity of yeasts. Temperature levels as high as 56°C were reported in Trinidad (Table 4: 1I) at 72 h and the lower temperature was around 43°C in Ghana fermentation (13I).

The genus *Bacillus* comprises a group of ubiquitous microorganisms in the environment, aerobic and facultative anaerobic, that display an enormous metabolic diversity as manifested by their ability to use a big diversity of carbon sources and to proliferate under very extreme conditions (Nazina et al., 2001). Towards the end of the fermentation, the major sources of carbon are organic acids such as AA and LA, and mannitol. Many *Bacillus* spp. are able to use those compounds as a source of energy, which may explain their growth during the later stages

(Sneath, 1986). The role of *Bacillus* spp. during cocoa beans fermentation has not been understood as of yet. The studies by Zak and Keeney (1976) suggested the involvement of *Bacillus subtilis* in the production of tetramethylpyrazines, while other studies associated the presence of *Bacillus* spp. with the occurrence of off-flavors that are regularly encountered towards the end of the fermentation, such as C3-C5 free fatty acids and 2,3-butanediol (Lopez and Quesnel, 1971; 1973; Schwan et al., 1986). More recently, Ouattara et al. (2008) demonstrated the ability of *Bacillus* spp. to produce polygalacturonase and pectin lyase, over a temperature range of 30–50°C and pH levels of 3 to 6 (Table 5). The observation that polygalacturonase production correlated positively with the temperature may indicate an important functional role of this microbial group during the fermentation. *Bacillus* spp. constitute the group that persists in commercial cocoa beans and some cocoa derived products, such as cocoa powder, due to their ability to differentiate into spores (Barrile et al., 1971; Mossel et al., 1974; Ostovar and Keeney, 1973).

Studies has reported that filamentous fungi do not play an important role during cocoa beans fermentation. When present, they are often seen at the surface of the cocoa beans mass, where the oxygen tension is high and the temperature and the concentration of metabolites like acetic acid are low. Mixing or turning the cocoa mass impairs their development (Rohan, 1964). However, in Indonesia fermentations (Table 3: 4-5H) molds constituted an important part of the microbiota at the start until 36 h, were strong polygalacturase producers, and all the isolates were able to produce amylolytic and proteolytic enzymes. Considering that the yeasts isolated during those fermentations did not show pectinolytic activity, a possible beneficial effect of molds during the fermentation is conceivable. Furthermore, the study of molds occurring during Brazil fermentation by Ribeiro et al. (1986), showed the ability of the isolates to assimilate both ethanol and mannitol, which might be crucial in the reduction of ethanol induced-stress.

Ethanol, LA, and AA, together with oxygen and temperature are key factors shaping the dynamics of the microbial development during cocoa beans fermentation. For instance, the heterogeneity of oxygen distribution throughout the cocoa beans mass has repercussions not only on the dominance of microbial groups, but also on the speed at which a particular group grows, thus influencing metabolic processes such as increase in the temperature. Consequently, it can be expected that cocoa beans mass is characterized by an inhomogeneous distribution of both microorganisms and their metabolites, an aspect which becomes more important with bigger volumes of the fermenting mass.

In a fermentation study conducted in Ghana, Jespersen et al. (2005) observed a slower increase in the yeast population in the outer part that lagged about 48 h in comparison to the development in the inner part. This spatially different development had implications for variables like the pH and the temperature. For example, while in the inner part a pH drop from 4.2 to 3.4 occurred within 24 h, in the outer part this level was still 4.1. Another situation of microbial heterogeneity during cocoa beans fermention can be found in the study conducted in Trinidad by Ostovar and Keeney (1973), where a considerable variability in the microbial levels and species at 5, 45, and 90 cm of depth was observed. In both Trinidad Estates a downward trend in the total viable counts along the fermentation box occurred, with higher levels registered at the top surface. Towards the end of the fermentation, the total microbial levels did eventually reach approximately the same number. More specifically, with respect to the microbial groups it is interesting to observe that in Centeno Estate at 5 cm depth, AAB comprised of 2 and 10% of the population, respectively, at 24 and 48 h, but were not detected at 45 or 90 cm. This indicates the extent of the modifications of the cocoa beans mass due to the production of AA. Also, the increase in temperature was localized at the top layer. This emphasizes the importance of turning and mixing practices during the fermentation.

Cocoa beans fermentation constitutes a man-made, ecological niche, with a remarkable diversity of microbial species (Tables 6–9). More than 100 species displaying different metabolic properties have been reported (Tables 6–9). Recently, new species of yeasts, LAB and AAB, have been detected, due to improved culturing strategies together with the use of molecular biology tools (e.g., Internally Transcribed Spacer-Polymerase chain reaction- ITS-PCR-, and DNA-DNA hybridizations) (Cleenwerck et al., 2007; 2008; Nielsen et al., 2007a; 2007b).

Species that appear to be indigenous to cocoa beans fermentation throughout the world are *Hanseniaspora guilliermondii, Issatchenkia orientalis,* and *Saccharomyces cerevisiae* in the yeasts group; *Lactobacillus fermentum,* and *L. plantarum* in the LAB group; *Acetobacter aceti* and *A. pasteurianus* belonging to the AAB group; and *Bacillus subtilis*, *B. licheniformis*, *B. pumilus,* and *B. cereus* in the *Bacillaceae* family (Tables 6–9). Many of these species were found during the fermentation of other food products.

During cocoa beans fermentation, shifts in species dominance take place. These are a result of the fermentation practices, combined with the idiosyncratic properties of the microbial strains. Regarding the yeasts population, a wider diversity of fermentative yeasts has occurred rather than respiratory yeasts (Table 6). However, studies referring to the dominance of respiratory yeasts are not scarce. In Indonesia, *Candida inconspicua* was the most prominent yeast throughout the fermentation, having been detected in samples taken at all time-points, while fermentative yeasts like *H. guillermondii* or *P. fermentans* were restricted to the first 72 h (Ardhana and Fleet, 2003). Likewise, in the investigations carried out in Ghana (Nielsen et al., 2007b), *P. membranifaciens*, another respiratory yeast, had the most notable occurrence during all fermentation systems, a role that was shared with *S. cerevisiae* towards the end, in the small heap fermentation, and *C. ethanolica*, in the big heap sampled in the center (Table 3). In these fermentations, yeasts like *H. guilliermondii, C. diversa, C. zemplinina,* and *C. silvae* were the predominant species but confined to the first 24–48 h.

Table 6 Yeast species reported in cocoa beans fermentation

Metabolism	1	2	3	4	5	6	7	8	9	10	11	12	13
Fermentative													
Candida bombi	N.P.	N.P.	+								N.P.	N.P.	N.P.
Candida diversa							+	+	+	+			
Candida glabrata (T)*/ Cryptococcus glabratus* (B)						+							
Candida michaelii								+					
Candida pelliculosa			+	+	+	+							
Candida quercitrusa(A)*/ Candida parapsilosis var. querci*(T)							+						
Candida rugopelliculosa			+										
Candida tropicalis				+	+								
Candida zemplinina							+	+	+	+			
Hanseniaspora valbyensis						+							
Issatchenkia occidentalis									+				
Issatchenkia orientalis/Candida krusei(A)						+	+	+	+	+			
Kloeckera Africana					+								
Kloeckera apis (A)*/ Hanseniaspora guilliermondii*			+	+	+	+	+	+	+	+			
Kloeckera javanica					+								
Kluyveromyces marxianus			+										
Kluyveromyces thermotolerans			+										
Lodderomyces elongisporus			+										
Pichia kluyveri									+	+			
Pichia pijperi							+			+			
Saccharomyces cerevisiae var. *chevalieri*			+										
Saccharomyces cerevisiae			+	+	+		+	+	+	+			
Schizosaccharomyces pombe								+	+	+			
Torulaspora delbreuckii (T)*/Torula colliculosa* (A)									+				
Torulaspora pretoriensis			+										
Weak Fermentative													
Candida cylindracea	N.P.	N.P.					+			+	N.P.	N.P.	N.P.
Candida humicola				+	+								
Candida silvae										+			
Candida sorboxylosa							+			+			
Issatchenkia hanoiensis								+		+			
Pichia fermentans			+			+							
Saccharomycopsis crataegensis									+				
Respiratory													
Candida ethanolica								+	+				
Candida inconspicua (T)*/*						+							
Candida rugosa			+										
Candida zeylanoides						+							
Pichia membranifaciens (A)*/ Candida valida* (T)							+	+	+	+			
Rhodotorula glutinis(T)*/ Cryptococcus glutinis* (A)					+								
Rhodotorula rubra				+									
Saccharomycopsis crataegensis								+					
Yarrowia lipolytica						+							

Note: Numbers correspond to the fermentations sites in table 3 with the respective references. 1-Trinidad (Centeno Estate), 2-Trinidad (San Louis Estate), 3-Brazil (Schwan et al., 1995; Schwan, 1998)., 4-Indonésia (Estate A), 5-Indonésia (Estate B), 6-The Dominican Republic, 7-Ghana (Heap 7B), 8-Ghana (Heap 8B), 9-Ghana (Heap 9B); 10-Ghana (Tray 10B), 11-Ghana (Heap 11B), 12-Ghana (Heap 12B), 13-Ghana (Heap 13B).

N.P.- Not performed; T- Teleomorph (the sexual reproductive stage of a fungi); A-Anamorph (an assexual reproductive stage of a fungi, often mold-like; B-Basionym (CBS, 2003b).

Table 7 Lactic acid bacteria species isolated in cocoa beans fermentation

Metabolism	1	2	3	4	5	6	7	8	9	10	11	12	13
Homofermentative													
Lactobacillus acidophilus	+												
*Lactococcus lactis (*B. *Streptococcus lactis)*	+	+	+				+						
Pediococcus acidilactici		+					+	+	+	+			
*Pediococcus damnosus (*B. *Pediococcus cerevisiae)*	+												
*Pediococcus dextrinicus (*B. *Lactococcus fermenti)*	+												
Streptococcus thermophilus	+	+											
Heterofermentative													
Lactobacillus bulgaricus	+	+											
Lactobacillus casei		+											
Lactobacillus fermentum (B. *L. cellobiosus; L. fermenti)*	+	+		+	+		+	+	+	+	+	+	+
*Lactobacillus ghanaensis**								+	+				
Lactobacillus mali											+		
Lactobacillus paracasei						+							
Lactobacillus paraplantarum						+							
Lactobacillus pentosus						+							
Lactobacillus plantarum	+		+	+	+	+	+	+	+	+	+		+
Lactobacillus pseudoficulneum							+						
Lactobacillus rossii									+				
Obligate Heterofermentative													
Lactobacillus brevis						+				+	+		+
Lactobacillus hilgardii				+				+	+				
Leuconostoc mesenteroides		+								+			
Leuconostoc pseudoficulneum								+	+	+			
Leuconostoc pseudomesenteroides							+		+	+	+		
Weissella cibaria											+		
Weissella kimchii											+		
Weissella paramesenteroides												+	

Note: Numbers correspond to the fermentations sites in table 3 with the respective references. 1-Trinidad (Centeno Estate), 2-Trinidad (San Louis Estate), 3-Brazil((Schwan et al., 1995; Schwan, 1998)., 4-Indonesia (Estate A), 5-Indonesia (Estate B), 6-The Dominican Republic, 7-Ghana (Heap 7B), 8-Ghana (Heap 8B), 9-Ghana (Heap 9B); 10-Ghana (Tray 10B), 11- Ghana (Heap 11B), 12-Ghana (Heap 12B), 13-Ghana (Heap 13B).

* New species discovered in cocoa beans fermentation (Nielsen et al., 2007a).

B.- Basionym (CBS, 2003a).

In general, characteristics like the ability to assimilate LA, to grow at 40°C or in the range 40 to 50°C, and to tolerate high ethanol concentrations have been related to the ability of yeasts to persist until the later stages of cocoa beans fermentation. Moreover, the fact that the majority of AAB occur during cocoa beans fermentation belong to the *Acetobacter* genus may constitute an important factor supporting the prevalence of yeasts until later stages of the fermentation, since an accumulation of ethanol to high toxic levels is precluded. Ardhana and Fleet (2003) reported the capacity of the *Candida* spp. and *Kloeckera* spp. to grow well in the presence of 10% and 5% ethanol, respectively, and Gálvez et al. (2007) described the ability of *C. inconspicua* to assimilate LA, while other yeasts that abounded at the start did not have this capacity.

Acetic acid is the type of acid that is usually present at higher concentrations at the end of the fermentation and known to exert a higher inhibitory effect on yeast growth than LA, propionic acid or CA (Deak, 2006). This might partly explain the reduction of yeast levels toward the end of the fermentation, for example, a decrease in 4 log cfu/g in Ghana fermentations (Table 3: 12-13B) after 120 h, although appreciable levels of sugars were still present (Table 4: 12–13K).

Heterofermentative LAB constitute the major group occurring during cocoa beans fermentations. In Indonesian fermentations (Table 3: 4-5E) *Lactobacillus fermentum* was the most predominant species between 36–48 h (60–80%) together with *L. plantarum*. A very similar pattern occurred in Ghanaian fermentations where *L. fermentum* was the most important species throughout the fermentation, accompanied by *L. plantarum* either during the first 48 hours of fermentation, or between 132 to 144 h (Camu et al., 2007; 2008b; Nielsen et al., 2007b). In the Dominican Republic fermentation, *L. fermentum* was not reported (Table 7). *L. plantarum* or *L. paraplantarum*, in conjunction with *L. paracasei* and *L. pentosus* had rather an important role. With respect to the properties of the LAB species, Camu et al. (2007) emphasized the aciduric and ethanol-tolerant character of *L. fermentum* as factors explaining the persistence of this LAB until the end of the fermentation, in comparison to

Table 8 Acetic acid bacteria species reported in cocoa beans fermentation

	1	2	3	4	5	6	7	8	9	10	11	12	13
Acetobacter													
Acetobacter aceti	+	+	+	+	+								
*Acetobacter ghanaensis**											+	+	+
Acetobacter lovaniensis						+							
*Acetobacter lovaniensis-like***													+
Acetobacter malorum								+	+	+			
Acetobacter pasteurianus				+	+		+	+		+	+	+	+
Acetobacter roseus[a]	+												
*Acetobacter senegalensis****												+	+
Acetobacter syzygii							+	+	+	+			
Acetobacter tropicalis							+	+	+	+			
Gluconobacter													
Gluconobacter oxydans (B. *Acetobacter oxydans)*	+		+					+					
Gluconobacter oxydans-like			+										

Note: Numbers correspond to the fermentations sites in table 3 with the respective. 1-Trinidad (Centeno Estate), 2-Trinidad (San Louis Estate), 3-Brazil (Schwan et al., 1995; Schwan, 1998), 4-Indonesia (Estate A), 5-Indonesia (Estate B), 6-The Dominican Republic, 7-Ghana (Heap 7B), 8-Ghana (Heap 8B), 9-Ghana (Heap 9B); 10-Ghana (Tray 10B), 11-Ghana (Heap 11B), 12-Ghana (Heap 12B), 13-Ghana (Heap 13B).

*New species discovered in cocoa beans fermentation closely related with A. *syzygii* (Cleenwerck et al., 2007).

**Recently described as new species: *Acetobacter fabarum* (Camu et al., 2008b; Cleenwerck et al., 2008).

***Recently described new species (Ndoye et al., 2007).

[a]Species not validated in current taxonomic description.

B.- Basionym (CBS, 2003a).

Leuconostoc pseudomesenteroides and some *Enterococcus casseliflavus* strains that played a small role. In the homofermentative group *Pediococcus acidilactici* was reported to occur in all fermentation systems studied by Nielsen et al. (2007b), while none was found in the investigation by Camu and co-workers (2007; 2008b). However, the proliferation of this species was always very restricted and constituted a minority of the total LAB population. On the contrary, in Trinidadian fermentation (Table 3: 1E) the detection of LAB until the end of the fermentation was due to the presence of *Streptococcus thermophilus* (a heat-tolerant species) that constituted 32% of the total viable counts at 120 h, 27% at 144 h, and 32.5% at 168 h, being the only species of LAB from 144 h. With respect to AAB, only the *Acetobacter* and *Gluconobacter* genera were reported in cocoa beans fermentation from the 10 existing genera of AAB (Cleenwerck and De Vos, 2008), with the *Acetobacter* genus being the most represented (Table 8). G. *oxydans* and *G. oxydans-like* spp. were the only *Gluconobacter* spp. reported. Indeed, in all fermentations

Table 9 *Bacillus* spp. species isolated from cocoa beans fermentation

	1	2	3	4	5	6	7	8	9	10	11	12	13
Bacillus cereus	+	+		+		+		+		N.D	N.R	N.R	N.R
Bacillus cereus var. *mycoides*	+												
Bacillus coagulans	+	+				+							
Bacillus licheniformis				+	+	+	+	+	+				
Bacillus megaterium	+	+					+	+	+				
Bacillus mycoides			+										
Bacillus pumilus		+		+	+	+		+	+				
Bacillus sphaericus					+	+			+				
Geobacillus stearothermophilus (B. *Bacillus stearothermophilus)*	+	+	+										
Bacillus subtilis	+	+	+	+		+		+	+				

Note: Numbers correspond to the fermentations sites in table 3 with the respective references. 1-Trinidad (Centeno Estate), 2-Trinidad (San Louis Estate), 3-Brazil (Schwan et al., 1995; Schwan, 1998), 4-Indonesia (Estate A), 5-Indonesia (Estate B), 6-The Dominican Republic, 7-Ghana (Heap 7B), 8-Ghana (Heap 8B), 9-Ghana (Heap 9B); 10-Ghana (Tray 10B), 11-Ghana (Heap 11B), 12-Ghana (Heap 12B), 13-Ghana (Heap 13B).

B.- Basionym (CBS, 2003a).

N.D.- Not detected; N.R.- Not reported.

conducted in Ghana, with the exception of the big heap (8B), and in the fermentations in Trinidad (San Louis Estate) and Brazil, no *Gluconobacter* species were found. It is interesting to point out that while many studies show a sharp decrease of the AAB population after the first 24 h (Ardhana and Fleet, 2003; Ostovar and Keeney, 1973), others report their persistence throughout fermentations even when turning was not performed (Camu et al., 2007; 2008b; Nielsen et al., 2007b). AAB strains of the species *A. pasteurianus, A. ghanaensis, A. senegalensis,* and *A. lovaniensis-like* were detected until the later stages of Ghana fermentation (Table 3: 11 and 13F), which reveals that AAB strains can have a remarkable ability to cope with a low oxyxen tension environment, despite an obligatory aerobic metabolism. *A. aceti* and *A. pasteurianus* strains with this property were also described in the anaerobic environment of wine sediments by Joyeux et al. (1984).

Cleenwerck et al. (2008) have discussed the difficulties associated with the isolation and culturing of AAB, where the use of a single growth medium proved to induce selective isolation of AAB. Therefore, we believe that future studies on AAB populations occurring during cocoa beans fermentation will benefit from the use of different isolation media, in addition to improved molecular strategies (Camu et al., 2007; 2008b).

Finally, with respect to *Bacillus* spp. it is interesting to highlight the isolates found during Indonesia fermentations that persisted throughout the process and were the dominant microflora after 48–72 h. These included *Bacilus pumilus*, *B. licheniformis, B. subtilis,* and *B. cereus*. Their simultaneous growth with that of yeasts, LAB and AAB, as observed in Indonesia (Table 3: 4-5G) and Ghana (Table 3: 7-9G) fermentations, may indicate that these strains had an exceptional ability to deal with ethanolic and acidic stresses. Concerning the relation with the temperature, thermotolerant species occur more frequently than thermophilic species, with *Geobacillus stearothermophilus* being the only thermophilic strain reported so far (Table 9).

With regard to the ecophysiology of these microorganisms, yeast species involved in cocoa beans fermentation have been isolated from workers hands, cutting utensils, fruit flies, surface of sound pods, and the interior of diseased pods. LAB have been associated with leaves and baskets, as well as cutting utensils, workers hands, and fruit flies (Camu et al., 2007; Jespersen, 2003). Fruit flies, at the same time, have been implicated as a source of inoculation for both AAB and *Bacillus* spp. groups (Ostovar and Keeney, 1973). From all these sources, the cocoa pod surface appears to be the most important for microbial pulp inoculation (Camu et al., 2008b).

Use of Culture-Independent Techniques

This decade has been marked by the use of culture-independent techniques designated PCR-DGGE (Denaturing Gradient Gel Electrophoresis). The sensitivity of this technique allowed the detection of species that played a more important role than those indicated by the results of culture-dependent techniques. This situation was reported for instance in studies during tray fermentation in Ghana, where in samples collected after 3 h the yeast *C. stellimalicola* had been only detected by means of DGGE (Jespersen et al., 2005). Nielsen et al. (2007b) found good correspondence between the DGGE results and culture-based techniques. Similarly, the lower detection limit of DGGE revealed that while based on culturing techniques the activity of the yeast *H. guilliermondii* was restricted to the first 24 h in the center of the large heap fermentation; the DGGE revealed that it was still present until approximately 96 h, at levels equivalent to 1% of the total population of yeasts. Likewise, DGGE profiles of LAB showed that *Leuconostoc pseudoficulneum* played a more important role during the fermentation in Ghana (Table 3: 7-10 E), since strong DGGE bands were obtained until the end of the fermentation, whereas according to the culturing techniques, the occurrence of this species would have been restricted to the first 24 h.

However, the DGGE method may be restricted by inhibitory compounds that interfere with the PCR reaction or difficulties associated with the bias introduced by the preferential amplification of the 16S rRNA (ribosomal ribonucleic acid) gene of certain groups or species (Camu et al., 2007; Prakitchaiwattana et al., 2004). This constitutes a reason for the importance of combining both culture dependent and culture independent techniques when studying such complex microbial fermentations.

Mechanisms of Cocoa Flavor Precursors Formation

The impact of the diffusion of metabolites into the beans can be classified into four main categories: acidification, subcellular decompartimentation, enzymatic degradation, and components leaching. These physico-chemical processes have been discussed in this section.

Citric acid is the main acid present in raw, unfermented cocoa cotyledons. Weissberger et al. (1971) found 9.8 $mg.g^{-1}$ of CA in experiments with beans from Trinidad. In Indonesia fermentations (Table 10: 4-5A) levels of 9.0 and 7.4 $mg.g^{-1}$ were quantified, while in Ghana (Table 10: 11A) these were in the order of 6 $mg.g^{-1}$. Succinic acid constitutes another acid that might be present at significant levels. In Ghana (Table 10: 11A) a level of 2.3 $mg.g^{-1}$ was reported, at the same time that the concentrations of LA and AA were null. In the course of the fermentation, while the concentration of CA decreased to 4, 3.5, and 2 $mg.g^{-1}$, respectively, in Indonesia (4A), Indonesia (5A), and Ghana (11A), the concentration of all the other acids increased (Table 10). With regard to the final pH of the beans, Ardhana and Fleet (2003) reported a decrease from 6.3 to 5.1 and from 6.5 to 5.0, respectively, in Indonesia (4A) and (5A) (Table 10).

The analysis of these metabolites in the pulp and beans reveals that LA diffusion occurs to a lesser extent in comparison with the other metabolites. This can be concluded from the lower amounts observed in the beans than in the pulp, while for the

Table 10 Cotyledons conditions during cocoa beans fermentation

	A Country/Ref.	J pH	K Sugars (mg g^{-1})	L Pulp metabolites (mg g^{-1})
4	Indonesia- Estate A (Ardhana and Fleet, 2003)	pH_{0h}: 6.3; $pH_{120h:}$: 5.1	F_{0h}: 1.0; F_{120h}: 0.4 G_{0h}: 0.7; G_{120h}: 0.1 S_{0h}: 19; S_{120h}: 0	CA_i: 9; CA_{120h}: 4 E_i: 0.2; E_{120h}: 0.4 LA_i: 0.1; LA_{120h}: 2.0 AA_i: 1.0; AA_{120h}: 25
5	Indonesia- Estate B (Ardhana and Fleet, 2003)	pH_{0h}: 6.5; $pH_{72h:}$: 5.0	F_{0h}: 0.8; F_{72h}: 0.3 G_{0h}: 0.6; G_{72h}: 0.1 S_{0h}: 18; S_{72h}: 0	CA_i: 7.4; CA_{72h}: 3.5 E_i: 0.2; E_{24h}: 52; E_{72h}: 1.6 LA_i: 0.1; LA_{72h}: 1.8 AA_i: 0.7; AA_{72h}: 15
11	Ghana (Camu et al., 2007)		F_{0h}: 0.7; F_f: 4.7 G_{0h}: 0.7; G_f: 5 S_{0h}: 12; S_f: 0	CA_i: 6; C_f: 2.0 SA_i: 2.3; SA_f: 5.0 E_i: 0; E_f: 7 LA_i: 0; LA_f: 2.8 AA_i: 0; AA_f: 6.4
12	Ghana- Heap 12 (Camu et al., 2008b)			E_i: 0; E_f: 1.8 AA_i: 0; A_f: 5.6
13	Ghana- Heap 13 (Camu et al., 2008b)			E_i: 0; E_f: 4.1 AA_i: 0; A_f: 7.0

Abbreviations: F- Fructose, G- Glucose, S- Sucrose, CA- Citric acid, E-Ethanol, LA- Lactic acid, AA- Acetic acid, M- Mannitol
Índices: i- Initial, f- Final.
Note: This table is the result of data conversion from graphs in the original articles, except for the work by Ardhana and Fleet (2003).

other metabolites the ratio pulp/beans was approximately equal or lower than one (Ardhana and Fleet, 2003; Camu et al., 2007; 2008a). We have not investigated the underlying reasons for this outcome.

The consequences of acidification and the accumulation of ethanol as well as exposure to an atmosphere low in oxygen and high temperature have been extensively studied by Biehl and coworkers (1997) along with leading investigations by researchers like Forsyth and Quesnel (Forsyth, 1952; Forsyth and Quesnel, 1957; 1963; Quesnal, 1965).

In vitro studies of fermentation-like conditions on the subcellular structure of raw, unfermented cocoa beans cotyledons revealed that an anaerobic atmosphere and incubation in the presence of 7.35 $mg.g^{-1}$ CA, pH 5.5 at 40°C, was able to induce seed germination (Biehl et al., 1982b). However, increasing concentrations of AA, ethanol, and rise in temperature, not only prevented seed germination, but also induced seed death, evidenced by the loss in the germination ability and diffusion of the purple pigments throughout the parenchyma cells (Quesnel, 1965). In particular, experiments with incubations of cocoa beans in acetic acid solution with concentrations found during cocoa beans fermentation, increased temperatures and varying atmosphere conditions (air vs nitrogen) were able to destroy the membranes of the hydrophilic compartments and induce fusion of lipid vacuoles. Such events resulted in subcellular reorganizations—a continuous lipid phase was formed which displaced water and organelles to the edges of the cell, and the components diffused out of their storage cells and spread across the parenchyma (Biehl et al., 1977; 1982a). Parameters modulating this effect appeared to be the amount of undissociated AA (higher levels get accumulated due to decreased pH in the seed) and the temperature. Quesnel (1965) demonstrated that, indeed, AA was the most effective agent inducing seed death, while the temperature was shown to become the dominant factor when levels as high as 53°C were reached in the cocoa beans mass (which happened during very few fermentations, as can be observed from Table 4). The role of LA in seed death is still unclear, but considering its low diffusion, it suggests that it will not have a preponderant effect (Biehl et al., 1985; Quesnel, 1965).

Due to the loss of membrane integrity, enzymes and their substrates, initially separated in individual compartments, converge, leading to a cascade of chemical reactions. It is in this apparent chaotic situation that resides one of the most valuable interests of the cocoa beans fermentation because this leads to the formation of the cocoa flavor precursors (Fig. 2). These precursors were shown to be reducing sugars, amino acids, and peptides that react together during roasting, mainly through Maillard and Strecker degradation reactions, generating compounds like pyrazines and Strecker aldehydes. These compounds contribute to the typical cocoa flavor (Reineccius et al., 1972; Rohan, 1963; 1964; 1967; Rohan and Stewart, 1966). In this context, we define "Cocoa flavor" as the totality of complex olfactory, gustative, or trigeminal sensations detected while roasting cotyledons of commercial cocoa (definition adapted from ISO 5492:1992 by Almeida (1999)). "Chocolate flavor" should be applied to the flavor resulting from the subsequent manufacturing operations and addition of other ingredients.

The composition of the enzymatic system of cocoa seeds is still not fully understood. In the literature contradicting information about the presence of enzymes like polygalacturonase, cellulase or lipase is found (Lopez et al., 1978; Lopez and Dimick, 1991). Difficulties associated with sample preparation caused by polyphenol-rich extracts have been pointed out to be one of the main reasons hampering the studies of enzyme activity in cocoa (Hansen et al., 1998; Lopez and Dimick, 1991).

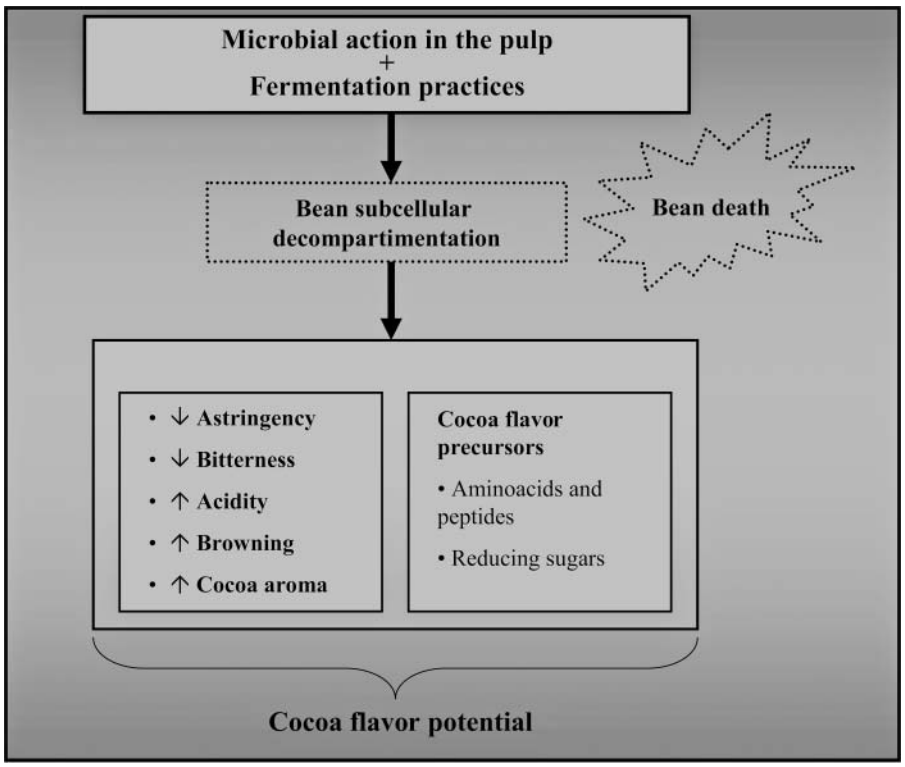

Figure 2 Changes in the cocoa beans during fermentation related to the cocoa flavor.

Nonetheless, there is a consensus on key enzymes responsible for the formation of cocoa flavor precursors, as well as some fermentative flavor compounds. These enzymes are invertase (EC 3.2.1.26), aspartic endoprotease (EC 3.4.23), carboxypeptidase (EC 3.4.17), polyphenol oxidase (EC 1.14.18.1), and glycosidases (Amin et al., 1998; Forsyth and Quesnel, 1957; Hansen et al., 1998; Lopez and Dimick, 1991; Sakharov and Ardila, 1999; Voigt et al., 1994a; 1994c).

It was shown that during the anaerobic phase of the fermentation, corresponding to the initial stages where the oxygen tension is low, due to the tightly packed structure of the beans, microbial oxygen utilization, and carbon dioxide production, the enzymatic reactions are mainly of a hydrolytic nature. Polyphenol oxidase becomes active during the later stages, when the pulp degradation creates conditions for the oxygen penetration in the mass. The terms "anaerobic hydrolytic phase" and "oxidative condensation phase" were employed by Forsyth and Quesnel (1963) to differentiate the period at which the enzymatic activities take place. However, in practice a considerable overlap between the anaerobic and the oxidative phases occurs, due to the heterogeneity of the cocoa beans mass.

Invertase activity is involved in the conversion of seed sucrose into fructose and glucose (although AA penetration in the cotyledons can also contribute to this conversion). Its location in the seed appears to be the shell (Lopez et al., 1978) and the cotyledons. It is active at pH levels between 4 and 5.5, with an optimum of 4.5 (Hansen et al., 1998). Cotyledon invertase appears to be very sensitive to the conditions during cocoa beans fermentation as only trace activity was detected after 2 days fermentation (Hansen et al., 1998). During the fermentation, the sucrose present in the cotyledons is substantially degraded at the same time that the levels of fructose and glucose increase due to sucrose hydrolysis (Table 10: 4-5 K, 11 K). In Ghana (Table 10: 11 K) the authors registered an increase in fructose and glucose levels from 0.7 to 5 $mg.g^{-1}$. However, in both Indonesia fermentations (Table 10: 4-5 K), instead of an increase, a decrease in the glucose and fructose levels was reported at the end of the fermentation. One could speculate on the possibility of degradation reactions of these sugars during the process or their exudation from inside the cotyledons, but surveillances on the quality of fermented commercial cocoa beans have been unanimous in describing appreciable levels of reducing sugars. For example, reducing sugar levels of 2.2 $mg.g^{-1}$ and 6 $mg.g^{-1}$ were described by Hashim et al. (1999) and Reineccius et al. (1972).

The major classes of seed proteins in unfermented cocoa beans are vacuolar storage proteins albumin and globulin, which constitute, respectively, 52 and 43% of the total seed protein (Biehl et al., 1982c; Voigt et al., 1993). The major albumin is a polypeptide with an apparent molecular weight of 19 kDa and the globulin fraction is comprised of polypeptides with an apparent molecular weight of 47, 31, and 14.5 kDa. In the globulin fraction, vicilin-type globulins are the only type found (Voigt et al., 1993). Voigt et al. (1993) observed that as a result of the fermentation the albumin fraction suffered a reduction of around 27%, whereas the decrease in the globulin fraction

was around 90%. In vitro proteolysis studies by Voigt et al. (1994a) showed that hydrophilic peptides and hydrophobic free amino acids were correlated with the formation of cocoa specific aroma. They found the enzymes aspartic endoprotease and the carboxypeptidase to be implicated in the generation of those products (Voigt et al., 1994a). In the sequence of this work, Voigt et al. (1994b) demonstrated in a very elegant manner that the proteinaceous fraction of the flavor precursors was specifically derived from degradation of vicilin-like globulin proteins by the aforementioned enzymes. These findings could explain the previous observations by Biehl et al. (1985), who showed that high concentration of proteolytic products was not necessarily correlated with high flavor potential. Interestingly, in vitro studies comparing the products of hydrolysis of globular storage proteins of different crops showed that cocoa-specific flavor failed to be produced from sources other than cocoa and that this was linked with the particular chemical structure of the vicilin-like globulin (Voigt et al., 1994d).

The enzymes aspartic endoprotease and carboxypeptidase, have different pH optima: this is 3 for the first, with an activity range between pH 3 to 7, and pH 6 for the latter (Hansen et al., 1998). Both proteases displayed considerable stability during the fermentation and drying (Hansen et al., 2000). The potential of prolonged activity might partly explain why reducing sugars are found to be the limiting factor of flavor formation during roasting (Lerceteau et al., 1999; Reineccius et al., 1972).

In what concerns variations of protein levels during the fermentation, de Brito et al. (2000) observed a decrease in protein levels in cotyledons from 220 $mg.g^{-1}$ to 18 $mg.g^{-1}$ after fermentation for 144 h and sun drying for 72 h, while the free amino acid content increased from 25.7 $mg.g^{-1}$ to 32.6 $mg.g^{-1}$. Hashim et al. (1998) reported an increase in free amino acids from 5.2 $mg.g^{-1}$ to 12.7 $mg.g^{-1}$ in fermentation experiments carried out in a rotary drum reactor, where the fraction of hydrophobic amino acids raised from 1.6 to 5.9 $mg.g^{-1}$. Rohsius et al. (2006) pointed out that the content of free amino acids in unfermented and well fermented cocoa beans were in the order of 2 to 4 $mg.g^{-1}$ in comparison to 8 to 25 $mg.g^{-1}$, respectively.

In the polyphenol group, the purple anthocyanidin fraction, constituted mainly by cyanidin-3-α-L-arabinoside- and cyanidin-3-β-D-galactoside, as well as the catechin and the proanthocyanidin fractions suffer modifications during the fermentation, due to enzymatic and non-enzymatic processes (Forsyth and Quesnel, 1957; Wollgast and Anklam, 2000). The glycosidase enzymes, present in cocoa cotyledons, hydrolyze the anthocyanidins to cyanidins and to the respective sugar moieties, galactose and arabinose (Forsyth and Quesnel, 1957). This results in the bleaching of the purple color and release of reducing sugars that can participate as flavor precursors. By the fourth to fifth day of fermentation, the pigments are usually found to be almost totally hydrolyzed (Ferrão, 1963). Hansen et al. (1998) found that glycosidases had an optimum pH of activity between 4 and 4.5 and were stable throughout the fermentation, as well as to the sun and artificial drying. The resulting cyanidin, besides being lost in the exudates, can also be decomposed, originating colorless compounds, or can undergo enzymatic oxidation in the oxidative phase (Forsyth and Quesnel, 1963).

By the time the oxygen starts to increase in the cotyledons, there are conditions for the activity of oxidase enzymes (mainly from polyphenol oxidase complex) and non-enzymatic oxidative reactions. The (-)-epicatechin is the major substrate for polyphenol oxidase, resulting in the production of quinones, very reactive compounds, which in turn can polymerize with other polyphenols or form complexes with amino acids and proteins. These processes have two important consequences: on the one hand a brown color is acquired by the beans, and on the other hand high molecular weight insoluble compounds are formed, reducing the astringency and the bitterness of the beans (Ferrão, 2002; Forsyth and Quesnel, 1963; Hansen et al., 1998). The loss of polyphenols in the exudates of the fermentation also contributes to a reduction of astringency and bitterness (Kim and Keeney, 1984). The participation of proteins in these reactions has a positive effect on the flavor. Thompson et al. (2007) cite works where it was observed that the lower availability of peptides and proteins during roasting was associated with the reduction of off-flavors.

The process of insolubilization of the polyphenols continues during the process of sun drying, yielding cocoa beans that have a "milder taste," when compared with beans that are dried by artificial means (Almeida, 1999; Ferrão, 2002). The polyphenol oxidase, which has an optimum pH of activity between 5.5 and 7, is however, gradually inactivated during both the fermentation and the drying processes.

In fermentations in Brazil, where cocoa beans had been fermented for 144 h and sun dryed for 72 h, the total polyphenol levels decreased from 231 $mg.g^{-1}$ to 157 $mg.g^{-1}$ (de Brito et al., 2000). On the other hand, in Ghana fermentation (11A) (Table 4) a reduction from 126 $mg.g^{-1}$ to 100 $mg.g^{-1}$ was observed after 144 h fermentation, being amplified during sun drying (10-14 days) to final levels of 22 $mg.g^{-1}$, which corresponded to a loss of 82% of polyphenols (Camu et al., 2008a). With respect to the specific groups of polyphenols, Camu et al. (2008a), in studies of heap fermentations in Ghana, reported a decrease in the (-)-epicatechin content from 9–12 $mg.g^{-1}$ to 3.5–1.5 $mg.g^{-1}$ at the end of the fermentation. In the same fermentations, the (+)-catechin decreased from 2.5–3.5 $mg.g^{-1}$ to levels of 1.0–2.5 $mg.g^{-1}$. After sun drying the level of (-)-epicatechin and (+)-catechin was further reduced by 50 and 60%, respectively. In fermentation studies conducted by Almeida and Leitão (1995) and Almeida (1999) in São Tomé and Príncipe, where samples of cocoa beans had been dried either by sun or by artificial means, they described a decrease in the (-)-epicatechin and procyanidins levels from 46 to 0.95 $mg.g^{-1}$ and from 81 to 12 $mg.g^{-1}$, respectively.

The fate of methylxanthines in the pigmented cells has reportedly been reduced due to loss in the exudates. Brunetto et al. (2007) registered a decrease in the level of theobromine from 9.2 to 6.4 $mg.g^{-1}$ in fermented dried samples, while the decrease in caffeine had been from 1.5 to 0.83 $mg.g^{-1}$ (a 45% drop). Although, Camu and co-workers (2008a; 2008b) detected lower

levels of theobromine, they found the same decreasing trend in fermented dried cocoa beans samples in Ghana fermentation (11A) (Table 4). On the contrary, caffeine levels remained approximately constant.

During the fermentation, the starch remains insoluble in the cotyledons. This is the reason why starch has been poorly degraded by amylase (de Brito et al., 2000; Holden, 1959; Roelofsen, 1958).

QUALITY ALONG THE CHAIN AND RESEARCH NEEDS

Farming System

Cocoa production faces many constraints worldwide, the most important of which are pests and diseases that cause an annual loss of production of 20 to 30% (Fowler, 1999; Gotsch, 1997). Witches broom, black pod, watery pod rot, and swollen shoot are some examples of these diseases, but also squirrels, rats, and monkeys can consume significant quantities of ripe pods. Control of pests and diseases is achieved by sanitation and application of pesticides, combined with the use of resistant planting material and good growing practices (Fowler, 1999).

While in some areas cocoa growing is not viable at all, in others, due to more dry climatic conditions, it is possible to conduct an organic farming production. Organic cocoa, whose market share is estimated to be less than 0.5% of the total production, has the advantage of attracting premium prices from consumers and rendering increased revenue to farmers (ICCO, 2007b). Madagascar, Tanzania, and Costa Rica are examples of cocoa suppliers for the organic market (ICCO, 2007b). Though the exact production methods may vary, general principles of organic production include the exclusion of most synthetic biocides and fertilizers, the management of soils through addition of organic materials, adequate growing practices (e.g., regulation of tree density and pruning), and use of crop rotation. In this type of production system, only copper-based fungicides, lime, and neem products are allowed (Prasad, 2005).

Several environmental benefits have been attributed to organic farming and were confirmed on the basis of available European literature in the area of soils, the farm ecosystem, ground and surface water protection, and farm inputs and outputs (Padel, 2001). Moreover, it is an agricultural system that ensures the supply of food for future generations (Cacek and Langner, 1986). For example, Gosling et al. (2006) reported that low input systems such as organic farming are generally more favorable to arbuscular mycorrhizal fungi development, which in turn have the potential to substitute for fertilizers and biocides which are not permitted in organic systems. This means that the impact of organic farming on the macro and microbiota may be very different compared with the conventional farming system. In particular, it can have important implications for the profile and dynamics of cocoa beans fermentation. Nothing is known about this yet. Investigation of these types of systems could make available reliable data on the magnitude of the benefits of organic agriculture and help to improve the performance of the organic methods.

Harvest

The harvest of cocoa pods is an operation that influences the quality and the yield of commercial cocoa. Linked to this operation is the assessment of pods ripeness, which is done using subjective factors, such as thorough color observation or shine loss (Ferrão, 2002). Unripe pods are difficult to depulp, contain fewer sugars, fat, and smaller nibs and compounds that contribute to the potential of flavor. In addition to these technological drawbacks, it has been noted that fermenting pods containing low pulp sugar content do not allow sufficient rise of the temperature in the cocoa mass, lead to slower and less vigorous fermentations, and do not produce the desired cocoa flavor (Dimick and Hoskin, 1999; Schwan, 1998). With respect to the impairment in flavor formation, it appears to be weakly linked to the enzymatic system of the beans, since they are found to be sufficiently developed in immature beans (Hansen et al., 2000). On the contrary, overripe pods are more vulnerable to decay, there is the risk of bean germination, and the pulp tends to become very liquid (Almeida, 1999; Ferrão, 2002). Given the fructification cycle of cocoa, the gathering of perfectly ripe fruits would imply very frequent harvests, which is economically infeasible. In practice, the harvest is performed during a more or less long time-frame depending as well on the availability of personnel. Therefore, pods with different degree of ripeness are often included in the fermentation (Ferrão, 2002; Lass, 1999). Due to this inevitable heterogeneity of fermenting batches, future studies should estimate the approximate proportion of unripe/ripe pods that can be included without compromising the quality of the final commercial cocoa beans.

Microbial Interactions

In cocoa beans fermentation, the information on the type of microbial interactions that take place and their role shaping the course of the microbial dynamics and quality of the commercial cocoa beans is still insufficient. Several publications on fermented foods and beverages have described an enhancement of LAB growth due to carbon dioxide production by yeasts and a supply of simple sugars or growth factors like vitamins. It is possible that the cooccurrence of yeasts and LAB, as observed in most fermentations in Table 3, might be a reflection of mutualistic and synergistic interactions. It is questionable whether this is the only type of interaction that occurs. In the literature the ability of yeasts to inhibit mold growth in natural ecosystems and under laboratory conditions, through nutrient competition, production of toxic compounds (organic acids or ethyl acetate), cell wall-lytic enzymes, and killer toxins is well documented. For instance, in maize dough fermentations, *Saccharomyces cerevisiae* and *Issatchenkia occidentalis* inhibited

the growth of mycotoxin producing molds *Penicillium citrinum*, *Aspergillus flavus,* and *A. parasiticus* (Jespersen et al., 1994). *S. cerevisiae* is a typical yeast involved in most cocoa beans fermentations, while *C. krusei* was found in fermentations in the Dominican Republic and Ghana (Table 6). This prompts the question as to whether the absence or the decline of mold populations in the fermentations summarized in Table 3 could have been influenced by the presence of these yeasts in particular, or by other yeast species.

Many recent articles have not made any reference to the occurrence of *Bacillus* spp. or reported the absence of the occurrence of *Bacillus* spp. (Table 3). Interestingly, in these fermentations, AAB, but especially LAB persisted throughout the fermentation and reached high levels, with LAB constituting the predominant group towards the later stages of the fermentation. On the contrary, the majority of the fermentations where *Bacillus* spp. were present were characterized by a comparatively less pronounced development of both LAB and AAB. While the technological practice of aeration appears not to prevent the presence of *Bacillus* spp. during cocoa beans fermentation (Ardhana and Fleet, 2003; Nielsen et al., 2007b), metabolites produced by LAB and AAB, namely LA and AA, could obstruct the growth of *Bacillus* spp. Moreover, it is known that many strains of LAB are able to produce bacteriocins, which can have a narrow spectrum, impairing the activity of closely related species, or a wide spectrum, inhibiting the growth of a diverse group of Gram-positive microorganisms (Klaenhammer, 1988). Whether the LAB strains occurring during those fermentations had the ability to produce bacteriocins active at the pH values found during the fermentation, is not known. This aspect raises the question of the significance of the absence of *Bacillus* spp. during cocoa beans fermentations, especially since in the tray fermentation (Table 3: 10G), which is claimed to produce commercial cocoa of better quality (Wood, 1975), *Bacillus* ssp. did not occur.

Camu et al. (2008a) found significant differences in the quality of commercial cocoa beans derived from fermentations performed with the same plant cultivar, during the same season, and with the same post-harvest practices (pod storage, fermentation method, type, and duration of drying). The stronger cocoa flavor was obtained from a heap (heap 7) characterized by the lowest levels of ethanol and LA in the pulp (3.53 $mg.g^{-1}$ for both metabolites) and an AA concentration that was among the lowest (4.83 $mg.g^{-1}$). When the microbial levels of the correspondent fermentation are compared with the levels of another heap (heap 5), which contained the highest levels of pulp ethanol and LA, surprisingly, there was no connection with microbial levels of yeasts or LAB, as they were the same in both heaps. On the contrary, in heap 7, AAB persisted throughout the fermentation and attained the highest levels at the end, while on heap 5 they corresponded to 10% of the level registered in heap 7. The high ethanol levels in the final cocoa beans from heap 5 (8.15 $mg.g^{-1}$ vs 3.53 $mg.g^{-1}$ in heap 7) are an indication of a less active metabolic activity of AAB. A hypothesis that can be raised is that the presence of high LA levels in heap 5 impaired the metabolic activity of AAB population, which did not occur in heap 7. Considering that LAB developed to the same levels in both heaps and that LA is a non-volatile acid, one might hypothesize about the presence of a possible microbial group that could have assimilated the LA as a source of carbon. This could have been the case of *Bacillus* spp. The influence of homofermentative LAB can be ruled out as the authors showed in a previous publication the occurrence of solely heterofermentative species in those fermentations; the same applies to the possible occurrence of different species of AAB, as the same species were found in these heaps.

Yeasts, LAB, AAB, and in some fermentations, *Bacillus* spp., constitute the most prominent microbial group, but other bacterial species (e.g., *Zymomonas mobilis*) and molds were also reported in a few fermentations, during a certain period of time (Table 3). It is possible that many other unidentified species might be present (Camu et al., 2008b). The extent to which metabolites or enzymes produced by these minority groups affect the development of the major microbial groups remains unclear.

Metagenomics is an advanced and sophisticated stage of culture-independent genomic tools (Sleator et al., 2008; Venter et al., 2004). It involves direct isolation of DNA from a certain ecological niche, followed by cloning of the complete genomes and analysis of the DNA library for sequences and functions of interest (Sleator et al., 2008). Once the data is collected it can be used in combination with the information derived from the culture dependent technique to answer concrete questions related with the occurrence of functional genes, viz., the presence of bacteriocins or production of cell wall lytic enzymes; or to have access to the microbiome profile of a fermentation (Gill et al., 2006). The application of metagenomics, combined with high-throughput sequencing techniques, may open new insights and research fields in the domain of cocoa bean fermentation, with repercussions for the quality of the final product.

Acidification during the Fermentation

Commercial cocoa beans from some countries tend to have more acidic characteristics than do others. Cocoa beans from Malaysia, Brazil, São Tomé and Príncipe and some crop seasons from Indonesia are examples of this situation (Ferrão, 2002; Fowler, 1999; Jinap and Dimick, 1990). This has an adverse effect in the development of their international market, since cocoa products made with acidic cocoa beans are weaker in its specific flavor (Duncan et al., 1989).

It is not yet totally understood what are the main causes are of the excessive acidification of cocoa beans and the type of microbial succession and species associated with it.

High acidity content of commercial cocoa beans has traditionally been associated with the content of acetic acid, due to the negative impact of excessive acidification by acetic acid of cocoa beans during the fermentation on proteolysis

reactions and development of cocoa flavor potential (Biehl et al., 1985). Later, Jinap and Dimick (1990) demonstrated a strong correlation between the concentration of acetic acid, the pH, and the titratable acidity of the fermented dried beans. However, studies on the acidity characteristics of fermented roasted beans showed that while acetic acid contributes to the pH and titratable acidity, this correlation was weaker with respect to the perceived acid flavor. Instead, the levels of lactic acid were found to be the determinant factor for the acid flavor of cocoa and chocolate flavor (Holm and Aston, 1993); therefore, attention should be given to the population of specifically lactic acid bacteria.

Genetic differences in the planting material cultivated in Malaysia and West Africa were considered to be responsible for the high acidity of the commercial cocoa beans. The upper Amazon hybrids from Malaysia are characterized by a larger amount of pulp, more so than the Amelonado type cultivated in West Africa (Carr, 1982). In Malaysia, when attempts were made to reduce the pulp content of cocoa beans prior to the fermentation, a reduction in the acidity of cocoa beans and an increase in cocoa flavor were obtained. Experimental approaches consisted of storing the pods for 9 to 12 days or sun drying the surface of the mucilaginous beans by spreading them in layers prior to fermentations in 0.32 to 0.42 m depth boxes (Biehl et al., 1990; Duncan et al., 1989; Meyer et al., 1989). One can explain the quality improvement by the increase in the ratio surface/volume, which promoted the respiratory metabolism of yeasts in detriment of the fermentative. In addition, the fact that during pulp pre-conditioning there was a decrease in the total amount of sugars, that would have contributed to a reduced Crabtree effect during the process of fermentation (Biehl et al., 1989). As a consequence, lower levels of ethanol, AA and LA were recorded in the pulp, there was a faster increase of the temperature of the fermenting mass and a decrease in beans pH to levels not below 5 (Biehl et al., 1989; 1990; Meyer et al., 1989). Similarly, in Brazil, it was reported that the removal of 20% of the cocoa pulp by mechanical means resulted in a faster microbial progression and in an increased temperature and pH value of the cotyledon (Schwan and Lopez, 1987). Notwithstanding the possible genotype contribution to differences in the acidic characteristics of commercial cocoa beans from Ghana and Malaysia, it is interesting to observe that post-harvest processing in Ghana also includes pod storage, that can take from 2 to 3 days (Camu et al., 2007) to as long as 18 days (Jespersen et al., 2005). An important question is how microbial successions of fermentations with or without pulp reduction differ. For example, it would be important to extend the analysis of the microbiota composition to other production countries in order to verify whether the trend of occurrence of fermentative yeasts over respiratory yeasts correlates with cocoa beans with higher levels of AA and impaired quality.

Turning of cocoa beans mass is an important operation influencing the activity of the acidifying microflora and supporting the enzymatic and non-enzymatic oxidation reactions in the beans. Studies conducted in Malaysia and Ghana revealed that turning of the beans mass after 48 h and 72 h, in box and heap fermentations, respectively, improved the quality of the resulting chocolate (Baker et al., 1994; Duncan et al., 1989). On the contrary, in recent studies in Ghana on the influence of heap turning in the cocoa flavor attributes (Table 4: 12-13M), higher cocoa sensory scores were obtained from beans derived from the non-turned heap. Although non-turned heaps are characterized by pulp and beans, AA levels were approximately the same as in turned heaps, in turned heaps there was an accelerated ethanol decrease in the pulp that reached levels approximately close to zero. Besides, in the non-turned heaps, the levels of ethanol inside the beans were 5 times higher. There were differences found in LAB and AAB species in the two heaps (Table 7 and 8). There was no mention as to what extent the different species contributed to the flavor attributes of the resulting cocoa and as to what was the contribution of the yeasts species.

Volatile Fraction of the Flavor

The sensory attributes of cocoa flavor are a combination of both a volatile and a non-volatile fraction. The non-volatile fraction of the flavor of fermented and roasted cocoa beans is conferred by the levels of polyphenols, methylxanthines, and organic acids (Almeida, 1999; Stark et al., 2006). The volatile fraction of the flavor is by far more rich and complex, with more than 500 volatile compounds having been detected to date (Nijssen et al., 1996). This fraction is a combination of compounds naturally present in the fresh seed and compounds formed and lost during the fermentation, drying steps, and roasting steps (Table 11).

Pyrazines are the main chemical group in the volatile fraction of the cocoa flavor, accounting for about 17% of the total amount of volatile compounds. Esters, acids, and hydrocarbons correspond to 12, 11, and 9%, respectively (Nijssen et al., 1996). Investigations aimed at characterizing the flavor potential of commercial cocoa beans have been concentrated on the content of free amino acids, oligopeptides, and reducing sugars as precursors for the typical cocoa aroma formation (Biehl et al., 1989; Dimick and Hoskin, 1999; Hashim et al., 1999; Mabrouk, 1979; Reineccius et al., 1972; Rohsius et al., 2006). However, additional volatile flavor characteristics such as “fruity” and “flowery,” flavors caused by the presence of esters and aldehydes formed via microbial synthesis are also of high importance in the flavor. Nevertheless, the impact of the fermentation practices and the influence of specific species on the cocoa flavor development have been overlooked and this should be taken into consideration.

Another noteworthy aspect is the origin of the pyrazine compounds in commercial cocoa beans. Dimethylpyrazines and tetramethylpyrazines are the main pyrazines quantified in commercial cocoa beans (Almeida, 1999; Hashim et al., 1997). The temperatures reached during the fermentation and drying, although moderate in comparison to the temperatures during

Table 11 Volatile constituents of raw, fermented and dried and roasted cocoa beans a,b,c

	Raw beans	Fermented and dried beans	Roasted beans
Hydrocarbons			
C4-alkylbenzene		x[a]	
Cyclohexane			x[a]
Methylcyclohexane			x[a]
Styrene	x[a]		tr[a]
Toluene		x[a]	x[a]
Trimethylbenzene		x[a,c]	
Alcohols			
1-Pentanol		x[a]	x[a]
1-Phenylethanol	x[b]	x[b]	x[b]
2-Methylpropanol		x[a]	
2-Phenylethanol	x[a, b]	x[a, b]	x[a, b]
3-Methyl-1-butanol	x[b]	x[a, b]	x[a, b]
3-Methyl-2-butanol		x[b]	x[b]
Benzylic alcohol	x[b]	x[b]	x[b]
Furfurilic Alcohol	x[b]	x[b]	x[b]
2-Heptanol		x[a]	
3-Hexanol			x[a]
Linalol		x[b]	x[b]
Aldehydes			
3- Methylbutanal	x[b]	x[b]	x[b]
5-Methyl-2-furfural			x[a]
5-Methyl-2-phenyl-2-hexanal			x[a]
Benzaldehyde	x[a, b]	x[a, b]	x[a, b]
Hexanal	x[b]	x[b]	x[b]
Pentanal			x[a]
Phenylacetaldehyde	x[a, b]	x[a,b]	x[a,b]
Ketones			
Acetophenone	x[a]	x[a]	x[a]
2-Hexadecanone	x[a]	x[a]	
Acids			
Hexadecanoic acid			x[a]
Tetradecanoic acid			x[a]
Esters			
2-Phenylethyl acetate	x [b]	x[a,b]	x[a,b]
3-Methyl-2-butanol acetate		x[b]	x[b]
3-Methylbutyl acetate		x[a]	
Benzyl isothiocyanate	x[a]	x[a]	x[a]
Benzyl thiocyanate	tr[a]		
Ethyl acetate	x[b]	x[b]	x[a,b]
Ethyl cinnamate		x[a]	
Ethyl dodecanoate	x[a]		
Ethyl dodecanoate		x[a]	
Ethyl hexadecanoate	x[b]	x[a, b]	x[a, b]
Ethyl octadecanoate		x[a]	x[a]
Ethyl tetradecanoate		x[a]	
Methyl octadecanoate		tr[a]	x[a]
Methyl phenylacetate	x[a]		
Bases			
Pyridine			x[a]
Acetylpyrrole	x[b]	x[b]	x[b]
Methylpyrrole			x[a]
n-Ethylpyrrole			x[a]
2,3-Diethylpyrazine			x[b]
2,3-Dimethylpirazine			x[b]
2,5-Diethyl-3-methylpyrazine			x[a]
2,5-Dimethyl-3-ethylpyrazine			x[a,b]
2,6-Diethyl-3-methylpyrazine			x[a]
2,6-Dimethylpyrazine			x[b]
2-Butyl-3,5-dimethylpyrazine			x[a]

(Continued on next page)

Table 11 Volatile constituents of raw, fermented and dried and roasted cocoa beans a,b,c *(Continued)*

	Raw beans	Fermented and dried beans	Roasted beans
2-Butyl-3-methylpyrazine			x[a]
2-Ethyl-3,5-dimethylpyrazine			x[b]
2-Ethyl-5-methylpyrazine			x[b]
2-Ethyl-6-pyrazine			x[b]
5-Methyl-2-furfural			x[a]
C5-alkylpyrazine			x[a]
Ethylpyrazine			x[b]
Methylpyrazine			x[a,b]
Tetramethylpyrazine		x[a,b,c]	x[a,b]
Trimethylpyrazine		x[a,b]	x[a,b]
S-compounds			
Dimethyl disulphide		tr[a]	x[a]
Nitriles and amides			
Dimethylformamide	x[a]	x[a]	x[a]
Phenylacetonitrile	tr[a]	x[a]	x[a]
Furans, furanones, Pyran, pyrones			
2-Ethylfuran			tr[a]
Methylfuran			x[a]

Tr- Trace

[a](Gill et al., 1984)- raw cocoa beans were derived from ripe fruits; no information is presented on the cultivar, fementation or roasting method.

[b](Almeida, 1999)- raw cocoa beans from Forastero cultivar (São Tomé amelonado); fermentation in boxes for 136 h with turning after 48 and 96 h, followed by sun drying; Roasting at 156°C for 27 min.

[c] (Hashim et al., 1997)- raw cocoa beans, derived from ripe fruit, from Forastero cultivar; fermentation in rotary drum reactor for 144 h.

roasting, can contribute to the final levels of pyrazines in commercial cocoa beans (Gill et al., 1984; Hashim et al., 1999; 1997). Pyrazines can also be formed via microbial synthesis, notably by *Bacillus* spp. The first evidence of microbial synthesis was provided by Kosuge and Kamiya (1962), who showed that tetramethylpyrazine could be produced by *B. subtillis*. A first proof of the implication of *Bacillus* spp. in the formation of pyrazines was reported by Zak et al. (1972), who found a similar trend between tetramethylpyrazine formation and the proliferation of *B. subtilis* in the cocoa beans mass. Similarly, Romanczyk et al. (1995) proved the ability of strains of *B. cereus* isolated from cocoa fementation of producing 2-acetyl-1-pyrroline and some alkylpyrazines. Hashim et al. (1997) studied optimum conditions for the maximum formation of total pyrazines, trimethyl, and tetramethylpyrazines in cocoa seeds fermenting for a period of six days in a rotary drum. They verified a considerable increase in the levels of tetramethyl and trimethhylpirazines after the third day of fermentation and a favorable influence of aeration. The influence of an increased aeration time in the increase of total pyrazines and trimethylpirazines could be related to the promotion of *Bacillus* spp. growth. Altogether, these data indicate the importance that *Bacillus* spp. might have during commercial cocoa beans fermentations, since pyrazines are a valuable attribute in the cocoa flavor (Table 11). However, the fact that these microbial species have also been implicated in the production of off-flavors, namely 2,3-butanediol, indicates the need of studying the properties of *Bacillus* and practices that could promote the formation of the desirable pyrazines, in detriment of off-flavors. Furthermore, with regard to the formation of butanediol, attention should be also given to members of *Leuconostoc* genus*, Lactococcus lactis* species, and *Saccharomyces cerevisiae* (identified for instance during fermentations in Trinidad and Brazil, Table 7: 1-3), since their ability to ferment citrate into butanediol has been reported in dairy and wine fermentations (Herold et al., 1995; Hugenholtz, 1993).

The Cocoa Butter Properties

Cocoa butter, a yellow fat extracted from fermented cocoa beans, is one of the most important ingredients of chocolate and it largely determines its physical properties. The most remarkable physical characteristic is the narrow melting range, between 32°C and 35°C. Its quick meltdown in the mouth produces a cool sensation, which is responsible for the pleasurable release of flavor (Hanneman, 2000).

Triglycerides are the main components of cocoa butter, representing around 97% of the total composition. The remaining fraction includes free fatty acids, mono- and diglycerides, phospholipids, glycolipids, and unsaponifiable matter (Pontillon, 1998). In terms of its fatty acid composition, cocoa butter is a relatively simple fat. Three fatty acids—palmitic, stearic, and oleic—generally account for over 95% of the fatty acids in cocoa butter. Of the remaining acids, linoleic acid is present at the highest level (Hernandez et al., 1991). The chemical composition of cocoa butter varies slightly according to the type of cocoa tree, the age of the plant, the country of origin, and the season of harvesting, but also on processing factors like duration of fermentation. Those factors determine the exact composition of cocoa butter resulting in specific melting point and solidification (crystallization) behavior (Meursing and Zijderveld, 1999; Talbot, 1999). Concerning the hardness,

besides the above-stated factors, the average daily temperature during the last few months of pod development also affects the characteristics of cocoa butter. Lower temperatures give butters that are softer or have a lower melting point (Fowler, 1999). Generally, cocoa butters made from Indonesian and Malaysian beans are harder than West African butters, which in turn are harder than Brazilian butters. These differences between cocoa butters can be found in the ratio of triglycerides of the type SOS/SOO (Table 12) (with S meaning "saturated fatty acid" and O meaning "oleic acid"). In very broad terms, SOS is a group of triglycerides that are solid at room temperature, whereas SOO is a group of triglycerides that are more liquid at room temperatures. Thus, Brazilian cocoa butter with a high level of SOO is less solid than Ghanaian cocoa butter, which in turn is less solid than Malaysian cocoa butter (Table 12) (Talbot, 1999).

Not much is known about the impact of fermentation on cocoa butter properties. In fact, it is not clear whether the fat suffers any quantitative and qualitative change in the storage cells. Making reference to practical field work developed in São Tomé and Príncipe in the 70s, Ferrão (2002) reported that quantitative modifications in the fat content due to fermentation and drying were found to be almost non-existent. The basis for that conclusion was the fact that fat is water insoluble and therefore it would not be lost in the fermentation sweatings. However, the same author states recent studies that suggest a modification in the relative proportion of quantitatively dominant fatty acids. Possible mechanisms involved in those modifications and the impact on the quality of the final product have not yet been fully explained. On the other hand, previous studies have confirmed that prolonged fermentation periods are associated with the increased formation of free fatty acids (FFA) (Lopez and Quesnel, 1973). When these are present at low concentrations they contribute to the normal flavor of cocoa, but at higher concentrations they contribute to a lower fat melting point and flavor deterioration. Given the fact that the risk of oxidation reactions in cocoa butter is negligible due to the low content of unsaturated fatty acids and the high content of natural antioxidants, the implication of (microbial) enzymes seems plausible (Lopez and Quesnel, 1973). In fact, certain filamentous fungi and *Bacillus* spp. are able to produce lipases and therefore they could hydrolize triglycerides in the fat (Ardhana and Fleet, 2003; Lehrian and Patterson, 1983; Lopez and Quesnel, 1973).

On account of the higher price of cocoa butter compared with other technological products such as cocoa powder, seeds with high fat content, even though not processed at optimum conditions, will always find interested buyers. If there are changes in the properties of cocoa butter, we may arrive at the conclusion that the fat characteristics on a certain day of fermentation might have a more desirable technological quality for the production of a particular product. Therefore, there is the need to investigate and elucidate the effect of fermentation on the triglyceride composition, melting, and solidification behavior of cocoa butter in different stages of the fermentation process, giving special attention to the correlation of those parameters with the occurring fermentative microbiota.

Quality Requirements of Commercial Cocoa Beans

Cocoa beans processors and chocolate manufacturers look for cocoa beans with a consistent ability to develop a strong cocoa flavor upon processing. In addition to the flavor potential attributes, the material yield and soundness constitute key criteria for the stage of commercialization. The quality aspects of cocoa beans are grouped into three main areas:

- Economic—relates to the content of useful material. The price manufacturers are willing to pay depends on parameters like bean size, fat content, or the presence of germinated and infested beans.
- Quality—relates to the flavor of the cocoa beans and include factors such as the absence of off-flavors, the presence of desirable ancillary flavors (e.g., floral, spicy, and fruity) and some physical properties, such as cocoa butter hardness, melting, and solidification behavior.
- Wholesomeness—relates to food safety (mycotoxins, pesticides, heavy metals, or foreign materials). The limits are regulated by the national food legislation in the country where the factory is located or through regulatory bodies like the European Union (EU) and the Food and Drug administration (FDA) in the United States.

Quality defects found in cocoa beans include moldy, smoky, acid, bitter, astringent, slaty, dull, putrid, lack of cocoa flavor,

Table 12 Triglyceride composition (%) of cocoa butters

Triglyceride	Brazil	Ghana		Malaysia	
SSS	1.0	1.4	2	2.3	ns
SOS	63.7	76.8	78	84.0	85.3
SSO	0.5	0.4	ns	0.5	ns
SLiS	8.9	6.9	ns	6.8	3.9*
SOO	17.9	8.4	ns	5.1	3.1
OOO	8.0	6.1	ns	1.3	0.7
References	(Talbot, 1999)	(Talbot, 1999)	(Torbica et al., 2006)	(Talbot, 1999)	(Segall et al., 2005)

S = Saturated fatty acid (mainly palmitic and stearic acids): O = oleic acid: Li = linoleic acid
ns- not specified; *- does not include the fraction of triglyceride formed by the fatty acids palmitic-linoleic -stearic acids.

insect damaged, germinated, and contaminated beans. The characteristics "bitter" and "astringent" are related to underfermentation problems. The term "slaty" beans refers to beans that were dried without undergoing fermentation; the terms "dull," "putrid," and "lack of cocoa flavor" are associated with overfermentation practices. "Contaminated" is a term used for beans that have absorbed off-flavors from other products, like rubber or oil based fuels (BCCCA, 1996; Fowler, 1999); and "smoky" refers to beans contaminated by smoke during artifical drying. Acid beans may result from fast (artificial) drying (BCCCA, 1996; Wood, 1975) and other not yet well known causes, as discussed previously.

Cocoa trading still lacks a single internationally accepted framework for grading of commercial cocoa beans. However, in cocoa physical markets, standard criteria are followed, which have been established by the Federation of Cocoa Commerce Ltd (FCC) and the Cocoa Merchants Association of America, Inc. (CMAA). For instance, the FCC makes a distinction between batches comprising less than 5% of moldy and less of 5% slaty cocoa beans (well-fermented cocoa beans) and batches with less than 10% moldy and less than 10% slaty cocoa beans, where in both cases the content of foreign material should be below 1.5%. According to this system, the classification of the cocoa beans is made by the use of the term "cut test." This test involves cutting 300 beans lengthwise; these beans are taken randomly from a sample, followed by the record of any defects and cotyledons color (Almeida, 1999). During cocoa beans fermentation, this test is especially useful since it allows the expeditious monitoring of the status of the fermentation and the determination of its end point since unfermented beans will show a purple color and fully fermented beans a brown color. However, a brown coloration is not always linked to well fermented cocoa beans, but instead may be associated with the occurrence of putrefaction reactions (Wood, 1975; Almeida and Leitão, 1995). In this context, the combination of the cut-test with analytical methods facilitates a better characterization of commercial cocoa bean batches, and may provide a way for a more fair remuneration among farmers.

We propose that other fermentation indexes for providing useful information on the flavor potential of the commercial cocoa beans could include the quantification of the following compounds:

- Volatiles produced during fermentation: especifically trimethyl- and tetramethylpyrazines (Table 11). Besides, certain compounds present in the raw seeds increase when fermented and these could also be used for this purpose. These include Phenyacetaldehyde, Benzaldehyde, and 2-phenylethyl acetate (Almeida, 1999; Gill et al., 1984; Hashim et al., 1997; Reineccius et al., 1972);
- The ratio of reducing/total sugars (Rohan, 1967);
- The ratio between polyphenol fractions (Gourieva and Tserevitinov, 1979; Pettipher, 1986a);
- The ratio soluble nitrogen/total nitrogen (Rohan and Stewart, 1967);

In addition, based on the work by Kirchhoff et al. (1989), the ratio of hydrophobic/total free amino acids could also provide useful information on the flavor potential of the commercial cocoa beans.

CONCLUSIONS

The primary factor influencing the quality attributes of cocoa beans, the principal raw material for chocolate production, is the cocoa tree cultivar and genotype. The harvest and post-harvest processing determine the final quality of the commercial cocoa beans. This means that even if the finest cultivars are selected when the subsequent processing is not properly controlled and good agricultural and manufacturing practices are employed, the final quality will be impaired. In this sequence of processing, fermentation is the most important step. Biochemical reactions inside the cocoa beans take place during fermentation leading to the formation of the cocoa flavor precursors and fermentative flavor. Later, during roasting, the cocoa flavor is fully developed.

During cocoa beans fermentation, different microbial groups are present. However, the comparison that we performed among different fermentations, showed that, contrary to what was believed so far, a well-defined succession of the microbial groups does not always take place; and even in the same country and region the type of microbial species active throughout the process are not necessarily the same. While the presence of yeasts, LAB and AAB, during fermentation gathers consensus in terms of their positive functional contribution for the final quality of commercial cocoa beans, the role of *Bacillus* spp. is still not well understood and constitutes a subject of controversy. This has major implications, considering that in many fermentations where *Bacillus* spp. are reported, they appear towards the later stages of the fermentation. It is pertinent to ask whether fermentations need to be halted at an earlier stage in order to avoid their proliferation, and how the technological procedures should be adjusted to ensure that the desirable biochemical reactions take place inside the bean. On the other hand, the role of microbial interactions during cocoa beans fermentation has been overlooked and in many aspects cocoa microbiologists rely on information derived from studies of other type of ecosystems. Cocoa beans fermentation is a domain that would benefit from the new insights that metagenomics bring, since it would allow the elucidation of not only specific genes that are being expressed in the time course of the fermentation, but also of the presence of microorganisms not detected by culturing techniques or culture independent techniques like PCR-DGGE. Such microorganisms could have a modulating effect on the dynamics of the major microbial groups and ultimately in the final quality of the fermented beans. The study of the functions of the microorganisms by use of metagenomics approaches could help in the future to devise strategies for the improvement of the fermentations by promoting specific microbial physiological traits.

In this review, we have also highlighted the need for better understanding the various aspects related to the microbial contribution to the acidic character of commercial cocoa beans. Our focus has also been on the volatile fraction of the flavor and the properties of the cocoa butter, together with the quantification of external factors to the fermentative microbiota, namely the degree of ripening of the pods and the type of farming system.

Since culture dependent techniques are still very important component in the study of cocoa beans fermentation, a platform for the standardization of microbiological methods should be established. This should include guidelines for the number and location of samples to be taken for the final composite sample, the media to be used for each microbiological group, and the respective incubation conditions. This would have the advantage of permitting reliable comparisons between countries and gaining of more knowledge leading to commercial cocoa beans of consistently better quality. This platform needs to extended to the use of culture independent techniques, covering the DNA extraction method to the conditions used to perform culture-independent analysis and amplicons identification.

Opportunities exist to elevate cocoa beans fermentation to levels similar to those of wine or beer fermentation (Schwan and Wheals, 2004). In fact, it was shown that controlled fermentations by use of starter cultures can produce commercial cocoa of good quality (Schwan, 1998). However, the fact that the practice of cocoa beans fermentation in the traditional way has allowed the production of cocoa beans of acceptable quality and price, it might contribute to resistances or delays in the investment in more modern farm infrastructures. Moreover, commercial cocoa beans belong to a category of raw commodities, since high value is added in the different industrial products (chocolate, cocoa powder, and cocoa butter). This contributes to the concentration of resources in the further stages of the chain.

Nevertheless, cocoa fermentation science is an exciting area, where fundamental research can result in important practical applications. Thus, findings leading to the improvement of the quality of commercial cocoa beans can gradually and successfully transform this field.

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REFERENCES

Afoakwa, E. O., Paterson, A., Fowler, M. F., and Ryan, A. (2008). Flavor formation and character in cocoa and chocolate: a critical review. *Critical Reviews in Food Science and Nutrition.* **48**: 840–857.

Almeida, M. H. G. and Leitão, M. C. A. (1995). Influência da tecnologia nas características do cacau comercial. Estudo de parâmetros de qualidade do cacau de São Tomé e Príncipe, in *1as. Jornadas sobre a Agricultura de São Tomé e Príncipe. 9–11 Dezembro de 1993*, Comunicações IICT, s. d. C. A.Lisboa, pp. 169–180.

Almeida, M. H. G. (1999). *Cacau. Technologia pós-colheita. A fracção volátil no flavor*, PhD thesis, Instituto Superior de Agronomia.

Amin, I., Jinap, S., and Jamilah, B. (1998). Proteolytic activity (aspartic endoproteinase and carboxypeptidase) of cocoa bean during fermentation. *Journal of the Science of Food and Agriculture.* **76**:123–128.

Ardhana, M. M., and Fleet, G. H. (2003). The microbial ecology of cocoa bean fermentations in Indonesia. *International Journal of Food Microbiology.* **86**:87–99.

Arlorioa, M., Locatelli, M., Travagliaa, F., Coïssona, J. D., Del Grossoa, E., Minassia, A., Appendinoa, G., and Martelli, A. (2008). Roasting impact on the contents of clovamide (*N*-caffeoyl-*L*-DOPA) and the antioxidant activity of cocoa beans (*Theobroma cacao* L.). *Food Chemistry.* **106**:967–975.

Axelsson, L. (2004). Lactic acid bacteria: Classification and physiology. *In:* Lactic Acid Bacteria: Microbiology and Functional Aspects, 3rd Ed., pp. 1–66. Salminen, S., von Wright, A. and Ouwehand, A., Eds., Marcel Dekker, New York.

Baker, D. M., Tomlins, K. I., and Gay, C. (1994). Survey of Ghanaian cocoa farmer fermentation practices and their influence on cocoa flavor. *Food Chemistry.* **51**:425–431.

Barnett, J. A., Payne, R. W., and Yarrow, D. (2000). Yeasts: Characteristics and Identification. Vol. 1, 3rd Ed.. Cambridge University Press, Cambridge.

Barrile, J. C., Ostovar, K., and Keeney, P. G. (1971). Microflora of cocoa beans before and after roasting at 150°C. *Journal of Milk and Food Technology.* **34**:369–371.

Bartley, B. G. D. (2005). The Genetic Diversity of Cacao and its Utilization. CABI Publishing, Oxfordshire, UK.

Bartowsky, E. J. and Henschke, P. A. (2008). Acetic acid bacteria spoilage of bottled red wine-A review. *International Journal of Food Microbiology.* **125**:60–70.

BCCCA (1996). Cocoa Beans. Chocolate Manufacturers' Quality Requirements. The Biscuit Cake Chocolate and Confectionary Alliance, London.

Biehl, B., Passern, U., and Passern, D. (1977). Subcellular structures in fermenting cocoa beans. Effect of aeration and temperature during seed and fragment incubation. *Journal of the Science of Food and Agriculture.* **28**:41–52.

Biehl, B., Passern, D., and Sagemann, W. (1982a). Effect of acetic acid on subcellular structures of cocoa bean cotyledons. *Journal of the Science of Food and Agriculture.* **33**:1101–1109.

Biehl, B., Quesnel, V. C., Passern, D., and Sagemann, W. (1982b). Water uptake by cocoa seeds during fermentation-like incubation. *Journal of the Science of Food and Agriculture.* **33**:1110–1116.

Biehl, B., Wewetzer, C., and Passern, D. (1982c). Vacuolar (storage) proteins of cocoa seeds and their degradation during germination and fermentation. *Journal of the Science of Food and Agriculture.* **33**:1291–1304.

Biehl, B., Brunner, E., Passern, D., Quesnel, V. C., and Adomako, D. (1985). Acidification, proteolysis and flavor potential in fermenting cocoa beans. *Journal of the Science of Food and Agriculture.* **36**:583–598.

Biehl, B., Meyer, B., Crone, G., Pollmann, L., and Said, M. B. (1989). Chemical and physical changes in the pulp during ripening and post-harvest storage of cocoa pods. *Journal of the Science of Food and Agriculture.* **48**:189–208.

Biehl, B., Bin Said, M., Meyer, B., and Samarakoddy, R. J. (1990). Bean spreading: A method for pulp preconditioning to impair strong nib acidification during cocoa fermentation in Malaysia. *Journal of the Science of Food and Agriculture.* **51**:35–45.

Brunetto, M. d. R., Gutiérrez, L., Delgado, Y., Gallignani, M., Zambrano, A., Gómez, Á., Ramos, G., and Romero, C. (2007). Determination of theobromine, theophylline and caffeine in cocoa samples by a high-performance liquid chromatographic method with on-line sample cleanup in a switching-column system. *Food Chemistry.* **100**:459–467.

Cacek, T. and Langner, L. L. (1986). The economic implications of organic farming. *American Journal of Alternative Agriculture.* **1**:25–29.

Camu, N., de Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P., Takrama, J. S., Vancanneyt, M., and de Vuyst, L. (2007). Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. *Applied and Environmental Microbiology.* **73**:1809–1824.

Camu, N., De Winter, T., Addo, S. K., Takrama, J. S., Bernaert, H., and De Vuyst, L. (2008a). Fermentation of cocoa beans: Influence of microbial activities and polyphenol concentrations on the flavor of chocolate. *Journal of the Science of Food and Agriculture.* **88**:2288–2297.

Camu, N., Gonzalez, A., de Winter, T., van Schoor, A., de Bruyne, K., Vandamme, P., Takrama, J. S., Addo, S. K., and de Vuyst, L. (2008b). Influence of turning and environmental contamination on the dynamics of lactic acid bacteria and acetic acid bacteria populations involved in spontaneous cocoa bean heap fermentation in Ghana. *Applied and Environmental Microbiology.* **74**:86–98.

Carr, J. G. (1982). Cocoa. In: Economic Microbiology. Vol. 7, pp. 275–292. Rose, A. H., Ed., Academic Press, London.

CBS (2003a). NCCB databases (Bacteria or Plasmids), Centraalbureau voor Schimmelcultures, URL: http://www.cbs.knaw.nl/databases/nccb/nccb_databases.aspx. Accessed: April 2008

CBS (2003b). Yeasts Database, Centraalbureau voor Schimmelcultures, URL: http://www.cbs.knaw.nl/yeast/BioloMICS.aspx. Accessed: April 2008

Cheesman, E. E. (1944). Notes on the nomenclature, classification and possible relationships of cacao populations. *Tropical Agriculture (Trinidad).* **21**:144–159.

Clapperton, J., Yow, S., Chan, J., Lim, D., Lockwood, R., Romanczyk, L., and Hammerstone, J. (1994). The contribution of genotype to cocoa (*Theobroma cacao* L.) flavor. *Tropical Agriculture (Trinidad).* **71**:303–308.

Cleenwerck, I., Camu, N., Engelbeen, K., de Winter, T., Vandemeulebroecke, K., de Vos, P., and de Vuyst, L. (2007). *Acetobacter ghanensis* sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa beans. *International Journal of Systematic and Evolutionary Microbiology.* **57**:1647–1652.

Cleenwerck, I. and De Vos, P. (2008). Polyphasic taxonomy of acetic acid bacteria: An overview of the currently applied methodology. *International Journal of Food Microbiology.* **125**:2–14.

Cleenwerck, I., Gonzalez, A., Camu, N., Engelbeen, K., De Vos, P., and De Vuyst, L. (2008). *Acetobacter fabarum* sp. nov., an acetic acid bacterium from a Ghanaian cocoa bean heap fermentation. *International Journal of Systematic and Evolutionary Microbiology.* **58**:2180–2185.

Coe, S. D. and Coe, M. D. (1996). *The True History of Chocolate*. Thames and Hudson, London.

Cook, L. R. (1982). *Chocolate Production and Use*. 3rd Ed., Magazines for Industry, Catalog and Book Div., New York.

Cros, E. and Jeanjean, N. (1998). Formation de l'arôme cacao. In: *Cacao et Chocolat: Production, Utilisation, Caractéristiques*. pp. 188–206. Pontillon, J., Ed., Technique et Documentation Lavoisier, Paris.

de Brito, E. S., Pezoa García, N. H., Gallão, M. I., Cortelazzo, A. L., Fevereiro, P. S., and Braga, M. R. (2000). Structural and chemical changes in cocoa (*Theobroma cacao* L) during fermentation, drying and roasting. *Journal of the Science of Food and Agriculture.* **81**:281–288.

Deak, T. (2006). Environmental factors influencing yeasts. In: *Biodiversity and Ecophysiology of Yeasts*. pp. 155–174. Rosa, C. A. and Peter, G., Eds., Springer, Berlin.

Del Boca, C. (1962). Cocoa beans: Quality requirements and methods of assessment. *Rev. Int. Chocolaterie.* **17**:218–221.

Dimick, P. S. and Hoskin, J. C. (1999). The chemistry of flavor development in Chocolate. In: *Industrial Chocolate Manufacture and Use*. 3rd Ed., pp. 137–152. Beckett, S. T., Ed., Blackwell Science, Oxford.

Duncan, R. J. E., Godfrey, G., Yap, T. N., Pettipher, G. L., and Tharumarajah, T. (1989). Improvement of Malaysian cocoa bean flavor by modification of harvesting, fermentation and drying methods - the Sime-Cadbury process. *The Planter.* **65**:157–173.

FAO (2007). FAO Statistical Year Book. Food and Agriculture Organization of the United Nations (FAO) statistics division. Accessed: 26 April 2007.

Ferrão, J. E. M. (1963). Cacaus de S. Tomé e Príncipe. Dos polifenóis durante a fermentação. *Anais do Instituto Superior de Agronomia.* **25**:17–222.

Ferrão, J. E. M. (2002). *Cacau:Tecnologia pós-colheita*. Instituto de Cooperação Portuguesa- Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Lisboa.

Forsyth, W. G. C. (1952). Cacao polyphenolic substances. 2. Changes during fermentation. *Biochemistry Journal.* **51**:516–520.

Forsyth, W. G. C. and Quesnel, V. C. (1957). Cacao glycosidase and color changes during fermentation. *Journal of the Science of Food and Agriculture.* **8**:505–509.

Forsyth, W. G. C. and Quesnel, V. C. (1963). The mechanism of cacao curing. In: *Advances in Enzymology and Related Areas of Molecular Biology*. pp. 457–492. Nord, F. F. Ed., John Wiley, New York.

Fowler, M. F. (1999). Cocoa beans: From tree to factory. In: *Industrial Chocolate Manufacture and Use*, 3rd Ed., pp. 8–35. Beckett, S. T., Ed., Blackwell Science, Oxford.

Fowler, M. S. (1994). Fine or flavor cocoas: Current position and prospects. *Cocoa Growers' Bulletin.* **48**:17–23.

Gálvez, S. L., Loiseau, G., Paredes, J. L., Barel, M., and Guiraud, J. P. (2007). Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. *International Journal of Food Microbiology.* **114**:124–130.

Gancedo, J. M. (1998). Yeast carbon catabolite repression. *Microbiology and Molecular Biology Reviews.* **62**:334–361.

Gauthier, B., Guiraud, J., Vincent, J. C., Parvais, J. P., and Galzy, P. (1977). Remarques sur la flore de levures de la fermentation traditionnelle du cacao en Côte d'Ivoire. *Revue des Fermentations et des Industries Alimentaires.* **32**:160–163.

Gill, M. S., Macleod, A. J., and Moreau, M. (1984). Volatile components of cocoa with particular reference to glucosinolate products. *Phytochemistry.* **23**:1937–1942.

Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., and Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science.* **312**:1355–1359.

Gosling, P., Hodge, A., Goodlass, G., and Bending, G. D. (2006). Arbuscular mycorrhizal fungi and organic farming. *Agriculture, Ecosystems, and Environment.* **113**:17–35.

Gotsch, N. (1997). Cocoa crop protection: An expert forecast on future progress, research priorities and policy with the help of the Delphi survey. *Crop Protection.* **16**:227–233.

Gourieva, K. B. and Tserevitinov, O. B. (1979). Method of evaluating the degree of fermentation of cocoa beans. USSR Patent Number 646254.

Hanneman, E. (2000). The complex world of cocoa butter. *The Manufacturing Confectioner.* **80**:107–112.

Hansen, C. E., Del Olmo, M., and Burri, C. (1998). Enzyme activities in cocoa beans during fermentation. *Journal of the Science of Food and Agriculture.* **77**:273–281.

Hansen, C. E., Mañe, A., Burri, C., and Bousbaine, A. (2000). Comparison of enzyme activities involved in flavor precursor formation in unfermented beans of different cocoa genotypes. *Journal of the Science of Food and Agriculture.* **80**:1193–1198.

Hashim, L., Selamat, J., Muhammad, K., and Ali, A. (1999). Effect of drying time, bean depth and temperature on free amino acid, peptide-N, sugar and pyrazine concentrations of Malaysian cocoa beans. *Journal of the Science of Food and Agriculture.* **79**:987–994.

Hashim, P., Selamat, J., Ali, A., and Kharidah, S. (1997). Pyrazines formation in cocoa beans : Changes during fermentation. *Journal of Food Science and Technology.* **34**:483–487.

Hashim, P., Selamat, J., Kharidah, S., and Ali, A. (1998). Changes in free amino acid, peptide-N, sugar and pyrazine concentration during cocoa fermentation. *Journal of the Science of Food and Agriculture.* **78**:535–542.

Hernandez, B., Castellote, A. I., and Permanyer, J. J. (1991). Triglyceride analysis of cocoa beans from different geographical origins. *Food Chemistry.* **41**: 269–276.

Herold, B., Pfeiffer, P., and Radler, F. (1995). Determination of the three isomers of 2,3-butanediol formed by yeasts or lactic acid bacteria

during fermentation. *American Journal of Enology and Viticulture.* **46**:134–137.

Holden, M. (1959). Processing of raw cocoa. III. Enzymic aspects of cocoa fermentation. *Journal of the Science of Food and Agriculture.* **10**:691–700.

Holm, C. S., and Aston, J. W. (1993). The effects of the organic acids in cocoa on the flavor of chocolate. *Journal of the Science of Food and Agriculture.* **61**:65–71.

Hugenholtz, J. (1993). Citrate metabolism in lactic acid bacteria. *FEMS Microbiology Reviews.* **12**:165–178.

ICCO (2007a). Growing Cocoa, International Cocoa Organization, URL: http://www.icco.org/about/growing.aspx. Accessed: 27 April 2007

ICCO (2007b). Chocolate industry, International Cocoa Organization, URL: http://www.icco.org/aboutshipping.aspx#. Accessed: 27 April 2007

ICCO (2008). Annual Report 2006/2007. International Cocoa Organization, London.

ISO (1992). ISO 5492 - Sensory Analysis - Vocabulary. International Organization for Standardazation, Geneva.

Jeanjean, N. D. (1995). Influence du génotype, de la fermentation et de la torréfaction sur le développement de l'arôme cacao: Rôle des précurseurs d'arôme. PhD Diss., Université de Montpellier II.

Jespersen, L., Halm, M., Kpodo, K., and Jakobsen, M. (1994). Significance of yeasts and molds occurring in maize dough fermentation for "kenkey" production. *International Journal of Food Microbiology.* **24**:239–248.

Jespersen, L. (2003). Occurrence and taxonomic characteristics of strains of *Saccharomyces cerevisiae* predominant in African indigenous fermented foods and beverages. *FEMS Yeast Research.* **3**:191–200.

Jespersen, L., Nielsen, D. S., Honholt, S., and Jakobsen, M. (2005). Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. *FEMS Yeast Research.* **5**:441–453.

Jinap, S. and Dimick, P. S. (1990). Acidic characteristics of fermented and dried cocoa beans from different countries of origin. *Journal of Food Science.* **55**:547–550.

Jonfia-Essien, W. A., West, G., Alderson, P. G., and Tucker, G. (2008). Phenolic content and antioxidant capacity of hybrid variety cocoa beans. *Food Chemistry.* **108**:1155–1159.

Joyeux, A., Lafon-Lafourcade, S., and Tibereau-Gayon, P. (1984). Evolution of acetic acid bacteria during fermentation and storage of wine. *Applied and Environmental Microbiology.* **48**:153–156.

Kattenberg, H. R. and Kemmink, A. (1993). The flavor of cocoa in relation to the origin and processing of the cocoa beans. **In:** Food Flavor, Ingredients and Composition. pp. 1–22. Charalambous, G., Ed., Elsevier, New York.

Kim, H. and Keeney, P. G. (1984). (-)-Epicatechin content in fermented and unfermented cocoa beans. *Journal of Food Science.* **49**:1090–1092.

Kirchhoff, P. M., Biehl, B., and Crone, G. (1989). Peculiarity of the accumulation of free amino acids during cocoa fermentation. *Food Chemistry.* **31**:295–311.

Klaenhammer, T. R. (1988). Bacteriocins of lactic acid bacteria. *Biochimie.* **70**:337–349.

Kosuge, T. and Kamiya, H. (1962). Discovery of pyrazines in a natural product: tetramethylpyrazine from cultures of a strain of Bacillus subtilis. *Nature.* **193**:776.

Lass, R. A. (1999). Cacao growing and harvesting practices. **In:** Chocolate and Cocoa: Health and Nutrition. pp. 11–42. Knight, I., Ed., Blackwell Science, Oxford.

Lehrian, D. W. And Patterson, G. R. (1983). Cocoa Fermentation. **In:** Biotechnology, A Comprehensive Treatise. Vol. 5, pp. 529–575. Reed, G., Ed., Verlag Chemie, Basel.

Lerceteau, E., Rogers, J., Pétiard, V., and Crouzillat, D. (1999). Evolution of cacao bean proteins during fermentation: A study by two-dimensional electrophoresis. *Journal of the Science of Food and Agriculture.* **79**:619–625.

Lopez, A. and Quesnel, V. C. (1971). An assessment of some claims relating to the prodution and composition of chocolate aroma. *Revue Internationale de la Chocolaterie.* **26**:19–20.

Lopez, A. and Quesnel, V. C. (1973). Volatile fatty acid production in cacao fermentation and the effect on chocolate flavor. *Journal of the Science of Food and Agriculture.* **24**:319–326.

Lopez, A. S., Lehrian, D. W., and Lehrian, L. V. (1978). Optimum temperature and pH of invertase of the seeds of *Theobroma cacao* L. *Revista Theobroma (Brazil).* **8**:105–112.

Lopez, A. S. and Dimick, P. S. (1991). Enzymes involved cocoa curing. **In:** Food Enzymology. Vol. 2, pp. 211–236. Fox, P. F., Ed., Elsevier Applied Science, London.

Mabrouk, A. (1979). A flavor of browning reactions products. **In:** Food Taste Chemistry. Vol. 115, pp. 205–245. Boudreau, J. C., Ed., American Chemical Society Symposium Series, Washington DC.

Meursing, E. H. and Zijderveld, J. A. (1999). Cocoa mass, cocoa butter and cocoa powder. **In:** Industrial Chocolate Manufacture and Use. pp. 101–114. Beckett, S. T., Ed., Blackwell Science, Oxford.

Meyer, B., Biehl, B., Said, M. B., and Samarakoddy, R. J. (1989). Post-harvest pod storage: A method for pulp preconditioning to impair strong nib acidification during cocoa fermentation in Malaysia. *Journal of the Science of Food and Agriculture.* **48**:285–304.

Minifie, B. W. (1980). *Chocolate, Cocoa and Confectionery: Science and Technology*. 2nd Ed., Avi Publishing Company, Inc., Westport, CT.

Mossel, D. A. A., Meursing, E. H., and Slot, H. (1974). An investigation on the numbers and types of aerobic spores in cocoa powder and whole milk. *Netherlands Milk Dairy.* **28**:149–154.

Motamayor, J. C., Risterucci, A. M., Lopez, P. A., Ortiz, C. F., Moreno, A., and Lanaud, C. (2002). Cacao domestication I: The origin of the cacao cultivated by the Mayas. *Heredity.* **89**:380–386.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., Lysenko, A. M., Petrunyaka, V. V., Osipov, G. A., Belyaev, S. S., and Ivanov, M. V. (2001). Taxonomic study of aerobic thermophilic bacilli: Descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*. *International Journal of Systematic and Evolutionary Microbiology.* **51**:433–446.

Ndoye, B., Cleenwerck, I., Engelbeen, K., Dubois-Dauphin, R., Guiro, A. T., Van Trappen, S., Willems, A., and Thonart, P. (2007). Acetobacter senegalensis sp. nov., a thermotolerant acetic acid bacterium isolated in Senegal (sub-Saharan Africa) from mango fruit (Mangifera indica L.). *International Journal of Systematic and Evolutionary Microbiology.* **57**:1576–1581.

Nielsen, D. S., Schillinger, U., Franz, C. M. A. P., Bresciani, J., Amoa-Awua, W., Holzapfel, W. H., and Jakobsen, M. (2007a). Lactobacillus ghanensis sp. nov., a motile lactic acid bacterium isolated from Ghanaian cocoa fermentations. *International Journal of Systematic and Evolutionary Microbiology.* **57**:1468–1472.

Nielsen, D. S., Teniola, O. D., Ban-Koffi, L., Owusu, M., Andersson, T. S., and Holzapfel, W. H. (2007b). The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods. *International Journal of Food Microbiology.* **114**:168–186.

Nijssen, L. M., Visscher, C. A., Maarse, H., Willemsens, L. C., and Boelens, M. H. (1996). *Cocoa*. TNO Nutrition and Food Research Institute, Zeist.

Ostovar, K. and Keeney, P. G. (1973). Isolation and characterization of microorganisms involved in the fermentation of Trinidad cacao beans. *Journal of Food Science.* **38**:611–617.

Ouattara, H. G., Koffi, B. L., Karou, G. T., Sangare, A., Niamke, S. L., and Diopoh, J. K. (2008). Implication of Bacillus sp. in the production of pectinolytic enzymes during cocoa fermentation. *World Journal of Microbiology and Biotechnology.* **24**:1753–1760.

Padel, S. (2001). Conversion to Organic farming: A typical example of diffusion of an inovation? *Sociologia Ruralis.* **41**:40–61.

Passos, F. M. L., Silva, D. O., Lopez, A. S., Ferreira, C. L. L. F., and Guimaraes, W. V. (1984). Characterization and distribution of lactic acid bacteria from traditional cocoa bean fermentations in Bahia. *Journal of Food Science.* **49**:205–208.

Pettipher, G. L. (1986a). An improved method for the extraction and quantitation of anthocyanins in cocoa beans and its use as an index of

degree of fermentation. *Journal of Science and Food Agriculture.* **37**:289–296.

Pettipher, G. L. (1986b). Analysis of cocoa pulp and the formulation of a standardised artificial cocoa pulp medium. *Journal of the Science of Food and Agriculture.* **37**:297–309.

Pontillon, J. (1998). Le beurre de cacao et les matières grasses en chocolaterie. **In:** Cacao et Chocolat: Production, Utilization, Caractéristiques. pp. 326–393. Pontillon, J., Ed., Lavoisier, Paris.

Prakitchaiwattana, C. J., Fleet, G. H., and Heard, G. M. (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Research.* **4**:865–877.

Prasad, R. (2005). Organic farming vis-'a-vis modern agriculture. *Current Science.* **89**:252–254.

Quesnel, V. C. (1965). Agents inducing the death of cacao seeds during fermentation. *Journal of the Science of Food and Agriculture.* **16**:441–447.

Reineccius, G. A., Keeney, P. G., and Weissberger, W. (1972). Factors affecting the concentration of pyrazines in cocoa beans. *Journal of Agriculture and Food Chemistry.* **20**:202–206.

Ribeiro, N. C. A., Bezerra, J. L., and Lopez, A. S. (1986). Micobiota na fermentação do cacau no Estado da Bahia, Brasil. *Revista Theobroma.* **16**:47–55.

Roelofsen, P. A. (1958). Fermentation, drying and storage of cocoa beans. *Advances in Food Research.* **8**:225–296.

Rohan, T. A. (1963). Precursors of chocolate aroma. *Journal of the Science of Food and Agriculture.* **14**:799–805.

Rohan, T. A. (1964). The Precursors of chocolate aroma: A comparative study of fermented and unfermented cocoa beans. *Journal of Food Science.* **29**:456–459.

Rohan, T. A. and Stewart, T. (1966). The precursors of chocolate aroma: Changes in the free amino acids during the roasting of cocoa beans. *Journal of Food Science.* **31**:202–205.

Rohan, T. A. (1967). The precursors of chocolate aroma: Application of gas chromatography in following formation during fermentation of cocoa beans. *Journal of Food Science.* **32**:402–404.

Rohan, T. A. and Stewart, T. (1967). The precursors of chocolate aroma: Production of free amino acids during fermentation of cocoa beans. *Journal of Food Science.* **32**:395–398.

Rohsius, C., Matissek, R., and Lieberei, R. (2006). Free amino acid amounts in raw cocoas from different origins. *European Food Research and Technology.* **222**:432–438.

Romanczyk Jr, L. J., McClelland, C. A., Post, L. S., and Aitken, W. M. (1995). Formation of 2-acetyl-1-pyrroline by several Bacillus cereus strains isolated from cocoa fermentation boxes. *Journal of Agricultural and Food Chemistry.* **43**:469–475.

Sakharov, I. Y. and Ardila, G. B. (1999). Variations of peroxidase activity in cocoa (*Theobroma cacao* L.) beans during their ripening, fermentation and drying. *Food Chemistry.* **65**:51–54.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., and Jiménez, L. (2005). Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition.* **45**:287–306.

Schwan, R. F., Vanetti, M. C. D., Silva, D. O., Lopez, A., and Moraes, C. A. (1986). Characterization and distribution of aerobic, spore-forming bacteria from cacao fermentations in Bahia. *Journal of Food Science.* **51**:1583–1584.

Schwan, R. F. and Lopez, A. (1987). Mudança no perfil da fermentação de cacau ocasionada pela retirada parcial da polpa da semente. **In:** 10th International Conference on Cocoa Research, Lagos, Nigeria: Cocoa Producers Alliance, Dominican Republic, pp. 227–282.

Schwan, R. F., Rose, A. H., and Board, R. G. (1995). Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. *Journal of Applied Bacteriology Symposium Supplement.* **79**:96S–107S.

Schwan, R. F., Cooper, R. M., and Wheals, A. E. (1997). Endopolygalacturonase secretion by Kluyveromyces marxianus and other cocoa pulp-degrading yeasts. *Enzyme and Microbial Technology.* **21**:234–244.

Schwan, R. F. (1998). Cocoa fermentations conducted with a defined microbial cocktail inoculum. *Applied and Environmental Microbiology.* **64**:1477–1483.

Schwan, R. F. and Wheals, A. E. (2004). The microbiology of cocoa fermentation and its role in chocolate quality. *Critical Reviews in Food Science and Nutrition.* **44**:205–221.

Segall, S. D., Artzb, W. E., Raslan, D. S., Ferraz, V. P., and Takahashi, J. A. (2005). Analysis of triacylglycerol isomers in Malaysian cocoa butter using HPLC-mass spectrometry. *Food Research International.* **38**:167–174.

Sleator, R. D., Shortall, C., and Hill, C. (2008). Metagenomics. *Letters in Applied Microbiology.* **47**:361–366.

Sneath, P. (1986). Endospore-forming Gram-positive rods and cocci. **In:** Bergey's Manual of Systematic Bacteriology. Vol. 2, pp. 1104–1206. Holt, J. G. and Sneath, P., Eds., Williams and Wilkins, Baltimore.

Sounigo, O., Umaharan, R., Christopher, Y., Sankar, A., and Ramdahin, S. (2005). Assessing the genetic diversity in the International Cocoa Genebank, Trinidad (ICG,T) using isozyme electrophoresis and RAPD. *Genetic Resources and Crop Evolution.* **52**:1111–1120.

Stark, T., Bareuther, S., and Hofmann, T. (2006). Molecular definition of the taste of roasted cocoa nibs (*Theobroma cacao*) by means of quantitative studies and sensory experiments. *Journal of Agricultural and Food Chemistry.* **54**:5530–5539.

Talbot, G. (1999). Vegetable fats. **In:** Industrial Chocolate Manufacture and Use. pp. 218–230. Beckett, S. T., Ed., Blackwell Science, Oxford.

Thompson, S. S., Miller, K. B., and Lopez, A. S. (2007). Cocoa and coffee. **In:** Food Microbiology: Fundamentals and Frontiers. 3rd Ed., pp. 837–850. Doyle, M. P. and Beuchat, L. R., Eds., ASM Press, Washington, D.C.

Torbica, A., Jovanovic, O., and Pajin, B. (2006). The advantages of solid fat content determination in cocoa butter and cocoa butter equivalents by the Karlshamns method. *European Food Research and Technology.* **222**: 385–391.

UNCTAD (2001). International Cocoa Agreement, United Nations Conference on Trade and Development, Geneva, Switzerland.

Valiente, C., Esteban, R. M., Mollá, E. F., and López-Andréu, F. J. (1994). Roasting effects on dietary fiber composition of cocoa beans. *Journal of Food Science.* **59**:123–124.

Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., Wu, D., Paulsen, I., Nelson, K. E., Nelson, W., Fouts, D. E., Levy, S., Knap, A. H., Lomas, M. W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y.-H., and Smith, H. O. (2004). Environmental Genome Shotgun Sequencing of the Sargasso Sea. *Science.* **304**:66–74.

Voigt, J., Biehl, B., and Kamaruddin, S. W. S. (1993). The major seed proteins of *Theobroma cacao* L. *Food Chemistry.* **47**:145–151.

Voigt, J., Biehl, B., Heinrichs, H., Kamaruddin, S., Marsoner, G. G., and Hugi, A. (1994a). In-vitro formation of cocoa-specific aroma precursors: Aroma-related peptides generated from cocoa-seed protein by co-operation of an aspartic endoprotease and a carboxypeptidase. *Food Chemistry.* **49**: 173–180.

Voigt, J., Heinrichs, H., Voigt, G., and Biehl, B. (1994b). Cocoa-specific aroma precursors are generated by proteolytic digestion of the vicilin-like globulin of cocoa seeds. *Food Chemistry.* **50**:177–184.

Voigt, J., Voigt, G., Heinrichs, H., Wrann, D., and Biehl, B. (1994c). *In vitro* studies on the proteolytic formation of the characteristic aroma precursors of fermented cocoa seeds: The significance of endoprotease specificity. *Food Chemistry.* **51**:7–14.

Voigt, J., Wrann, D., Heinrichs, H., and Biehl, B. (1994d). The proteolytic formation of essential cocoa-specific aroma precursors depends on particular chemical structures of the vicilin-class globulin of the cocoa seeds lacking in the globular storage proteins of coconuts, hazelnuts and sunflower seeds. *Food Chemistry.* **51**:197–205.

Weissberger, W., Kavanagh, T. E., and Keeney, P. G. (1971). Identification and quantitation of several nonvolatile organic acids of cocoa beans. *Journal of Food Science.* **36**:877–879.

Wollgast, J. and Anklam, E. (2000). Review on polyphenols in *Theobroma cacao*: Changes in composition during the manufacture of chocolate and methodology for identification and quantification.*Food Research International.* **33**:423–447.

Wood, G. A. R. (1975). Cocoa. 3rd Ed., D. Rhind, Whitstable.

Zak, D. L., Ostovar, K., and Keeney, P. G. (1972). Implications of *Bacillus subtilis* in the synthesis of tetramethilpyrazine during fermentation of cocoa beans. *Journal of Food Science.* **37**:967–968.

Zak, D. L. and Keeney, P. G. (1976). Changes in cocoa proteins during ripening of fruit, fermentation, and further processing of cocoa beans. *Journal of Agricultural and Food Chemistry.* **24**:483–486.