GILBERTO VINÍCIUS DE MELO PEREIRA

MICROBIOLOGICAL AND PHYSICOCHEMICAL PERFORMANCE OF COCOA BEAN FERMENTATIONS CARRIED OUT IN DIFFERENT SYSTEMS AND SCREENING OF YEAST AND BACTERIA STRAINS TOWARD DEVELOPMENT OF A STARTER CULTURE

LAVRAS – MG

2012

GILBERTO VINÍCIUS DE MELO PEREIRA

MICROBIOLOGICAL AND PHYSICOCHEMICAL PERFORMANCE OF COCOA BEAN FERMENTATIONS CARRIED OUT IN DIFFERENT SYSTEMS AND SCREENING OF YEAST AND BACTERIA STRAINS TOWARD DEVELOPMENT OF A STARTER CULTURE

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Mestre.

Orientadora

Dra. Rosane Freitas Schwan

LAVRAS – MG

2012

Ficha Catalográfica Preparada pela Divisão de Processos Técnicos da Biblioteca da UFLA

Pereira, Gilberto Vinícius de Melo.
Microbiological and physicochemical performance of cocoa bean fermentations carried out in different systems and screening of yeast and bacteria strains toward development of a starter culture / Gilberto Vinícius de Melo Pereira. – Lavras : UFLA, 2012.
178 p. : il.

Dissertação (mestrado) – Universidade Federal de Lavras, 2012.
Orientador: Rosane Freitas Schwan.
Bibliografia.

1. Chocolate. 2. Cacau. 3. Fermentação. 4. Bactérias do ácido lático. 5. Bactérias do ácido acético. 6. Leveduras. I. Universidade Federal de Lavras. II. Título.

CDD – 576

GILBERTO VINÍCIUS DE MELO PEREIRA

MICROBIOLOGICAL AND PHYSICOCHEMICAL PERFORMANCE OF COCOA BEAN FERMENTATIONS CARRIED OUT IN DIFFERENT SYSTEMS AND SCREENING OF YEAST AND BACTERIA STRAINS TOWARD DEVELOPMENT OF A STARTER CULTURE

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Mestre.

APROVADA em 14 de fevereiro de 2012.

Dr. Disney Ribeiro Dias	UFLA
Dra. Irene da Silva Coelho	UFRRJ
Dra. Priscilla Efraim	UNICAMP

Dra. Rosane Freitas Schwan

Orientadora

LAVRAS – MG

2012

AGRADECIMENTOS

A DEUS, pela vida e pela força para enfrentar todas as dificuldades;

À Universidade Federal de Lavras (UFLA) e ao Programa de pós-graduação em Microbiologia Agrícola do Departamento de Biologia, pela oportunidade concedida para realização do mestrado;

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa de estudo;

Ao apoio financeiro dado pelo Instituto de Pesquisa 'Mars Center of Cocoa Sciencie (MCCS)' – Almirante cacau;

À professora Rosane, não só pela influência em minha jornada científica, mas pela paciência e dedicação na orientação, pela amizade, carinho e confiança a mim depositada;

À minha mãe, Antonia, um exemplo de força, perseverança, garra, sabedoria e amor. Agradeço a ela os ensinamentos que me tornaram o que sou, ao sustento, ao amor, e a força que me incentivou a chegar até aqui;

Às minhas irmãs Kênia e Uyara e meu pai Gilberto, que me acompanharam nesta jornada com carinho e dedicação;

Ao meu amigo Prof. Euziclei, pela ajuda na excução deste trabalho, pelo bom convívio e por me ensinar a grande virtude da paciência;

Às amigas Karina e Cíntia, por estarem sempre perto quando precisei, dando bons conselhos, me defendendo, enfim, sendo grandes amigas.

À professora Irene, por sua dedicação e exemplo de responsabilidade, que foram fundamentais para o desenvolvimento de parte deste trabalho na Bahia;

À estagiária de iniciação científica Roberta, por sua competência, responsabilidade e comprometimento com este trabalho;

Aos integrantes e ex-integrantes do laboratório de Microbiologia, Cláudia, Cidinha, Carla, Profa. Cristina, Profa. Patrícia, Prof. Eustáquio, Prof. Romildo, Prof. Disney, Ivani, Cidinha, Gabriela, Rose, Maiara, Mariana, Magda, Vanessa, Beatriz, Monique, Andreia, Igor, Marcela, Dani, pelo momentos de descontração e convívio;

A todos da empresa Almirante Cacau, em especial: Iara, Alício, Ana Claudia, Soraia, Irene, Uilson, Almir, Ailton, Renilton, Lajana, Breno, Leoni, pelo apoio e acolhida durante a realização da primeira etapa deste trabalho;

Aos moradores e ex-moradores da República Labirinto, pelas amizades e pelo convívio durante cinco anos de existência.

RESUMO

Fermentações espontâneas de cacau, realizadas em cinco diferentes tipos de biorreatores, foram estudadas através de uma abordagem polifásica que implicou em análises microbiológicas através de métodos dependentes e independentes de cultivo da fermentação da polpa de cacau e análises físico-químicas da polpa de cacau e do cotilédone. As fermentações compreenderam em um micro-reator de polipropileno com capacidade para 400 g de amêndoas de cacau, reator de aço inoxidável com capacidade para 10 Kg de amêndoas de cacau, reator de aço inoxidável com capacidade para 40 Kg de amêndoas de cacau, reator de madeira com capacidade para 40 Kg de amêndoas de cacau e reator de madeira com capacidade para 600 Kg de amêndoas de cacau (Anexo 1). Ambas as abordagens microbiológicas revelaram que as espécies predominantes foram as mesmas em todos os processos fermentativos. Estas espécies consistiram das leveduras *Saccharomyces cerevisiae* e *Hanseniaspora* sp., das bactérias do ácido lático *Lactobacillus fermentum* e *L. plantarum* e da bactéria do ácido acético *Acetobacter tropicalis*. Outros grupos microbianos, tais como: *Bacillus*, *Staphylococcus*, *Enterobactérias* e leveduras não-*Saccharomyces*, foram detectados durante os diferentes processos fermentativos, entretanto, maior diversidade foi observada nas fermentações realizadas em caixas de madeira. Adicionalmente, uma potencial nova espécie de bactéria do ácido acético, pertencente ao gênero *Asaia*, foi isolada durante a fermentação em caixa de madeira com capacidade para 40 Kg de amêndoas de cacau. A análise de agrupamento, realizada através dos perfis gerados pelo gel de DGGE de levedura e bactérias, revelou uma complexa microbiota referente às fermentações em caixa de madeira, indicando que a ecologia microbiana dos processos fermentativos foi específica para o método adotado (caixas de madeira *vs.* tanques de aço inoxidável). Em geral, um perfil semelhante de consumo do substrato (sacarose, glicose, frutose e ácido cítrico) e da produção de metabólitos (ácido lático, ácido acético e etanol) ocorreu nos diferentes processos fermentativos. Entretanto, diferenças nas concentrações de alguns metabólitos produzidos na polpa, bem como suas difusões para o cotilédone, foram observadas. Como exemplo, menores concentrações de etanol e de ácido acético, bem como elevadas concentrações de ácido cítrico e ácido lático, foram observadas na micro-fermentação em recipiente de polipropileno, quando a mesma foi comparada com a fermentação em tanque de aço inoxidável com capacidade para 10 Kg de amêndoas de cacau. Além disso, transformação de carboidratos em etanol, e depois de etanol em ácido acético, foi mais eficientemente alcançada no biorreator de tanque de aço inoxidável com capacidade para 40 Kg de amêndoas de cacau, quando o mesmo foi comparado com as fermentações realizadas em caixas de madeira. Com novos

aperfeiçoamentos, os modelos de tanques de aço inoxidável podem ser úteis na elaboração de novos biorreatores para a otimização do processo de fermentação de cacau. O micro-fermentador de polipropileno pode fornecer uma analogia adequada em comparação a fermentações em grande escala, pelo menos no que diz respeito à ecologia microbiana. Entretanto, mais estudos devem ser realizados para avaliar o efeito da excessiva produção de alguns metabólitos na polpa de cacau, e suas difusões para o cotilédone, sobre a qualidade tecnológica e sensorial dos tanques de aço inoxidável e do recipiente de polipropileno. Em uma segunda etapa deste estudo, o potencial biotecnológico de leveduras, bactérias do ácido lático e bactérias do ácido acético foi avaliado, abrangendo capacidade de crescimento sob condições de e*stresse* tolerância e parâmetros cinéticos de crescimento em meio sintético de polpa de cacau. Em geral, as linhagens de *S. cerevisiae* UFLA CHYC7.04 (eficiente produtora de etanol e resistente à altas concentrações de etanol, bem como a condições de baixa acidez e altas temperaturas), *L. fermentum* UFLA CHBE8.12 (fermentadora de ácido cítrico, produtora de ácido lático, e resistente à altas concentrações de ácido lático e etanol, bem como a condições de baixa acidez e altas temperaturas) e *A. tropicalis* UFLA CHBE16.01 (etanol e ácido láctico oxidantes, produtora de ácido acético, e resistente a altas concentrações de ácido acético e etanol, bem como a condições de baixa acidez e altas temperaturas) foram selecionados para o coquetel microbiano que pode levar a um melhor controle do processo fermentativo de cacau. Além disso, os resultados referentes a cinco linhagens de leveduras não-*Saccharomyces* (*Debaromyces etchelslsii* UFLA CYB5.56, *Candida humilis* UFLA CYD14.32, *Pichiak Kluyeri* UFLA CYC2.02 e *Issatchenkia orientalis* UFLA CYB6.02 e UFLA CYC6.02) indicou que as mesmas podem ser testadas em associação com a levedura *S. cerevisiae* em estudos futuros.

Palavras-chave: Chocolate. Fermentação de cacau. Bactérias do ácido lático. Bactérias do ácido acético. Leveduras.

ABSTRACT

Spontaneous cocoa bean fermentations carried out in 500-g plastic containers, 10-Kg stainless steel tank, 40-kg stainless steel tank, 40-kg wooden box and 600-kg wooden box (Anex 1) were studied using a multiphasic approach that entailed culture-dependent and -independent microbiological analyses of fermenting cocoa pulp-bean samples and target metabolite analyses of both cocoa pulp and cotyledons. Both microbiological approaches revealed that the dominant species of major physiological roles were the same in all the fermentation processes. These species consisted of *Saccharomyces cerevisiae* and *Hanseniaspora* sp. in the yeast group; *Lactobacillus fermentum* and *L. plantarum* in the lactic acid bacteria (LAB) group; and *Acetobacter tropicalis* belonging to the acetic acid bacteria (AAB) group. A range of other microbial groups, such as *Bacillus*, *Staphylococcus*, *Enterobacteria* and non-*Saccharomyces* yeast were detected during the different fermentation processes, but the highest diversity was observed in the case of box fermentations. Additionally, a potentially novel AAB belonging to the genus *Asaia* was isolated during fermentation in 40-kg wooden box. Cluster analysis of the rRNA genes-PCR-DGGE profiles revealed a complex picture on the DGGE gels of the box samples, indicating that bacterial and yeast ecology were fermentation-specific processes (wooden boxes *vs.* stainless steel tanks). Generally, a similar course of the substrate consumption (sucrose, glucose, fructose, and citric acid) and metabolite production kinetics (ethanol, lactic and acetic acid) occurred both in the pulp and beans in all fermentations processes. However, differences in the concentrations of some metabolites produced in the pulp were observed for different fermentation processes. For instance, lowest concentration of ethanol and acetic acid, and higher of citric acid and lactic acid, were recovered during 500-g plastic containers fermentations when it was compared to 10-Kg stainless steel tank. In addition, the carbohydrate-ethanol-acetic fermentation was achieved with greater efficiency in 40-kg stainless steel tank, when it was compared to 40-kg wooden box and 600-kg wooden box. With further refinements, the stainless steel tank models may be useful in designing novel bioreactors for the optimisation of cocoa fermentation with starter cultures. In addtion, the plastic containers may provided a suitable model system analogy to larger-scale fermentations, at least with regards to microbial ecology. However, further studies should be evaluate to assess the impact of excessive production of the some metabolites in the pulp on the technological and sensorial quality of the stainless steel tank and plastic containers. In a second step of this study, the technological potential of yeast, LAB and AAB isolates were evaluated through a poly-phasic selection study, encompassing stress-tolerant growth ability and fermentation kinetic parameters in cocoa pulp simulation media. Overall, the

strains of *S. cerevisiae* UFLA CHYC7.04 (ethanol producer, and acid-, heat-, and ethanol-tolerant), *L. fermentum* UFLA CHBE8.12 (citric acid fermenting, acid lactic producer, and heat-, acid-, lactic acid-, and ethanol-tolerant) and *A. tropicalis* UFLA CHBE16.01 (ethanol and lactic acid oxidizing, acetic acid producer, and acid-, heat-, acetic acid-, and ethanol-tolerant) were selected as one mixed-strain starter cultures that should lead to better controlled and more reliable cocoa bean fermentation processes. In addition, the results regarding the five non-*Saccharomyces* strains (*Debaromyces etchelslsii* UFLA CYB5.56, *Candida humilis* UFLA CYD14.32, *Pichia Kluyeri* UFLA CYC2.02 and *Issatchenkia orientalis* UFLA CYB6.02 and UFLA CYC6.02) indicated that these can be tested in association with *S. cerevisiae* in futures studies into cocoa starter cultures.

Keywords: Chocolate. Cocoa fermentation. Lactic acid bacteria. Acetic acid bacteria. Yeast.

SUMÁRIO

PRIMEIRA PARTE

ASPECTS THAT CONTRIBUTE TO THE QUALITY OF COMMERCIAL COCOA BEANS, WITH SPECIAL REFERENCE TO THE IMPACT OF FERMENTATION

1 INTRODUÇÃO

Durante a transformação tecnológica de sementes de cacau em chocolate, quatro etapas são essenciais para o desenvolvimento do sabor típico de chocolate: fermentação, secagem, torração e conchagem (AFOAKWA et al., 2008). As sementes de cacau são também denominadas amêndoas de cacau - denominação reservada às sementes que já perderam a capacidade de germinação, o que ocorre durante a fermentação. A semente é composta de casca, membrana prateada ou endosperma, dois cotilédones e eixo embrionário (FORSYTH; QUESNEL, 1957; KIM; KEENEY, 1984). A matéria prima para a produção do chocolate é o cotilédone, que após passar pelos processos de fermentação, secagem, quebra e separação da casca recebe o nome de *nibs*. O processo fermentativo ocorre por ação de uma sucessão de diferentes grupos microbianos na polpa envoltória das sementes, que é rica em carboidratos (principalmente sacarose, frutose e glicose) e ácido orgâncios (principalmente ácido cítrico). Os álccois (principalmente etanol) e os ácidos orgânicos (principalmente ácido lático e ácido acético) produzidos pelo metabolismo microbiano, associados à alta temperatura da massa de cacau em fermentação, são fatores que contribuem para a morte do gérmen e desencadeiam uma série de alterações químicas, essenciais para o desenvolvimento do "flavor" do chocolate (ARDHANA; FLEET, 2003; BIEHL et al., 1993; LEHRIAN; PATTERSON,

1983; QUESNEL, 1958; SCHWAN, 1998; SCHWAN; ROSE; BOARD, 1995; SCHWAN; WHEALS, 2004).

Leveduras, bactérias do ácido lático, bactérias do ácido acético, e em alguns casos, bactérias formadoras de esporos, são os principais grupos microbianos presentes durante o processo de fermentação. O tempo requerido para a fermentação é variável, geralmente 3 a 7 dias (SCHWAN; WHEALS, 2004). Inicialmente, o alto conteúdo de açúcares e baixo pH, associados com a baixa tensão de oxigênio, favorecem o desenvolvimento de leveduras que dominam o processo fermentativo até geralmente 48 horas a partir de seu início. As leveduras hidrolisam e fermentam os açúcares produzindo etanol e CO_2. As condições ocasionadas pelo crescimento das leveduras estimulam o desenvolvimento de bactérias do ácido lático (microaerofílicas tolerantes à acidez). Estas bactérias também fermentam os açúcares e utilizam o ácido cítrico presente na polpa, apresentando crescimento máximo em torno de 16 à 48 horas. Com a desintegração da polpa que envolve as amêndoas e o revolvimento da massa em fermentação, a aeração se torna maior, favorecendo o crescimento das bactérias do ácido acético. Tais bactérias promovem a oxidação do etanol - produzidos inicialmente pelas leveduras a ácido acético, em uma reação extremamente exotérmica, elevando a temperatura da massa fermentativa para patamares de 45 a 50ºC (ARDHANA; FLEET, 2003; CAMU et al., 2007; SCHWAN; ROSE; BOARD, 1995; SCHWAN; WHEALS, 2004; THOMPSON; MILLER; LOPEZ, 2007; VUYST et al., 2010).

A diversidade de leveduras, bactérias do ácido lático e bactérias do ácido acético é decorrente das condições ambientais resultantes do proprio desenvolvimento de cada um destes grupos no substrato de fermentação (a polpa de cacau) (SCHWAN; ROSE; BOARD, 1995). As mudanças de pH, na temperatura, nas concentração de açúcares e nos produtos da fermentação, exercem uma pressão de seleção sobre determinados grupos e espécies,

favorecendo linhagens que são melhores adaptadas a este ambiente. A avaliação do crescimento de bactérias e leveduras sob estas condições de bruscas mudanças ambientais (condição de *estresse*) pode fornecer informações úteis sobre suas capacidades de realizarem o processo fermentativo. A adaptação de células microbianas não é possível se as mesmas não forem capazes de modificar sua própria fisiologia ou comportamento em resposta a *estresses* ambientais (GASCH; WERNER-WASHBURNE, 2002).

Processadoras de cacau requerem um fornecimento constante de amêndoas de cacau, que deve obedecer a critérios de qualidade (SCHWAN, 1998). Entretanto, o processo fermentativo de cacau é ainda realizado de forma rudimentar, principalmente sob folhas de bananeira ou em caixas de madeira. A inconstância ou a falta de padronização destes processos geram amêndoas de qualidades variadas (LAGUNES-GÁLVEZ et al., 2007). O conceito de industrialização de processos fermentativos tradicionais para melhor desempenho e eficiência não é novo. Por exemplo: vinho, cerveja, queijo e iogurte eram anteriormente feitos usando processos tradicionais (WOOD, 1998). Agora, essas fermentações são desenvolvidas em processos altamente eficientes e controlados, em modernos fermentadores e com o uso de culturas iniciadoras (STEINKRAUS, 2004). A fermentação de cacau ainda está em seu primeiro passo. Embora experimentos preliminares de aplicação de culturas iniciadoras mostraram resultados satisfatórios (DZOGBEFIA; BUAMAH; OLDHAM, 1999; LEAL JÚNIOR et al., 2008; LEFEBER et al., 2011; SAMAH; PTIH; SELAMAT, 1992; SCHWAN, 1998), elas não têm sido implementadas nas condições de campo. Uma limitação à introdução de culturas iniciadoras no processo de fermentação de cacau é que as formas atuais como este processo é conduzido facilitam a contaminação por microbiota natural. A demanda por práticas mais higiênicas aumentou o apelo do uso de tanque de aço inoxidável em diferentes bioprocessos (por exemplo, como para a produção de vinho e

cerveja). Como a fermentação de cacau envolve uma complexa sucessão microbiológica e bioquímica, o uso de tanque inoxidável é uma perspectiva desafiadora, e exaustivos estudos devem ser realizados antes de sua implementação em condições de campo (SCHWAN,1998).

O primeiro objetivo deste estudo foi investigar as mudanças físico-químicas microbiológicas durante fermentações espontâneas de cacau realizadas em cinco diferentes biorreatores, denominados: (1) micro-reator de polipropileno com capacidade para 400gr de amêndoas de cacau, (2) reator de aço inoxidável com capacidade para 10Kg de amêndoas de cacau, (3) reator de aço inoxidável com capacidade para 40Kg de amêndoas de cacau, (4) reator de madeira com capacidade para 40Kg de amêndoas de cacau e (5) reator de madeira com capacidade para 1000Kg de amêndoas de cacau. As análises microbiológicas foram realizadas através de métodos dependentes e independentes de cultivo, que foram aplicados para avaliar a dinâmica e estrutura de comunidades de bactérias e leveduras durante os diferentes processos fermentativos. Em paralelo, uma relação entre os substratos e metabólitos presentes na polpa e no cotilédone foi estabelecida com os microrganismos identificados.

Em uma segunda etapa deste estudo, o potencial biotecnológico de leveduras, bactérias do ácido lático e bactérias do ácido acético foi avaliado através de um estudo de triagem polifásica, abrangendo capacidade de crescimento sob condições de e*stresse* tolerância e parâmetros cinéticos de crescimento em meio sintético de polpa de cacau.

2 LITERATURE REVIEW

2.1 Characteristics of the cocoa pod and cocoa beans

Commercial cocoa is derived from the seeds (beans) of the ripe fruit (pods) of the plant *Theobroma cacao* (Fig. 1).

Figure 1 (a) Cocoa plantation of Bahia State, Brazil; (b) Cocoa fruit pod growing directly out of the bark on a trunk of a cocoa tree; (c) Opened cocoa pod with beans embedded in a mucilaginous pulp. Source: Anabel Mascarenhas, private pictures, reprinted with permission

The Swedish botanist, Carolus Linnaeus, classified it in the 1700s, 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). The natural habitat of the cocoa tree 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). Their popularity would later lead to the spread of cocoa tree rootstocks to other European colonies bearing their growth (LASS, 1999; THOMPSON; MILLER; LOPEZ, 2007).

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). The seeds of cocoa pods basically consist of two parts: an outer part comprising the skin or testa (seed coat or shell), surrounding the bean, and an inner part comprising the embryo (germ) and two cotyledons contained within the testa and uniformed at a small embryonic axis (Fig. 2a).

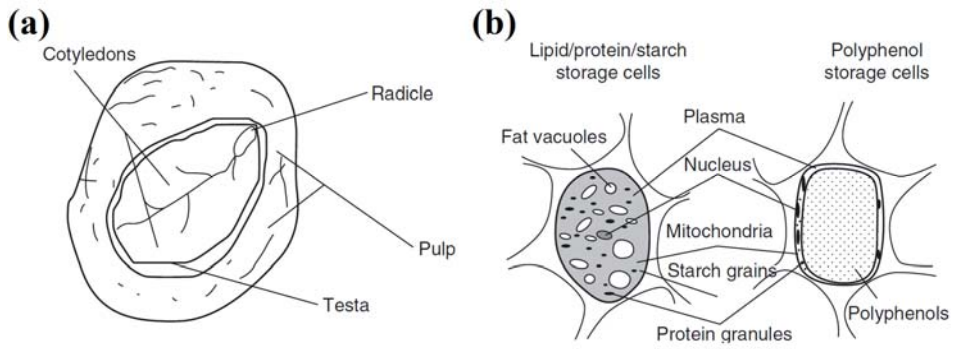

Figure 2 (a) Cocoa bean. (b) Cocoa bean storage cells. Source: Vuyst et al. (2010)

The cotyledons comprise of two types of cells, namely white lipid/protein/ starch-containing cells and purple polyphenol-containing cells, with parenchyma plasma forming a grid between the two types of cells (Fig. 2b). The cotyledons are referred to as the nibs in the cured beans. In the cotyledons, the fat is the most important component, representing about half the weight of the dry seed (LIMA et al., 2011). 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. The total amount of polyphenols in dried fresh cocoa beans may vary between 12 and 20% (w/w) and these are responsible for its high astringency, contributing to their bitterness as well (FORSYTH; QUESNEL, 1957; KIM; 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; KEENEY, 1984; WOLLGAST; ANKLAM, 2000). Within the catechins group, the (-)-epicatechin is the predominant fraction amounting to 35% of the total polyphenol content (KIM; KEENEY, 1984).

Currently, cocoa is produced in South America (Ecuador, Colombia, Brazil, Venezuela and Guiana), Central America, the West Indies, West Africa (Nigeria and Ghana), Ceylon, Autralia, Indonseia and Java. A large number of

cocoa tree varieties exist, which are grouped according to morphological (fruits and seeds) and geographical (genetic origin) characteristics. This large cocoa variety is due to genetic factors and human intervention as agricultural practices (CARR; DAVIS; DOUGAN, 1979). In principle, all cocoa varieties originate from the Criollo and Forastero varieties, which are considered indigenous to the Upper Amazon basin (Amazonian region, Venezuela). Of the Forastero varieties, the melon - shaped Amelonado (Lower Amazon) is the most widely grown (Brazil and West Africa). Trinitario - a third cocoa group often referred to in the literature - is considered a hybrid between Criollo and Forastero (most often Lower Amazon) varieties, which is particularly suitable for cultivation (CARR; DAVIS; DOUGAN, 1979). The characters of the cocoa genotype have often been used in classifying cocoa. Today, Criollo, Forastero and Trinitario varieties represent approximately 1%, 94%, and 5% of the world production of cocoa. The Criollo and Trinitattynrio types are the source of the original ‘fine’ cocoa, which has almost disappeared from the market because of its susceptibility to disease and lower productivity (THOMPSON; MILLER; LOPEZ, 2001). Forastero is much less prone to various diseases and overcomes several other weaknesses (yield, bearing), giving a more stable crop from year to year. Thus, the Forastero is preferred by farmers throughout the world, yielding bulk cocoa (for milk and dark chocolates and other confectionary products) (THOMPSON; MILLER; LOPEZ, 2001).

2.2 The on-farm cocoa processing

Raw cocoa seeds have an astringent, unpleasant and bitter flavor and must be processed after harvest before they can be converted into good - tasting and flavorsome chocolate. Postharvest processing of cocoa beans involves an

on-farm processing, known as curing, which determine the quality of the final cocoa beans (Fig. 3).

Figure 3 Stages that contribute to the quality of commercial cocoa beans during on-farm processing. Source: Anabel Mascarenhas, private pictures, reprinted with permission

Curing involves fermentation followed by drying of the cocoa beans (BIEHL et al., 1993; LEHRIAN; PATTERSON, 1983). Cocoa pods are indehiscent and do not abscise. So they must be manually removed from the trees. A range of harvesting is employed in different countries, but usually pods are removed using a machete, hooked knives or secateurs. Although the possibility of mechanized pod harvesting has been explored, it is made extremely difficult by the strong attachment of the pod to the tree, and the irregular fruiting on trunk and branches (WOOD; LASS, 1985).

After harvesting, the pod-breaking operation begins. The pods are broken open with machetes or cutlasses (although a wooden club should be preferred), on site (at the farm in or at the edge of the plantation), or at a processing plant (fermentary) (NIELSEN et al., 2007). The husk and placenta of the pod are discarded and the wet beans are scooped out of the broken pods by hand, which is followed by an immediate and spontaneous fermentation, preferably within 24 h. Mechanical pod breaking and bean extraction are used on some large estates in West-African countries, Mexico and Brazil. Surveys by Baker, Tomlins and Gray (1994) and Carr, Davis e Dougan (1979) found that many farms will store harvested, unopened, pods for few days to up two weeks prior breaking. In some cases this was to allow a small producer time enough to gather sufficient pods for fermentation over several weeks of harvest. A more commonly cited reason for pod storage was that it improved initiation of fermentation, and gave better quality beans (BIEHL et al., 1989; TOMLINS; BAKER; DAPLYN, 1993). The same surveys also revealed significant variation in individual practices between, and often within, different countries (BAKER; TOMLINS; GRAY, 1994; CARR; DAVIS; DOUGAN, 1979; TOMLINS; BAKER; DAPLYN, 1993). Studies have confirmed that fermentation may be affected by ripeness (ARDHANA; FLEET, 2003; SCHWAN; WHEALS, 2004; WOOD; LASS, 1985). Therefore, a lack of control at the harvest, storage and

breaking stages may be clearly seen to contribute to the variable quality of cocoa beans produced worldwide (DIRCKS, 2009).

The fruits are opened and the seeds, embedded in the whole pulp or roughly separated from it, are allowed to ferment. Fermentation occurs in heaps, boxes, baskets, or on trays; the process lasts 3-7 days, and the temperature is not allowed to rise above 50°C. After, the fermented cocoa beans are subjected to drying process, which reduces the moisture content to less than 8% (AFOAKWA et al., 2008; HASHIM et al., 1998). The drying of cocoa is carried out by sun or artificial methods. Drying reduces levels of acidity and the astringency in cocoa, decreasing volatile compounds content (AFOAKWA et al., 2009). However, different authors reported that those volatile and non-volatile compounds were maintained after the fermentation, drying and roasting processes (BAILEY et al., 1962; FRAUENDORFER; SCHIEBERLE, 2006).

The origin of the 'curing' process has been lost in antiquity, but it was believed at one time that fermentation was conducted simply to aid in removing the mucilaginous pulp surrounding the seed so as to facilitate drying and storage (THOMPSON; MILLER; LOPEZ, 2001). This fact is true, but the main reason for cocoa fermentation is to induce biochemical transformations within the bean, that lead to formation of the color, aroma, and flavor precursors of "chocolate" (SCHWAN; ROSE; BOARD, 1995).

2.3 Fermentation methods

Various cocoa bean fermentation systems have been developed worldwide. The actual methods of fermentation vary in different cocoa-producing countries and regions, and even from one cocoa grower to another within a region or country (SCHWAN; WHEALS, 2004). Fermentations may be performed in heaps (*e.g.*, Ghana and Ivory Coast), boxes (*e.g.*, Brazil and

Malaysia), baskets (*e.g.*, Nigeria and Ghana), trays (*e.g.*, Ghana), sacks (*e.g.*, Ecuador) and platforms fermentation (*e.g.*, Ecuador).

The methods mainly used are the box and heap methods. Approximately one-half of the cocoa is fermented in some kind of wooden box, for many years and worldwide, and the remaining half is fermented by using heaps or other traditional methods that are slowly disappearing (HOWAT; POWELL; WOOD, 1957; THOMPSON; MILLER; LOPEZ, 2007; WOOD; LASS, 1985). The box fermentation system is widely used in *e.g.,* Brazil, Indonesia and Malaysia (WOOD; LASS, 1985). As illustrated in Fig. 3, a number of boxes are arranged, each having a capacity of generally 1,000 kg cocoa beans, which are turned to another box after 1-2 days, and finally after 2-3 days of further fermentation into the next box. The box fermentation systems facilitates turning, as the movement of the beans is aided by gravity and cocoa of good quality can be produced (SCHWAN et al., 1990; THOMPSON; MILLER; LOPEZ, 2007; WOOD; LASS, 1985). However, a problem occasionally encountered during box fermentation is uneven temperature and oxygen distribution through the fermenting mass with the corners and areas around aeration holes being better aerated and occasionally colder and less acidic than the rest of the fermenting mass enabling moulds to grow abundantly (THOMPSON; MILLER; LOPEZ, 2007).

Ghanaian farmers traditionally ferment the cocoa using the heap method in the field among the trees close to where the pods were harvested (TOMLINS; BAKER; DAPLYN, 1993). Heap fermentation is carried out on banana leaves on the ground, and the heap of cocoa beans is covered with banana leaves and sticks. It is a cheap method that produces well fermented beans, when it is done properly. Among small holders in Ghana, heap fermentation is a popular method. It does not require permanent equipment, and therefore, it suits well to

family holders with small production. The duration of the heap process fermentation is around from 4 to 7 days (BAKER; TOMLINS; GRAY, 1994).

Differences in the chemistry of the heap and box methods have been noted (CARR; DAVIS; DOUGAN, 1979). Carr, Davis and Dougan (1979) found that the bottom of the box was more aerobic than the bottom of the heap such that, in the former, the sugars were consumed faster with a higher maximum ethanol and acetic acid concentrations. It has been noted (ANON, 1981) that physical (temperature) and chemical (lactic and acetic acids, pH and oxygen) profiles of the heap were more uniform than the box, the profiles for both methods only being similar at the centre location. In both investigations, the sensory characteristics of the finished cocoa from either heap or box fermentations were similar. Moreover, the chocolate was inferior and more acidic than standard commercial Ghana cocoa (LIMA et al., 2011).

As turning of the fermenting cocoa bean mass seems to be a determining factor for good fermentation practice, fermentations in appropriate bioreactors have been proposed (SCHWAN; WHEALS, 2004). Although mechanical turning is not a realistic alternative and forced aeration requires careful control, drum fermentors and stainless steel vessels have been tried. However, as fermentation of solid material is difficult to control, in particular with respect to homogeneity and gradient formation, and physical and chemical parameters, new designs of fermentors are needed to have some of these parameters under control between certain limits (for instance through turning and cooling) and to speed up and control the cocoa bean fermentation process (SCHWAN; WHEALS, 2004).

Several attempts have been previously used for rapid and effective means of curing small quantities of cacao beans (less than 20Kg) in order to research aspects of cocoa fermentations, such as the effects of cultivar, pod storage, pulp removal and acid production (CLAPPERTON et al., 1994;

QUESNEL, 1958; QUESNEL; LOPEZ, 1975). In most of these studies, it was noted that the temperature of cocoa-bean mass did not increase normally, often only reaching 35-37°C. Correct bean temperature was maintained during small scale fermentation by better insulation of vessel or conducting the fermentations in an incubator (QUESNEL; LOPEZ, 1975).

2.4 Microbial succession and biochemical transformations: The spontaneous three–phase cocoa bean fermentation process

Cocoa pulp is composed of 82% – 87% water, 10% – 15% sugars, 1% – 5% pectin, 1% – 3% citric acid, 0.1% – 0.4% other non-volatile acids (*e.g.*, malic acid), 0.5% – 0.7% proteins and 8% – 10% minerals and oligoelements (ARDHANA; FLEET, 2003; LEHRIAN; PATTERSON, 1983; QUESNEL, 1957; SCHWAN, 1998; SCHWAN; ROSE; BOARD, 1995). Of the sugars present, about 60% is sucrose and 39% is a mixture of glucose and fructose. The concentration of sucrose, glucose and fructose is a function of cultivar and fruit age; unripe pods contain a higher proportion of sucrose, and ripe pods contain mainly fructose and glucose (LEHRIAN; PATTERSON, 1983; THOMPSON; MILLER; LOPEZ, 2007). During fermentation, microbial activity leads to the formation of a range of metabolic end-products. The former includes pectin depolymerisation by yeasts. The latter encompasses anaerobic yeast fermentation of sugars to ethanol, microaerophilic fermentation of sugars and citric acid to lactic acid, acetic acid and mannitol by lactic acid bacteria, and aerobic exothermic bioconversion of ethanol into acetic acid by acetic acid bacteria. These microbial activities result in the death of the bean due to diffusion of mainly ethanol and acetic acid through the testa into the cotyledons, and the creation of an environment (*i.e.,* a decrease of internal pH from 6.5 to 4.8, an increased of cocoa mass temperature up to 50 °C, and a damaged internal

cocoa bean structure) for development of flavor precursors and pigment degradation by endogenous enzymes, such as invertase, glycosidases, proteases and polyphenol oxidase (BIEHL et al., 1989). As a result of these biochemical reactions in the cocoa beans an additional number of flavour precursors are formed, in particular reducing sugars, peptides and amino acids, that are further modified through Maillard reactions during roasting of well-fermented, dried cocoa beans (AFOAKWA et al., 2008; THOMPSON; MILLER; LOPEZ, 2007). For instance, storage proteins are hydrolysed by an endogenous aspartic endoprotease and carboxypeptidase with different pH and temperature, releasing hydrophilic oligopeptides and free hydrophobic amino acids. The sugars come from sucrose and its hydrolysis products, glucose and fructose, by both cotyledon and pulp invertase activity, in addition to being released from glycosides (SCHWAN; WHEALS, 2004).

The importance of cocoa bean fermentation in contributing to chocolate quality has been recognised for over 100 years, and numerous studies have been conducted in different countries to determine the microbial species associated with this process (CAMU et al., 2007; GARCIA-ARMISEN et al., 2010; PAPALEXANDRATOU et al., 2011; SCHWAN et al., 1986; SCHWAN; ROSE; BOARD, 1995; SCHWAN; WHEALS, 2004). A microbial succession of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) takes place during fermentation (Fig. 4a).

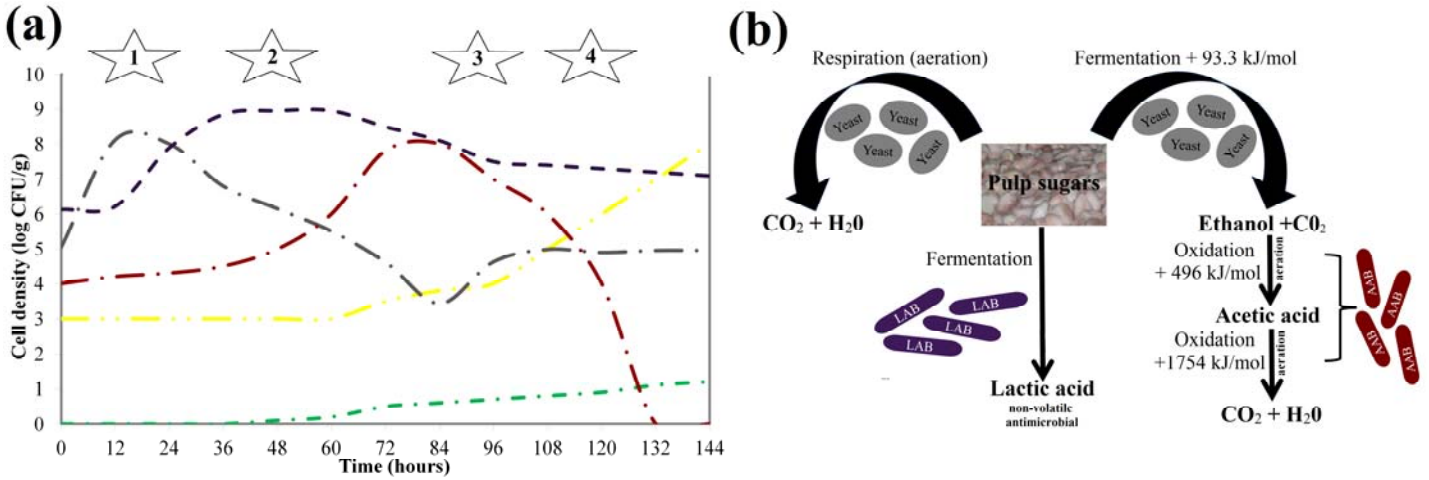

Figure 4 (a) Schematic of microbial succession during cocoa bean fermentations. Adapted from Schwan and Wheals (2004). LAB (purple); AAB (red); yeast (gray); spore-forming bacteria (yellow); and filamentous fungi (green). The numbers inside the stars indicate maximal concentrations of ethanol (1), lactic acid (2), and acetic acid (3), and the maximum temperature (4). (b) Microbial activities during cocoa bean fermentation

Although overlap often occurs, three main fermentation phases can be considered consecutively: phase (1) or the anaerobic growth of yeasts; phase (2) or the growth of LAB; and phase (3) or the growth of AAB (ARDHANA; FLEET, 2003; CAMU et al., 2007; SCHWAN; ROSE; BOARD, 1995; SCHWAN; WHEALS, 2004; THOMPSON; MILLER; LOPEZ, 2007; VUYST et al., 2010). During the first 24 – 48 h of fermentation (phase 1), yeasts convert sugars (sucrose, glucose and/or fructose) into alcohol (ethanol) under conditions of high carbohydrate concentrations, limited oxygen availability (due to tight packing of the beans) and a pH below 4.0 (due to the high citric acid content in cocoa pulp) (Fig. 4b). They grow from an initial $10^2 – 10^7$ to a maximum $10^7 – 10^9$ colony-forming unit (CFU)/g of cocoa pulp. Thus, the yeast conduct an alcoholic fermentation (Fig. 4b) and involve the sequential growth of *Kloeckera* and its teleomorphic form *Hanseniaspora*, *Saccharomyces*, *Candida*, *Pichia* and *Kluyveromyces* species. As pulp is drained away, ethanol formation proceeds and both temperature and pH increase, which creates ideal conditions for the growth of LAB (phase 2) and latter of AAB (phase 3). The LAB also ferment pulp sugars (Fig. 4b) and involve the growth of *Lactobacillus*, *Leuconostoc* and *Lactococcus* species. From 24 to 112 h of fermentation, aerobic AAB, which occur from the very early stages of fermentation and hence survive the initial steps of this process, persist until the end when the conditions for AAB growth are optimal. Furthermore, turning of large heaps and moving of the beans during box fermentation favor AAB growth and increase the maximum fermentation temperature (CAMU et al., 2008a; CARR; DAVIS; DOUGAN, 1979; NIELSEN et al., 2007).

Figure 5 shows the association of the cocoa microbiota through scanning electron microscopy, performed during a process of spontaneous fermentation in wooden boxes (unpublished data). The beans were fixed in Karnovisk's fixative solution and post-fixed in 10 g/l osmium tetroxide, according to the procedures

performed by Magalhães et al. (2010). After, samples were critical-point dried and coated with gold using a Bal-tec SDC 050 (Capovani Brothers Inc. Scotia, NY, USA). The preparations were observed using a scanning electron microscope (LEO EVO 040) (A Carl Zeiss SMT AG Company, Germany). It showed the succession of bacteria and yeast through the fermentation course. The microbiota in the early stages of fermentation (24 h) was dominated by bacilli (short and curved long) cells growing in association with lemon-shaped yeast cells (Fig. 5c and d). In the middle of fermentation (60 h), although yeast cells were still visible, bacilli cells become dominates (Fig. 5e). Finally, after 132 hours of fermentation yeast cells were no longer visible, and short bacilli become the only visible cells (Fig. 5f).

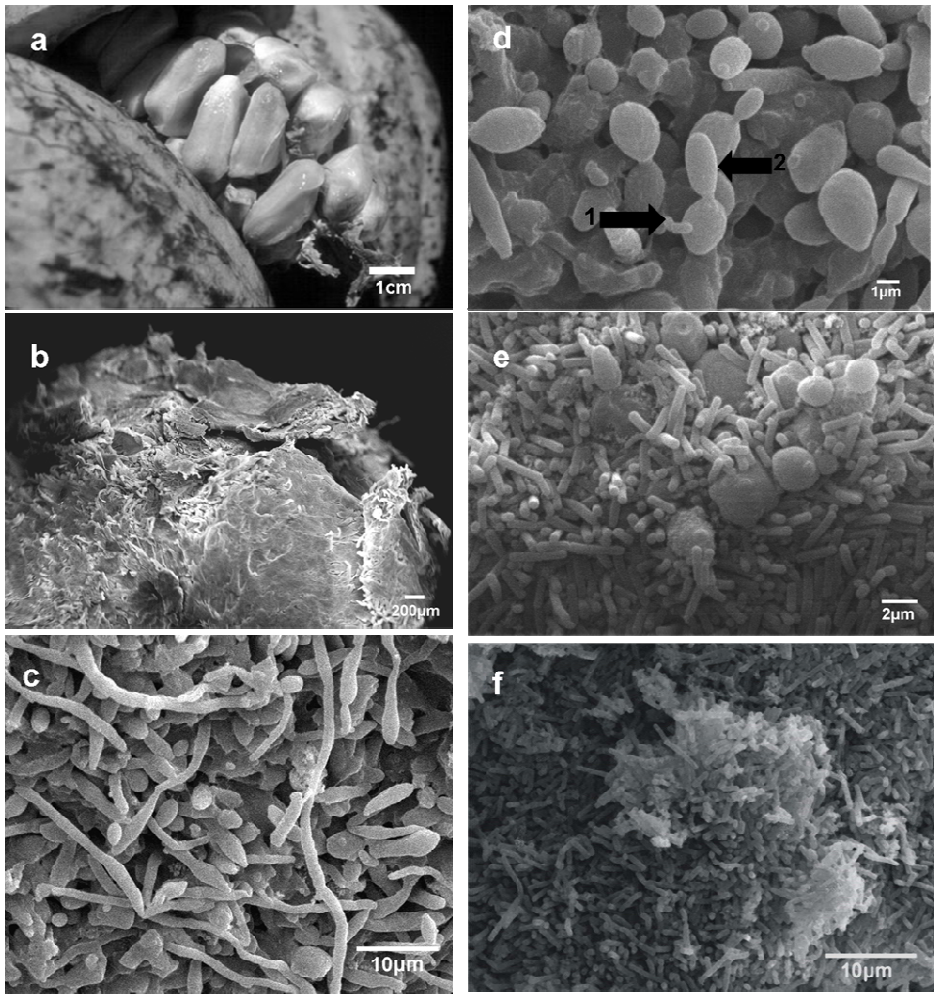

Figure 5 Analysis by Scanning Electron Microscopy of cocoa beans during a spontaneous fermentation in wooden boxes (unpublished data). (a) Cocoa beans viewed with the naked eye; (b) external surface of cocoa beans; (c) and (d) cocoa beans after 24 h of fermentation; (e) cocoa beans after 60 h of fermentation; and (f) cocoa beans after 132 h of fermentation.

Species of *Bacillus* develop when the pH of the bean mass becomes less acid and its temperature increases to 40–50 ºC. *Bacillus* species were isolated during the first three days of fermentation with populations around 10^3 CFU/g of cocoa pulp, but their numbers remain virtually unchanged. From the third day of fermentation the number of spore-forming bacteria begins to increase to such an extent that they form over 80% of the microbiota (SCHWAN; ROSE; BOARD, 1995). This phase in the succession coincides with increases in oxygen tension, temperature and pH of the fermenting mass. Filamentous fungi are also found in small numbers throughout the fermentation, most commonly in the aerated and cooler, superficial areas of the fermenting mass.

At the end of the fermentation the beans are usually transferred to platforms and sun-dried. During this process, (*e.g.,* commencing after 156 hours), there is a sharp decrease in the total microbial population. During sun drying cocoa beans are often humidified to help the workers remove the rest of the mucilage with their feet but eventually only microorganisms which are able to form spores, bacilli and filamentous fungi, can survive (ARDHANA; FLEET, 2003; SCHWAN; WHEALS, 2004; THOMPSON; MILLER; LOPEZ, 2001).

During the last decade, knowledge about the spontaneous cocoa bean fermentation process has increased (ARDHANA; FLEET, 2003; CAMU et al., 2007, 2008a, 2008b; GARCIA-ARMISEN et al., 2010; JESPERSEN et al., 2005; KOSTINEK et al., 2008; NIELSEN et al., 2005, 2007). Some of these and all older studies have typically involved cultivation of microorganisms, colonies being counted to obtain a quantitative picture of the populations, followed by their isolation and identification by reference to phenotypic and genotypic characteristics (ARDHANA; FLEET, 2003). Such conventional isolation procedures inevitably require some amount of prior knowledge about the types of microorganisms that may be present in the population to be studied. Selective media and culture conditions used in these studies will enhance recovery of

some species, but others may be poorly recovered and some may not be recovered at all on standard laboratory media (GIRAFFA; NEVIANI, 2001). In recent years, researchers have started to use culture-independent techniques to identify possibly uncultivable but potentially important players in the cocoa bean fermentation process (CAMU et al., 2007, 2008a, 2008b; GARCIA-ARMISEN et al., 2010; NIELSEN et al., 2005, 2007). These methods are based on nucleic acids (*e.g.,* 16S or 18S rRNA genes in the case of bacteria and yeast, respectively) to obtain both a qualitative and a semi quantitative picture of a microbial community without the need to isolate its single components. Access to rRNA gene databases and comparative gene sequence analysis software has simplified the identification of microorganisms (PACE et al., 1986). A widely used method, denaturing gradient gel electrophoresis (DGGE), has shown to be of great potential for the study of microbial population dynamics in food (ERCOLINI, 2004; ERCOLINI et al., 2006; FONTANA; COCCONCELLI; VIGNOLO, 2006; JANY; BARBIER, 2008). Polymerase chain reaction (PCR)-DGGE analysis is based on the separation of PCR amplified rDNA genes fragments in a gradient of denaturing agents, providing a 'genetic fingerprint' that is representative of the bacterial population in a sample. This technique has also been used as part of a polyphasic analysis of the bacteria and yeast populations associated with cocoa bean fermentation (CAMU et al., 2008b; GARCIA-ARMISEN et al., 2010; NIELSEN et al., 2005, 2007). These techniques led to a number of discoveries, including the description of several new species.

2.5 Diversity and Roles of Yeast

In the early 20th century, cocoa fermentation was attributed primarily to the activity of yeasts. Nicholson (1913) and Smith (1913) were the first taxonomic study of cocoa yeast, which identified *Saccharomyces theobromae* (now classified as *S. cerevisiae*), *S. ellipsoidens* and *S. apiculatus* (now classified as *Kloeckera* apiculata) as the major yeasts found. These early studies also suggested that it was these yeasts that converted the pulp sugars into ethanol and CO_2 during early stages of fermentation. More recently, there has been a focus on the application of non-cultural PCR-DGGE method, to examine the diversity of yeast specie present throughout fermentation (NIELSEN et al., 2005; PAPALEXANDRATOU; VUYST, 2011).

Twelve relevant taxonomic studies of cocoa yeast, dated in the last 40 years, from Australia, Trinidad, Brazil, Ivory Coast, Dominican Republic, Malaysia and Ghana are reported in Table 1.

Table 1 Yeast species reported in cocoa beans fermentation

Metabolism	A	B1	T1	T2	IV	D	M	G1	G2	G3	G4	G5
Fermentative												
S. cerevisiae	+	+	+	+	+		+	+	+	+	+	+
K. ohmeri												+
C. bombi		+										
C. diversa								+	+	+	+	
C.glabrata (T)/ *Cr. glabratus* (B)						+						
C. michaelii									+			
C. pelliculosa		+	+	+		+						
C. quercitrusa(*A*)/ *C. parapsilosis var. querci*(T)	+							+				
C. rugopelliculosa		+										
C. tropicalis			+	+								+
C. zemplinina								+	+	+	+	
C. sake					+							
H. valbyensis						+						
I. occidentalis	+					+				+		
I. orientalis/ C. krusei(A)					+			+	+	+	+	+
K. corticis					+							
K. apiculata/ (A) H. uvarum					+							
K. Africana				+								
K. apis (A)*/ H. guilliermondii*	+	+	+	+		+		+	+	+	+	
H. opuntiae												+
K. javanica				+								
Ky. marxianus		+										
Ky. thermotolerans		+										
L. elongisporus		+										
P. kluyveri										+	+	+
P. pijperi								+			+	
P. mexicana												+
Sz. pombe									+	+	+	
T. delbreuckii (T)*/T. colliculosa*										+		
T. pretoriensis		+										

Table 1, continuation

Weak													
Fermentative													
Z. hellenicus(T)*/ C. hellenica* (A)	+												
C. carpophila													+
K. wickerhamii						+							
C. orthopsilo													+
C. cylindracea								+				+	
C. lactativorus						+							
C. humicola			+	+									
I. hanoiensis										+		+	
C. silvae												+	
C. sorboxylosa								+				+	
P. burtonii	+												
P. anomala	+												
P. fermentans		+											
P. manshurica													+
P. caribbica													+
Sy. crataegensis							+				+		
Respiratory													
C. sorbisivorans	+												
C. ethanolica										+	+		+
C. inconspícua							+						
C. rugosa		+											
C. zeylanoides							+						
P. membranifaciens (A)*/ C. valida (*T*)*	+					+		+		+	+	+	
*R. glutinis(*T*)/ Cy. glutinis (*A*)*					+								
R. rubra			+										
Sy. crataegensis										+			
Sy. ludwigii													+
Y. ipolytica							+						

A: Australia (DIRCKS, 2009); T1: Trinidad (OSTOVAR; KEENEY, 1973) Centeno Estate); T2: Trinidad (OSTOVAR; KEENEY, 1973), San Louis Estate; B1: Brazil (SCHWAN, 1998; SCHWAN; ROSE; BOARD, 1995); IV: Ivory Coast (SANCHEZ et al., 1985); IV: Ivory Coast (PAPALEXANDRATOU et al., 2011); D: Dominican Republic (LAGUNES-GÁLVEZ et al., 2007); M: Malaysia (CARR; DAVIS; DOUGAN, 1979); G1: Ghana- Small heap (NIELSEN et al., 2007); G2: Ghana- Big heap (NIELSEN et al., 2007); G3: Ghana- Big heap (NIELSEN et al., 2007); G4: Ghana- Tray (NIELSEN et al., 2007); G5: Ghana (DANIEL et al., 2009). Abbreviations: S.: *Saccharomyces*; K.: *Kloeckera*; C.: *Candida*; H.: *Hanseniaspora*; I.: *Issatchenkia*; L.: *Lodderomyces*; P.: *Pichia*; Ky.: *Kluyveromyces*; Sz.: *Schizosaccharomyces*; T.: *Torulaspor*a; Z.: *Zygoascus*; R.: *Rhodotorul*a; Cy.: *Cryptococcus*; Sy.: *Saccharomycopsis*; Y.: *Yarrowia*

A wider diversity of fermentative yeasts has occurred rather than respiratory yeasts. The species most worldwide found are *S. cerevisiae* and *Hanseniaspora guilliermondii* and a succession between them is generally observed. During the first 24-36 h of fermentation the apiculate yeasts *H. guilliermondii* rapidly grow to maximum populations of 10^7 – 10^8 CFU/g of cocoa pulp. There still questions as to why this apiculate yeast grows and dominate the early stages of fermentation so consistently (DIRCKS, 2009). As the concentration of ethanol and temperature increase, *H. guilliermondii* dies off, and is replaced by more ethanol- and heat-tolerant yeast, *S. cerevisiae*. Others ethanol- and heat-tolerant non-*Saccharomyces* yeasts also have been reported to grow at this stage. These species reach maximum populations of 10^6 – 10^7 CFU/g of cocoa pulp between 36-48 h, and may persist at these levels of the bean mass exceeds 48°C after approximately 72 h, causing even the thermo-tolerant yeasts to decline rapidly.

Although the global dominance of fermentative yeast is notorious (Table 1), 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; FLEET, 2003). Likewise, in the investigations carried out in Ghana (NIELSEN et al., 2007), *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. 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.

The microbial diversity of yeast during cocoa bean fermentations has been shown to vary with location and process parameters, such as nutrient

availability, temperature, pH, and oxygen tension, and with metabolic activities. In Ivory Coast, Sanchez et al. (1985) have found an abundant and varied yeast population, the dominant yeast species being *K. apiculata*, *K. corticis* and *S. chevalieri*, now recognized as *S. cerevisiae*. Ardhana and Fleet (2003) in East Java, Indonesia, *K. apis*, *S. cerevisiae* and *C. tropicalis* were found to be the most significant yeast species. In Bahia, Brazil, Camargo, Leme e Magalhães Filho (1963) found the most frequent yeast species to be *C. krusei*, *Geotrichum candidum* and *C. mycoderma* (now recognised as *C. vivi*). Later, Schwan, Rose and Board (1995) in Bahia, Brazil, identified amongst several other yeasts, the dominant species to be *S. cerevisiae*, *K. apiculata*, *Kluyveromyces marxianus* and *C. rugosa*.

In recognition of the artificial nature of several yeast genera, a series of taxonomic changes have gradually transformed the current phenotype-based classification towards a phylogeny-based classification (DANIEL et al., 2009). Some species of yeast found during cocoa fermentation has passed for this reorganization taxonomic. The encountered taxa concerned by these changes are the genus *Issatchenkia*, which has been shown by multigene sequence analyses to be closely related to the genus *Pichia*, into which it has been integrated (KURTZMAN; ROBNETT, 1998); *Pichia caribbica*, which, together with *P. guilliermondii*, has been assigned to the new genus *Meyerozyma* (KURTZMAN; SUZUKI, 2008); and *Pichia mexicana*, which has been confirmed by Kurtzman and Suzuki (2008) to be appropriately classified in the genus *Yamadazyma* (BILLON-GRAND, 1989). Thus, new studies have been conducted to identify yeasts associated with cocoa bean fermentation by using molecular tools. Jespersen et al. (2005) identified yeast isolates from heap and tray fermentations in Ghana by conventional microbiological analyses and by amplification of their ITS1-5.8S ITS2 rDNA regions and/or D1/D2 domain of the 5' end of the large subunit (26S) rDNA. *C. krusei* was found to be the dominant species during

heap fermentation, followed by *P. membranifaciens*, *P. kluyveri*, *H. guilliermondii* and *Trichosporon asahii*, whereas *S. cerevisiae* and *P. membranifaciens* were found to be the dominant species during tray fermentation followed by low numbers of *C. krusei*, *P. kluyveri*, *H. guilliermondii* and some yeast species of minor importance. The authors concluded that fermentation of cocoa beans is a very inhomogeneous process with great variations in both yeast counts and species composition. The variations seem to depend especially on the processing procedure, but also the season and the post-harvest storage are likely to influence the yeast counts and the species composition (JESPERSEN et al., 2005).

Daniel et al. (2009) studied the yeast diversity of Ghanaian cocoa bean heap fermentations through a multiphasic approach, encompassing an initial grouping by PCR-fingerprinting with the primer M13, followed by an identification using the D1/D2 region of the large subunit rRNA gene, internal transcribed spacer sequences (ITS), and partial actin gene sequences. *P. kudriavzevii* (*I. orientalis*), *S. cerevisiae* and *H. opuntiae* formed the major components of the yeast community. *H. opuntiae* was identified conclusively for the first time from cocoa fermentations. Among the less frequently encountered species, *C. carpophila*, *C. orthopsilosis*, *Kodamaea ohmeri*, *Meyerozyma (Pichia) caribbica*, *P. manshurica*, *Saccharomycodes ludwigii*, and *Yamadazyma* (*Pichia*) *mexicana* were identified. *H. opuntiae* was preferably growing during the earlier phase of fermentation, while no specific temporal distribution was recognized for *P. kudriavzevii* and *S. cerevisiae*.

A study performed by Nielsen et al. (2005) evaluated the yeast diversity culture-independently associated with heap and tray cocoa fermentations from Ghana by DGGE of 26S rRNA gene fragments obtained through Polymerase Chain Reaction (PCR) with universal eukaryotic primers. The authors found that the DGGE profiles were relatively complex, underlining that the fermentation of

cocoa is a complex microbial process. *H. guilliermondii*, *C. krusei* and *P. membranifaciens* were detected from most fermentations, indicating their possible important role in the fermentation of Ghanaian cocoa. *S. cerevisiae* and *C. zemplinina* were almost exclusively detected during tray fermentations. A comparison was made between the developed DGGE protocol and traditional culture-based isolations. The results were comparable but slightly different, as one yeast species (*C. zemplinina*) was only detected using DGGE. On the other hand, *Trichosporon asahii* yielded only faint bands in the denaturing gels, despite the fact that it was detected using culture-based methods. Analysis of pure cultures showed that the targeted region of the 26S rRNA gene was poorly amplified in *T. asahii*, whereas all other investigated isolates were amplified efficiently using the chosen PCR approach. The authors therefore concluded that the DGGE technique seems to offer a relatively fast and reliable method for studying yeast population dynamics during cocoa fermentations (NIELSEN et al., 2005).

More recently, the yeast species composition of 12 cocoa bean fermentations carried out in Brazil, Ecuador, Ivory Coast and Malaysia were investigated culture-independently by DGGE of 26S rRNA gene fragments (PAPALEXANDRATOU; VUYST, 2011). The most frequent yeast species were *Hanseniaspora* sp., followed by *P. kudriavzevii* and *S. cerevisiae* - independent of the origin of the cocoa. Exceptionally, the Ivorian cocoa bean box fermentation samples showed a wider yeast species composition, with *Hyphopichia burtonii* and *Meyerozyma caribbica* among the main representatives. Yeasts were not detected in the samples when the temperature inside the fermenting cocoa pulp-bean mass reached values higher than 45 °C or under early acetic acid production conditions.

The most important roles of the yeasts seem to be (SCHWAN; ROSE; BOARD, 1995): (i) Production of ethanol under low-oxygen and high-sugar

conditions. Ethanol disappears upon fermentation due to oxidation to acetic acid by AAB or even oxidative consumption by aerobically growing yeasts as well as through sweating and evaporation. (ii) Breakdown of citric acid in the pulp, which together with losses in the sweatings leads to an increase of the pH of the pulp allowing growth of bacteria; although a few yeasts assimilate citric acid, citric acid-consuming yeasts have been isolated from the cocoa bean fermenting mass, such as *P. fermentans* and certain isolates of *C. krusei* (JESPERSEN et al., 2005). (iii) Production of organic acids (acetic, malic, oxalic, phosphoric, succinic, and tartaric acids), which rather have a buffering capacity and will tend to reduce fluctuations in pH. (iv) Production of some volatile organic compounds, principally fusel alcohols, fatty acids, and fatty acid esters, which may contribute either to cocoa flavor or, more likely, to precursors of cocoa flavor. Yeasts such *as K. apiculata* and *S. cerevisiae* var. chevalieri produce large amounts of aroma compounds (SCHWAN; WHEALS, 2004). (v) Secretion of pectinases that reduce the viscosity of the pulp and cause its drainage. Some of the yeasts produce pectinolytic enzymes that break down the walls of cells in the pulp, among which *Kluyveromyces marxianus* and *S. cerevisiae* var. chevalieri show substantial activity (JESPERSEN et al., 2005; SANCHEZ et al., 1985; SCHWAN et al., 1997; SCHWAN; ROSE; BOARD, 1995). The spaces formed between the beans, due to collapse of the parenchyma cells in the pulp between beans, allow air to enter; aeration of the pulp mass is important for the growth of AAB. To speed up the fermentation process and enhance the quality of the final product, pectinases may be added to the pulp or strains over-producing pectinolytic enzymes may be used (SCHWAN; WHEALS, 2004).

2.6 Diversity and Roles of lactic acid bacteria (LAB)

The LAB phase in the microbial sequence of the cocoa fermentations was earlier considered insignificant because it is brief and releases by-products which served as substrates for others bacteria (ROELOFSEN, 1958). Studying cocoa fermentations in Trinidad, Forsyth and Rombouts (1952) incubated plates anaerobically, resulting in improved recovery of LAB isolates. Between 24-36 h of fermentation, the authors observed isolates reached populations of $10^7 – 10^8$ CFU/g of cocoa pulp, which represented only 20% of the total microflora at that time. This, and their apparent transience, meant that isolates were considered unimportant and remained until some years later (ROMBOUTS, 1952). Study performed by Ostovar and Keeney (1973) constituted the first thorough taxonomic study of LAB associated with cocoa fermentations. A realization that residual lactic acid may be responsible for flavor defects in Asian and Brazilian cocoa beans led to increased attention being given to the role of LAB. Carr, Davis and Dougan (1979) and Passos et al. (1984) investigated the role of LAB in determining the quality of cocoa beans produced in Malaysia and Ghana, and Brazil, respectively. During the last decade, knowledge about the LAB diversity in spontaneous cocoa bean fermentation process has increased (ARDHANA; FLEET, 2003; CAMU et al., 2007, 2008a, 2008b; JESPERSEN et al., 2005; KOSTINEK et al., 2008; NIELSEN et al., 2005, 2007; SCHWAN; WHEALS, 2004).

LAB increased in numbers when part of the pulp and “sweatings” had drained away, and the yeast population was declining. Yeast metabolism favors the growth of acidoduric, LAB (SCHWAN; WHEALS, 2004). Table 2 provides an updated summary of the major taxonomic studies undertaken in the last 40 years.

Table 2 LAB species isolated in cocoa beans fermentation

Metabolism	A	T1	T2	B1	B2	B3	I1	I2	IV	D	N	G1	G2	G3	G4	G5	G6	G7	V
Homofermentative																			
L. nagelii					+	+													
L. acidophilus		+																	
Lac. lactis		+	+	+								+							
P. acidilactici	+		+		+	+					+	+	+	+	+				
P. damnosus		+																	
P. dextrinicu		+																	
S. thermophilus		+	+																
V. carniphilus					+														
Heterofermentative																			
L. vaginalis					+														
L. bulgaricus		+	+																
L. cacaonum					+														
L. casei			+																
L. fermentum	+	+	+		+	+	+	+	+		+	+	+	+	+	+	+	+	+
L. ghanaensis													+	+					
L. mali					+	+										+			
L. paracasei										+									
L. paraplantarum										+									
L. pentosus										+									
L. plantarum	+	+		+	+	+	+	+		+	+	+	+	+	+	+		+	+
L. pseudoficulneum												+							
L. rossii														+					
F. pseudofilculneus																			+

Table 2, continuation

Obligate Heterofermentative																			
L. durianis					+	+													
L. brevis										+	+				+	+		+	
L. hilgardii					+		+						+	+					
Lec. mesenteroides			+												+				
Lec. pseudoficulneum													+	+	+				
Lec. pseudomesenteroides	+				+	+			+			+		+	+	+			
W. cibaria																+			
W. kimchii																+			
W. paramesenteroides																	+		

A: Australia (DIRCKS, 2009); T1: Trinidad (OSTOVAR; KEENEY, 1973) Centeno Estate; T2: Trinidad (OSTOVAR; KEENEY, 1973), San Louis Estate; B1: Brazil (SCHWAN, 1998; SCHWAN; ROSE; BOARD, 1995); B2: Brazil (GARCIA-ARMISEN et al., 2010); B3: Brazil (PAPALEXANDRATOU et al., 2011); I1: Indonesia (ARDHANA; FLEET, 2003), Estate A; I2: Indonesia (ARDHANA; FLEET, 2003), Estate B; IV: Ivory Coast (PAPALEXANDRATOU et al., 2011); D: Dominican Republic (LAGUNES-GÁLVEZ et al., 2007); N: Nigeria (KOSTINEK et al., 2008); G1: Ghana- Small heap (NIELSEN et al., 2007); G2: Ghana- Big heap (NIELSEN et al., 2007); G3: Ghana- Big heap (NIELSEN et al., 2007); G4: Ghana- Tray (NIELSEN et al., 2007); G5: Ghana (CAMU et al., 2007); G6: Ghana- Heap 12 (CAMU et al., 2008b); G7: Ghana- Heap 13 (CAMU et al., 2008a); V: fermentation in vessels (LEFEBER et al., 2011). Abbreviations: L.: *Lactobacillus*; Lac.: *Lactococcus*; P.: *Pediococcus*; S.: *Streptococcus*; Lec.: *Leuconostoc*; W.: *Weissella*; V. *Vagococcus*

In general, heterofermentative LAB constitute the major group occurring during cocoa beans fermentations, mainly represented by the species *Lactobacillus fermentum* and *L. plantarum*. In Indonesian fermentations, *L. fermentum* was the most predominant species between 36–48 h (60–80%) together with *L. plantarum* (ARDHANA; FLEET, 2003). 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, 2008a; NIELSEN et al., 2007). In Bahia (Brazil), six *Lactobacillus* spp. and two species of the genus *Pediococcus* together with and *Lactococcus lactis* and *Leuconostoc mesenteroides* were also isolated (SCHWAN; ROSE; BOARD, 1995).

As shown in Table 2, cocoa bean heap fermentations carried out in Ghana have been studied most extensively, encompassing both culture-dependent and culture-independent methodologies. In general, through classical microbiological analysis, the main LAB species described are *L. brevis*, *L. fermentum* and *L. plantarum* (CARR; DAVIS; DOUGAN, 1979; KOSTINEK et al., 2008). Recently, it has been shown by both culture-dependent and culture-independent methods that *L. plantarum* and *Leuconostoc pseudomesenteroides* dominate at the onset of Ghanaian cocoa bean heap fermentation and *L. fermentum* toward the end, while *Fructobacillus pseudoficulneus* (formerly *Leuconostoc pseudoficulneum*) and *Weissella ghanensis* (only in one heap fermentation out of seven heaps studied) constitute a small part of the LAB community, whether or not as opportunistic species (CAMU et al., 2007; NIELSEN et al., 2007). Polyphasic, bacterial analyses of Ghanaian cocoa bean fermentation processes have revealed also the occurrence of the new LAB species, such as *L. cacaonum* (BRUYNE et al., 2009a), *L. fabifermentans*

(BRUYNE et al., 2009a), *W. fabaria* (BRUYNE et al., 2009b) and *W. ghanensis* (BRUYNE et al., 2008).

The biochemical roles of LAB during cocoa fermentations have been described by some researchers (CAMU et al., 2008; PASSOS et al., 1984; SCHWAN; WHEALS, 2004; THOMPSON; MILLER; LOPEZ, 2007). The homofermentative species (*i.e., Pediococcus acidilactici* and *Lactococcus lactis*) utilize glucose via the Embden-Meyerhof pathway yielding lactic acid. The heterofermentative species (*i.e.*, *L. plantarum*, *L. fermentum*. *Leuconostoc* spp. and *Weissella* spp.) utilize glucose via the hexose monophosphate pathway producing 50% lactic acid, and ethanol, acetic acid, glycerol, mannitol and CO_2. Their relative proportion will thus change the composition of the pulp substrate and may consequently change the microbial succession. The citric acid conversion at the onset of fermentation confirms the role of LAB during a natural cocoa bean fermentation process (LEBEFER et al., 2011). As consumption of citric acid results in the production of organic acids with a higher pKa value, it hence increases the pH of the environment, which allows better bacterial growth and microbiological control of the environment. Also, the initial growth on citric acid, avoids competition with depectinizing (citrate-negative) yeasts that degrade carbohydrates to ethanol anaerobically. LAB are virtually non-proteolytic and their ability to ferment amino acids (precursor molecules of chocolate flavor) is also restricted with only two: serine and arginine.

In addition to being affected by changing aeration (mixing), temperature and ethanol contents, the growth of LAB in cocoa fermentations may also be affected by other forms of interactions. In cooperation with other micro-organisms, produce ethanol, organic acids, volatile compounds and enzymes and, thus, are important for the flavor and aroma production in beans used for chocolate production. Studies of sourdough fermentation using pure cultures of

L. plantarum showed that LAB can produce a vast array of volatile compounds (HANSEN; HANSEN, 1994). Some key metabolites identified were higher alcohols (*e.g.,* hexanol), esters (*e.g.,* ethyl acetate) and lactate, as well as n-hexanal and 2-pentyl-furan. While such studies have been conducted in wine (FLEET, 2003) and cheese (ADDIS et al., 2001), no study of a similar nature has been performed in fermentation of cocoa. Even though these micro-organisms participate in the development of desirable changes in the beans, the physiological roles of each species in each group remain to be clarified.

2.7 Diversity and Roles of acid acetic bacteria (AAB)

Taxonomic studies of AAB associated with cocoa beans fermentation were historically surveyed. After the decline in the populations of yeasts and LAB, the characteristic vinegar-like aroma of cocoa bean fermentations lead early investigators to conclude and demonstrate that AAB were significant contributors to the process (BAINBRIDGE; DAVIES, 1912; ROELOFSEN; GIESBERGER, 1947; ROHAN, 1964). In the early part of this century, researches attributed this acetic acid production to the presence of small rod shaped bacteria, following what they described as the 'alcoholic' phase of fermentation (SMITH, 1913). Roelofsen and Geisberger (1947) made first taxonomic and quantitative study of the AAB involved in cocoa fermentation. Carr, Davis and Dougan (1979) made a detailed comparison on the populations and diversity of species in fermentation in Ghana and Malaysia. Today, the growth behaviour of AAB during different stages of cocoa fermentation has been the focus of many researchers and new species are being described by polyphasic approaches (CAMU et al., 2007, 2008; LAGUNES-GONZÁLEZ et al., 2005; NIELSEN et al., 2007). The species *Acetobacter fabarum* (CLEENWERCK et al., 2008), *A. ghanensis* (CLEENWERCK et al., 2007) and

A. senegalensis (CAMU et al., 2007; NDOYE et al., 2007) were recently described.

The diversity of AAB species in cocoa fermentation is much less than the diversity of yeast or LAB. While 56 yeast (Table 1) and 31 LAB (Table 2) species have been described to date, only 15 different AAB species were found during cocoa fermentation (Table 3).

Table 3 AAB species isolated in cocoa beans fermentation

Metabolism	A	T1	T2	B1	B2	I1	I2	D	M	G1	G2	G3	G4	G5	G6	G7	V
P. E. (*Acetobacter* species)																	
A. pasteurianus	+					+	+			+	+		+	+	+	+	+
A. xylinum									+								
A. rancens									+								
A. aceti	+	+	+	+		+	+										
A. ghanaensis[a]														+	+	+	+
A. lovaniensis								+	+								
A. lovaniensis-like[b]																+	
A. malorum											+	+	+				
A. roseus[c]		+															
A. senegalensis[d]															+	+	+
A. syzygii										+	+	+	+				
A. tropicalis	+									+	+	+	+				
*A. ghanensis/syzygii**					+												
*A. pasteurianus/pomorum**					+												
*A. lovaniensis/fabarum**					+												

Table 3, continuation

P.S. (*Gluconobacter* species)																	
G. oxydans	+	+		+					+		+						
G. oxydans-like				+													
Gc. entanii					+												
*G. europaeus/swingsii**					+												

A: Australia (DIRCKS, 2009); T1: Trinidad (OSTOVAR; KEENEY, 1973) Centeno Estate; T2: Trinidad (OSTOVAR; KEENEY, 1973), San Louis Estate; B1: Brazil (SCHWAN, 1998; SCHWAN; ROSE; BOARD, 1995); B2: Brazil (GARCIA-ARMISEN et al., 2010); I1: Indonesia (ARDHANA; FLEET, 2003), Estate A; I2: Indonesia (ARDHANA; FLEET, 2003), Estate B; D: Dominican Republic (LAGUNES-GÁLVEZ et al., 2007); M: Malaysia (CARR; DAVIS; DOUGAN, 1979); G1: Ghana- Small heap (NIELSEN et al., 2007); G2: Ghana- Big heap (NIELSEN et al., 2007); G3: Ghana- Big heap (NIELSEN et al., 2007); G4: Ghana- Tray (NIELSEN et al., 2007); G5: Ghana (CAMU et al., 2007); G6: Ghana- Heap 12 (CAMU et al., 2008a); G7: Ghana- Heap 13 (CAMU et al., 2008a); V: fermentation in vessels (LEFEBER et al., 2010). Abbreviations A.: *Acetobacter*; G.: *Gluconobacter*. Gc.: *Gluconacetobacter*; P.F.: Preference for ethanol as carbon source; P.S.: Preference for sugar as carbon source. [a]New species discovered in cocoa beans fermentation closely related with *A. syzygii* (CLEENWERCK et al., 2007). [b]Recently described as new species: *Acetobacter fabarum* (CAMU et al., 2008a; CLEENWERCK et al., 2008). [c]Recently described new species (NDOYE et al., 2007). [d]Species not validated in current taxonomic description. * These species were not discriminated by the microbiological methods employed

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. Enumeration, isolation, identification and preservation of AAB are not easy. Not all the media support growth of AAB equally and they are selective for one strain to another (GULLO et al., 2006). Although there are lots of media, they mainly consist of the same ingredients with varying proportions, which cause different reactions on the plate (SENGUN; KARABIYIKLI, 2011). Thus, future studies on AAB populations occurring during cocoa beans fermentation will benefit from the use of different isolation media (CAMU et al., 2007, 2008b; LIMA et al., 2011).

Strains of *Acetobacter* and *Gluconobacter* are mostly found (SCHWAN; WHEALS, 2004). The genus *Acetobacter* was first introduced in 1898 with a single species, *A. aceti*. The genus *Gluconobacter* was proposed in 1935 for strains with intense oxidation of glucose to gluconic acid rather than oxidation of ethanol to acetic acid and no oxidation of acetate (YAMADA; YUKPHAN, 2008). In particular, the members of genus *Acetobacter* were found more frequently than those of *Gluconobacter*. The species of *Gluconobacter* prefer sugars as carbon source than *Acetobacter*, because these bacteria can obtain energy more efficiently by the metabolisation of the sugars *via* pentose phosphate pathway. They oxidize glucose *via* two alternative pathways. One operates inside the cell followed by oxidation *via* the pentose phosphate pathway and second operates outside the cell and involves the formation of gluconic acid and ketogluconic acid (KULKA; WALKER, 1954; OLIJVE; KOK, 1979). The former is carried by NADP+-dependent glucose dehydrogenase, and the latter is performed by NADP+ independent glucose dehydrogenase and is also called as "direct glucose oxidation" pathway (KITOS et al., 1958).

Although the cocoa pulp contains a high initial concentration of sugars, the relatively high content of pectin makes the pulp viscous limiting diffusion of

air, making it impossible to use of these sugars by *Gluconobacter* species. After the decline in the population of yeast and LAB, the fermenting mass becomes more aerated. This creates conditions suitable for the development AAB. These are responsible for the oxidation of ethanol to acetic acid and further oxidation of the letter to carbon dioxide and water. AAB have two enzymes which play a role in oxidation process: alcohol dehydrogenase and aldehyde dehydrogenase. The alcohol dehydrogenase activity of *Acetobacter* is more stable under acetic conditions than that of *Gluconobacter* (MATSUSHITA; TOYAMA; ADACHI, 1994), which explains the strong predominance of *Acetobacter* in cocoa fermentations.

A. pasteurianus strains have previously been reported to form a significant part of the AAB community during cocoa fermentations in Ghana (heap), Indonesia (box), Australia (heap and tray) and Brazil (box) (Table 3). In addition, *A. aceti* was often found in some cocoa fermentation, but it was not identified by Nielsen et al. (2007) in Ghanaian cocoa fermentations (Table 3). The worldwide dominance of *A. pasteurianus* can probably due to the fact that this AAB species is better adapted to the cocoa bean fermentation ecosystem, for instance to the increase of the temperature upon fermentation (CAMU et al., 2007). In addition, 16 S rDNA library sequencing approach allowed the detection of *Glucono(aceto)bacter* species for the first time during Ghanaian cocoa bean fermentation (GARCIA-ARMISEN et al., 2010). Interestingly, whereas *Glucono*(*aceto*)*bacter* prefers glucose as the energy source, which has been depleted by both yeasts and LAB in the early phases of cocoa bean fermentation, *Acetobacter* preferentially oxidizes ethanol into acetic acid, a key metabolic trait during cocoa bean fermentation (CAMU et al., 2008b). Thus, the presence of (unfermented) glucose is necessary for later growth of *Glucon*(*aceto*)*bacter* compared to LAB, which may reflect suboptimal

fermentation conditions of the "pulp-bean mass" (GARCIA-ARMISEN et al., 2010).

Amplification of repetitive bacterial DNA elements through the polymerase chain reaction (rep-PCR fingerprinting) using the $(GTG)_5$ primer was recently found as a promising genotypic tool for rapid and reliable speciation of AAB associated with cocoa bean fermentation (VUYST et al., 2008). The $(GTG)_5$-PCR fingerprinting allowed to differentiate four major clusters among the fermented cocoa bean isolates, namely *A. pasteurianus, A. syzygii*- or *A. lovaniensis*-like, and *A. tropicalis*-like, being that the species *A. syzygii*-like and *A. tropicalis*-like strains were reported for the first time during cocoa bean fermentations (VUYST et al., 2008).

The main activity of AAB is the oxidation of ethanol into acetic acid. This volatile acid diffuses into the beans and this, in combination with heat produced by this exothermic bioconversion, causes the death of the seed embryo as well as the end of fermentation. In turn biochemical changes in the beans are initiated, leading to the formation of precursor molecules that are necessary for the development of a characteristic aroma, flavor, and color of the beans (HANSEN; DEL-OLMO; BURRI, 1998). These properties are further developed during drying, roasting and final processing of well-fermented cocoa beans (THOMPSON; MILLER; LOPEZ, 2001). Thus, the acidity of cocoa beans, the high temperature in the fermenting mass and the diffusion and hydrolysis of protein in the cotyledons have been attributed to the metabolism of the AAB (SCHWAN; WHEALS, 2004). The activity of AAB is thus essential for the production of high-quality cocoa.

2.8 Origin of yeast, LAB and AAB in cocoa fermentation

The ecophysiology of yeast, LAB and AAB has been studied. 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). Fruit flies, at the same time, have been implicated as a source of inoculation for both AAB and *Bacillus* spp. (OSTOVAR; KEENEY, 1973). From all these sources, the cocoa pod surface appears to be the most important for microbial pulp inoculation (CAMU et al., 2008b).

Ostovar and Keeney (1973) and Rombouts (1952) isolated a wide range of yeasts involved in the fermentation of cocoa from knives used for pod breaking, fermentation boxes, pod surfaces, dried pulp and workers hands. Faparusi (1974) investigated the occurrence of yeasts associated with cocoa at different stages from flower to ripe pods. A number of yeasts often present in high numbers during the actual fermentation such as *C. krusei* (imperfect form of *I. orientalis*), *K. apiculata* (imperfect form of *H. uvarum*) and *P. membranifaciens* were detected at the different stages of maturation. Jesperson et al. (2005) demonstrated that certain yeast species isolated during cocoa fermentation in Ghana come from the surface of pods, and from the trays used for fermentation.

The fruit fly *Drosophila melanogaster* and other insects such as ants are another possible (and possibly underestimated) source of inoculation (OSTOVAR; KEENEY, 1973). During cocoa fermentations *D. melanogaster* is present in numbers so high, that it is even referred to as “the cocoa fly” in a few early publications (NICHOLLS, 1913). Early experiments by Nicholls (1913) suggested that *D. melanogaster* plays an important role in inoculating the cocoa

pulp. Bainbridge and Davies (1912) state that *D. melanogaster* is the main responsible for inoculating the fermenting mass with acetic acid bacteria. Ostovar and Keeney (1973) isolated 3 different AAB species, 4 different *Bacillus* spp., 4 different LAB species and various yeasts from 4 fruit flies collected at a cocoa farm on Trinidad. All isolates were isolated from fermenting cocoa at the farm as well. In Brazil, it has been found that *Drosophila* spp. normally carries *C. krusei* and is an important vector for transferring microorganisms between ecological nieces (MARAVALHAS, 1966).

2.9 Multivariate analysis of global microbial diversity in cocoa bean fermentation

The updated of the major cocoa taxonomic studies summarized in Tables 1, 2, and 3 were submitted to a multivariate statistic analysis [principal component analysis (PCA)] to obtain a more simplified view of the relationships among microbial diversity and cocoa-producing countries. A plot of the results is shows in Fig. 6.

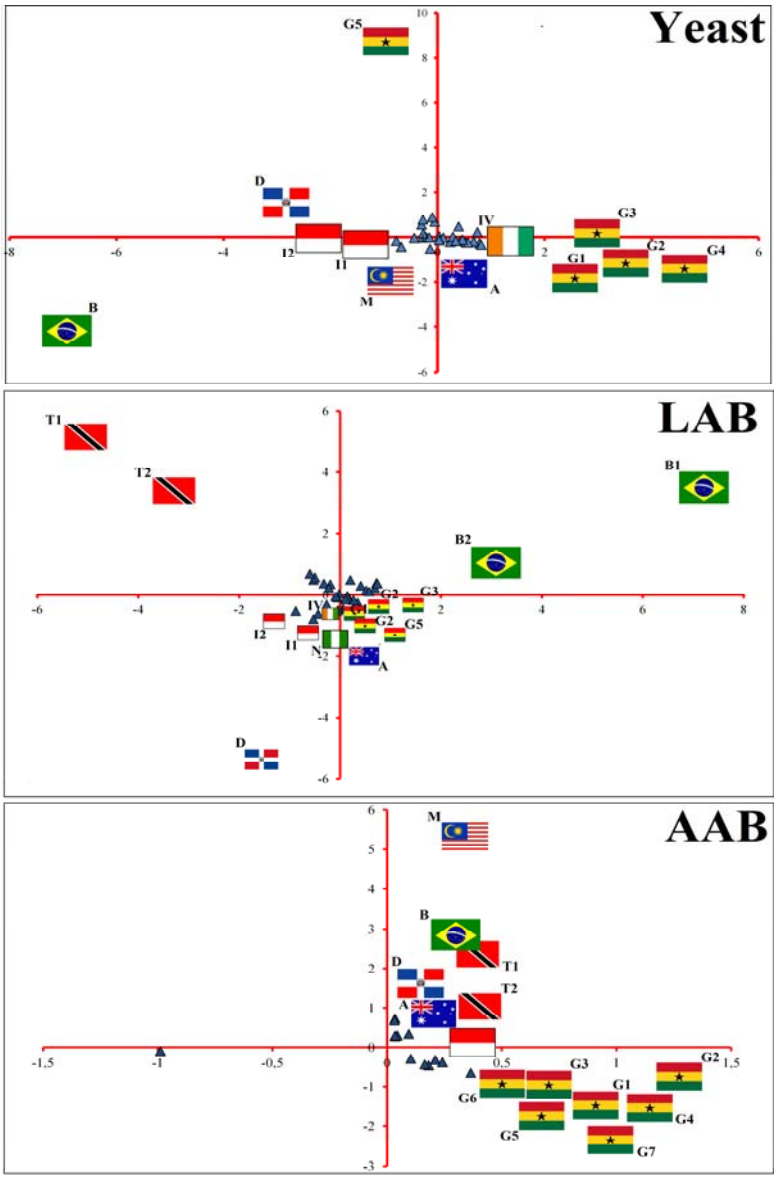

Figure 6 Principal Component Analysis (PCA) of the major cocoa taxonomic studies summarized in Tables 1, 2, and 3. Statistical analysis was carried out with Statistica software version 9.0 (StatSoft Inc., Tulsa, OK, USA). Ghana; Brazil; Dominican Republic; Indonesia; Malaysia; Australia; Ivory Coast; Nigeria; yeast, or LAB, or AAB isolates reported in Tables 1, 2, and 3. For yeast analysis: A: Australia (DIRCKS, 2009); T1: Trinidad (OSTOVAR; KEENEY, 1973) Centeno Estate; T2:

(...continue...)

Trinidad (OSTOVAR; KEENEY, 1973), San Louis Estate; B1: Brazil (SCHWAN, 1998; SCHWAN; ROSE; BOARD, 1995); I1: Indonesia (ARDHANA; FLEET, 2003), Estate A; I2: Indonesia (ARDHANA; FLEET, 2003), Estate B; D: Dominican Republic (LAGUNES-GÁLVEZ et al., 2007); M: Malaysia (CARR; DAVIS; DOUGAN, 1979); G1: Ghana- Small heap (NIELSEN et al., 2007); G2: Ghana- Big heap (NIELSEN et al., 2007); G3: Ghana- Big heap (NIELSEN et al., 2007); G4: Ghana- Tray (NIELSEN et al., 2007); G5 Ghana (DANIEl et al., 2009). For LAB: A: Australia (DIRCKS, 2009); T1: Trinidad (OSTOVAR; KEENEY, 1973) Centeno Estate; T2: Trinidad (OSTOVAR; KEENEY, 1973), San Louis Estate; B1: Brazil (GARCIA-ARMISEN et al., 2010); B2: Brazil (PAPALEXANDRATOU et al., 2011); I1: Indonesia (ARDHANA; FLEET, 2003), Estate A; I2: Indonesia (ARDHANA; FLEET, 2003), Estate B; IV: Ivory Coast (PAPALEXANDRATOU et al., 2011); D: Dominican Republic (LAGUNES-GÁLVEZ et al., 2007); N: Nigeria (KOSTINEK et al., 2008); G1: Ghana- Small heap (NIELSEN et al., 2007); G2: Ghana- Big heap (NIELSEN et al., 2007); G3: Ghana- Big heap (NIELSEN et al., 2007); G4: Ghana- Tray (NIELSEN et al., 2007); G5: Ghana (CAMU et al., 2007). For AAB analysis: A: Australia (DIRCKS, 2009); T1: Trinidad (OSTOVAR; KEENEY, 1973) Centeno Estate; T2: Trinidad (OSTOVAR; KEENEY, 1973), San Louis Estate; B: Brazil (GARCIA-ARMISEN et al., 2010); I1: Indonesia (ARDHANA; FLEET, 2003), Estate A; I2: Indonesia (ARDHANA; FLEET, 2003), Estate B; D: Dominican Republic (LAGUNES-GÁLVEZ et al., 2007); M: Malaysia (CARR; DAVIS; DOUGAN, 1979); G1: Ghana- Small heap (NIELSEN et al., 2007); G2: Ghana- Big heap (NIELSEN et al., 2007); G3: Ghana- Big heap (NIELSEN et al., 2007); G4: Ghana- Tray (NIELSEN et al., 2007); G5: Ghana (CAMU et al., 2007); G6: Ghana- Heap 12 (CAMU et al., 2008b); G7: Ghana- Heap 13 (CAMU et al., 2008a)

Typically, available reports on the bacteria and yeast ecology were found to be a country–specific fermentation process. For example, different fermentation systems studied by Nielsen et al. (2007) in Ghana, and by Ostovar and Keeney (1973) in Trinidad, were always grouped closely in the graphical representation, and apart from others countries (Fig. 6). The same was seen for LAB analysis in Brazil (GARCIA-ARMISEN et al., 2010; PAPALEXANDRATOU et al., 2011) and in Indonesia (ARDHANA; FLEET, 2003); Estate A and Estate B.

Species that appear to be indigenous to cocoa beans fermentation throughout the world are *H. guilliermondii*, *I. orientalis* and *S. cerevisiae* in the

yeasts group; *L. fermentum*, and *L. plantarum* in the LAB group; and *A. pasteurianus* and *A. aceti* belonging to the AAB group (Table 1, 2, and 3). With few exceptions, these species did not influence the discrimination of different fermentation processes since they were reported worldwide. However, in the Dominican Republic fermentation, *L. fermentum* was not reported (Table 2), which explain the distance of Dominican Republic apart from the other countries (Fig. 6). In addition, *L. plantarum* or *L. paraplantarum*, and *L. paracasei* and *L. pentosus*, seems to have an important role in this country. The species *Vagococcus carniphilus* and *L. vaginalis* were only reported in Brazil, as well as the species *L. acidophilus*, *P. damnosus*, *P. dextrinicu*, *Streptococcus thermophilus, L. bulgaricus* and *L. casei* were reported in Trinidad; it supports the distance of these countries apart from others in the PCA analysis. In this same sense, the species of *P. acidilactici*, *L. fermentum*, *L. plantarum*, *Lec. pseudoficulneum* and *Lec. pseudomesenteroides* were reported to occur in all fermentation systems studied by Nielsen et al. (2007).

In the yeast PCA analysis, the great distancing of Brazil from the other countries in the graphical representation can be explained by the high diversity found; the species *C. bombi*, *Kluyveromyces thermotolerans*, *Lodderomyces elongisporus*, *Torulaspora pretoriensis* and *C. rugosa* were only reported during Brazilian fermentations. In addition, *S. cerevisiae*, *C. diversa*, *C. zemplinina*, *I. orientalis*, *K. apis*, *P. kluyveri*, *Schizosaccharomyces pombe* and *P. membranifaciens* were found in most of Ghana fermentations study by Nielsen et al. (2007).

Regarding to AAB analysis, only the *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* genera were reported in cocoa beans fermentation from the 10 existing genera of AAB, with the *Acetobacter* genus being the most represented (Table 3). *G. europaeus*/*swingsii*, *G. oxydans*, and *G. oxydans*-like spp. were the only *Gluconobacter* spp. reported, while *Gluconacetobacter*

entanii was yhe single *Gluconacetobacter* species found. Indeed, in all fermentations conducted in Ghana, with the exception of the one big heap (NIELSEN et al., 2007) no *Gluconobacter* species were found. 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), that would explain the proximity of the fermentations conducted in Ghana for PCA analysis of AAB (Fig. 6)

The country-specific fermentation profile may also has been driven by the different taxonomic methods employed in different studies. It is possible that traditional methods may have misidentified microbial species. For instance, the study conducted in Ghana by Nielsen et al. (2007) was performed using culture-dependent and culture-independent methods, which increase the chances of a better allocation taxonomic compared to studies using only classical microbiological. Daniel et al. (2009) in Ghana fermentation, was positioned so far from the others studies in the yeast graphical representation. In this study, the yeast isolates were typed by M13-based PCR-fingerprinting and identified using the D1/D2 region of the large subunit rRNA gene, internal transcribed spacer sequences and partial actin gene sequences. The sequence comparisons of representative isolates, using not only rRNA gene cluster sequences, but also a faster evolving protein-coding gene, provided the currently most comprehensive identification of the yeasts involved in the cocoa bean heap fermentations (DANIEL et al., 2009). Thus, 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 increasing the 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 (LIMA et al., 2011). A contribution of the standardization of microbiological methods may be in understanding the problem of acidity in some countries. Commercial cocoa beans from some countries tend to have more acidic characteristics than to others. Cocoa beans from Malaysia, Brazil, São Tomé and Príncipe, and some crop seasons from Indonesia are examples of this situation (FOWLER, 1999; JINAP; 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.

2.10 Diversity and Functional Roles of *Bacillus* ssp.

The association of *Bacillus* species with cocoa bean fermentations has been reported for some time (BAINBRIDGE; DAVIES, 1912; NIELSEN et al., 2007; ROMBOUTS, 1952; SCHWAN; ROSE; BOARD, 1995; OUATTARA et al., 2008, 2011). Ostovar and Keeney (1973) and Schwan et al. (1986) found a broad diversity of *Bacillus* species during fermentation in Trinidad and Brazil, respectively. However, molecular studies have revealed a much lower diversity which indicates that only traditional methods may have misidentified *Bacillus* species and overestimated its diversity (OUATTARA et al., 2008). In general, the species *B. subtilis*, *B. cereus*, *B. pumilus*, *B. licheniformis* and *B. megaterium* are the most frequently isolated in Brazilian (SCHWAN et al., 1986), Trinidad (OSTOVAR; KEENEY, 1973) Australia (DIRCKS, 2009) and Ghanaian (CARR; DAVIES, 1980; NIELSEN et al., 2007) cocoa fermentations.

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 and Ghana, only a few references have 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 (LIMA et al., 2011). Only in Ghana the authors clearly reported the absence of *Bacillus* spp. during tray fermentation (NIELSEN et al., 2007), while other studies into Ghana fermentations the authors stated that in addition to yeasts, LAB and AAB, no other major group participated in the process (CAMU et al., 2007, 2008b).

Cocoa fermentations conducted in Trinidad (OSTOVAR; KEENEY, 1973) and Ghana (NIELSEN et al., 2007) were characterized by a late appearance of *Bacillus* spp., typically between 60 and 96 h. Towards the end of the cocoa fermentation, the major sources of carbon are organic acids, such as acetic acid and lactic acid, 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). On the contrary, in the Indonesia fermentations (ARDHANA; FLEET, 2003), *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 (NIELSEN et al., 2007), the pattern of occurrence of *Bacillus* spp. was intermediate between that of Indonesia (ARDHANA; FLEET, 2003) and Trinidad (OSTOVAR; KEENEY, 1973) fermentations, since this group was detected in samples taken from 48 h. In Brazil fermentation (SCHWAN et al., 1986), 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 (OSTOVAR; KEENEY, 1973), Indonesia (ARDHANA; FLEET, 2003), and Ghana (NIELSEN et al.,

2007), which might indicate unfavorable conditions for the development of this group of microorganisms.

The role of *Bacillus* spp. during cocoa beans fermentation has not been understood yet. Zak and Keeney (1976) suggested the involvement of *B. 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 C_3-C_5 free fatty acids and 2,3-butanediol (LOPEZ; QUESNEL, 1971; 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. Pectinolytic enzymes are known to be indispensible for the normal course of the fermentation process and for the development of the quality of fermented cocoa (BHUMIBHAMON; JINDA, 1997; SCHWAN; WHEALS, 2004). Pectinolytic enzyme production by some *Bacillus* strains from soil (SOARES; SILVA; GOMES, 1999; SORIANO; DIAZ; JAVIER-PASTOR, 2005), water retting process (TAMBURINI et al., 2003) and fermented food (SILVA et al., 2005) have been reported. This suggests that *Bacillus* present in the fermenting cocoa mass might contribute to the yield of pectinolytic enzymes. Ouattara et al. (2008) found that a large proportion (91.83%) of the *Bacillus* strains isolated from different cocoa fermentation methods (tarpaulin, wooden box and banana leaves) showed pectinolytic activity. Pectinolytic enzymes are classified in two main groups according to their mode of attack on the pectin molecule: (i) *de-esterifying* enzymes (pectin methylesterase EC 3.1.1.11) which removes the methoxy group from pectin, and (ii) *depolymerases* enzymes that cleave the b-(1, 4) glycosidic bonds between galacturonate units either by hydrolysis (polygalacturonase EC 3.2.1.15) or by trans-elimination (pectin lyase EC 4.2.2.10 and pectate lyase EC 4.2.2.2). The pH variation from pH 3 to 6 and oxygen availability proved to

have less effect on polygalacturonase synthesis by *Bacillus* strains isolated from cocoa fermentation, but the amount of pectin lyase was heightened near neutral pH and totally inhibited in conditions of oxygen limitation (OUATTARA et al., 2008). This suggests that pectin lyase could be synthesized in the latter stage of fermentation, when the fermenting mass is more aerated and the pH is less acid, while the production of polygalacturonase could be more suitable at the beginning of the fermentation process, in anaerobiosis conditions. Thus at least one enzyme could be specifically synthesized at a particular stage of cocoa fermentation.

2.11 Other microbial groups

Yeasts, LAB and AAB constitute the most prominent microbial group, but other bacterial species (e.g., *Pseudomonas*, *Pantoea*, *Tatumella*, *Klebsiella*, *Erwinia*, *Micrococcus*, *Microbacterium*, *Frateuria, Acinetobacter*, *Chryseobacterium*, *Zymomonas*, *Brevundimonas* and *Xanthomonas*) and filamentous fungi were also reported in a few fermentations, during a certain period of time. 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.

The most frequently mentioned bacterial species are those belonging to the family *Enterobacteriaceae* and species of *Staphylococcus*. During the early stages of successful heap and box fermentations carried out in Ivory Coast, *Erwinia* spp. (*E. soli*, *E. tasmaniensis*) and *Pantoea* sp. were detected culture-independently (PAPALEXANDRATOU et al., 2011). Garcia-Armisen et al. (2010) have found that 2.8 -3.3% of the clones obtained from Ghanaian (heap at 60 h) and Brazilian (box at 48 h) fermentation samples represent enterobacterial

species (*Tatumella* sp., *Pantoea punctata* and *Erwinia tasmaniensis*), showing their decline upon fermentation.

The technological role that these microorganisms play in the course of the cocoa bean fermentation is not known, although they should have some beneficial and several negative fermentation characteristics. Among the latter, they may use different carbohydrates present in the cocoa pulp creating a competitive association "bacteria-yeast" during fermentation. Moreover, they may produce a plethora of by-products as a consequence of their metabolism and impart off-flavors to the beans (SCHWAN; WHEALS, 2004). On the other hands, some these enterobacterial species needs more consideration because they can showed benefices effects to cocoa fermentation, for instance contributing to depectinization of the pulp, breakdown of pulp citrate and/or gluconic acid production (PAPALEXANDRATOU et al., 2011).

Studies have shown a steady and marked presence of filamentous fungi during the cocoa fermentation process and during the drying and storage steps (ARDHANA; FLEET, 2003; MARAVALHAS, 1966; MOUNJOUENPOU et al., 2008; RIBEIRO; BEZERRA; LOPEZ, 1986; SANCHEZ-HERVAS et al., 2008). When present, they are often found 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). A study of filamentous fungi occurring during Brazil fermentation by Ribeiro, Bezerra and Lopez (1986) showed the ability of the isolates to assimilate both ethanol and mannitol, which might be crucial in the reduction of ethanol induced-stress. It is known that filamentous fungi can cause hydrolysis of the pulp, produce acids, off-flavors, and alter the taste of the cocoa beans (SCHWAN; WHEALS, 2004). Besides the deteriorative potential and consequent influence on sensorial quality

of cocoa and chocolate, the presence of fungi in food is also a public health issue due to the possibility of mycotoxin production (COPETTI et al., 2011).

2.12 Use of starter cultures for fermentation of cocoa

Pos storage, fermentation system and mixing frequency all indirectly affect the microbial ecology of fermentation and thus the quality of cocoa beans produced (AFOAKWA et al., 2008; BIEHL et al., 1989; THOMPSON; MILLER; LOPEZ, 2007). However, it is also important to consider how the microbiology of fermentation may be controlled more directly. Improved understanding of the microbiology of cocoa fermentation has led to the investigations into the use of starter cultures. The first published was done by Sanchez et al. (1985), which fermented cocoa beans using a pure culture of yeasts, including *S. chevalieri* (now classified as *S. cerevisiae*), *C. zeylanoides*, and *Ky. fragilis* (now classified as *K. marxianus*). Each yeast culture was inoculated at 10^6 CFU/g of cocoa pulp and it was observed that the strain *S. cerevisiae var chevalieri* rapidly degraded the pulp and effectively fermented the beans, and the chocolate made from cocoa beans fermented by this strain was comparable to quality of traditionally fermented beans (SANCHEZ et al., 1985).

Former research on starter culture development for controlled cocoa bean fermentations mainly focussed on the introduction of pectinolytic yeasts for enhanced cocoa pulp juice production (BUAMAH; DZOGBEFIA; OLDHAM, 1997; DZOGBEFIA; BUAMAH; OLDHAM, 1999; LEAL JÚNIOR et al., 2008; SAMAH; PTIH; SELAMAT, 1992). Buamah, Dzogbefia and Oldham (1997) and Dzogbefia, Buamah and Oldham (1999) used pectinolytic yeast *Kluyveromyces fragilis*, *C. norvegensis* (now classified as *P. norvegensis*), *T. candida* (now classified as *C. saitoana*), and *S. chevalieri* in controlled fermentation of cocoa beans in sterile funnels under laboratory conditions. *S.*

chevalieri and *K. fragilis* both significantly increased the yields of cocoa sweatings, which could be commercialized as jam, marmalade, or syrup (BUAMAH; DZOGBEFIA; OLDHAM, 1997). Furthermore, the inoculation did not alter the physic-chemical properties of the degraded pulp. The exclusion of AAB from the fermentation also resulted in low levels of acetic acid (DZOGBEFIA; BUAMAH; OLDHAM, 1999). More recently, a controlled inoculation of cacao bean fermentation using a *Kluyveromyces marxianus* hybrid yeast strain, with an increased pectinolytic activity, would improve an earlier liquid drainage from the fermentation mass, developing a superior final product quality (LEAL JÚNIOR et al., 2008). Introduction of the hybrid yeast affected the profile of total seed protein degradation evaluated by polyacrylamide gel electrophoresis, with improved seed protein degradation, and reduction of titrable acidity. The increase in mass aeration during the first 24 h seemed to be fundamental for the improvement of fermentation quality, demonstrating the potential application of this improved hybrid yeast strain with superior exogenous pectinolytic activity (LEAL JÚNIOR et al., 2008).

Whereas fermentative and pectinolytic yeast starter cultures have been tried before to speed up cocoa bean fermentation with respect to pulp volume and air ingress, the use of a mixed starter culture of LAB and AAB strains for this fermentation is promising as well. Successful use of a defined microbial starter culture composed of yeast (*S. chevalieri*, synomym of *S. cerevisiae*), LAB (*L. delbrueckii subsp. lactis* and *L. plantarum*), and AAB (*A. aceti* and *G. oxydans*) has been reported in the case of Brazilian cocoa bean box fermentations (SCHWAN, 1998). It was possible to mimics the traditional cocoa fermentation using only representatives of these three groups of microorganisms.

The functional roles and the physiological adaptations of LAB and AAB species most frequently found in the cocoa pulp ecosystem have been reported through laboratory fermentations of selected cocoa-specific LAB and AAB

strains in cocoa pulp simulation media (LEFEBER et al., 2011). The data surveyed for this study could explain the competitiveness of particular cocoa-specific strains, namely, *L. plantarum* 80 (homolactic and acid tolerant), *L. fermentum* 222 (heterolactic, citric acid fermenting, mannitol producing, and less acid tolerant), and *A. pasteurianus* 386B (ethanol and lactic acid oxidizing, acetic acid overoxidizing, acid tolerant, and moderately heat tolerant), during the natural cocoa bean fermentation process. For instance, it turned out that the capacity to use citric acid, which was exhibited by *L. fermentum* 222, is of the utmost importance. Also, the formation of mannitol was dependent not only on the LAB strain but also on environmental conditions (LEFEBER et al., 2011).

Although these preliminary experiments of the application of defined starter cultures show satisfying results, they have not been introduced in the field. The major difficultly associated with inoculated cocoa fermentations is the removal of natural microbiota. In small-scale laboratory fermentations, this could be achieved by washing the cocoa pods with hypochlorite treated water and using sterilized equipment, as by Schwan (1998). However, as on-farm fermentations usually involve large amounts of beans, washing cocoa pods becomes a laborious and ineffective process and require intensive training of farmers. Recently, cocoa bean fermentations controlled by means of starter cultures were introduced on several farms in two different cocoa-producing regions (West Africa and Southeast Asia) (LEFEBER et al., 2011). In this study, two starter culture mixtures were tested, namely one composed of *S. cerevisiae* H5S5K23, *L. fermentum* 222, and *A. pasteurianus* 386B (three heaps and one box), and another composed of *L. fermentum* 222 and *A. pasteurianus* 386B (seven heaps and one box). In all starter culture-added cocoa bean fermentation processes, the inoculated starter culture species were able to outgrow the natural contamination of the cocoa pulp-bean mass and they prevailed during cocoa bean fermentation. This on-farm implementation of starter culture-added cocoa

bean fermentations enabled the production of chocolates with a respective consistent chocolate flavour, independent of cocoa-producing region and fermentation method. It overcomes the variability in fermentation degree and flavour deviations typically seen in the case of spontaneous cocoa bean fermentations (LEFEBER et al., 2011).

3 FINAL CONSIDERATIONS

The world's cocoa beans processing remains uncontrolled and largely unchanged from how it was performed centuries before. This contrasts to others fermented foods such as bread, wine and cheese which have all been successfully industrialized. The industrialization of any fermented food requires that three main factors be understood and controlled: (i) the substrate and its properties; (ii) the physical processes or until operations carried out; (iii) and the microbiology of the fermentation. With regards to cocoa fermentation, some of these areas have been well addressed, while there remain significant gaps in others. Cocoa research performed in the last century has described the basic physiology and ecology of cocoa fermentation, as well as the biochemical changes occurring during this process leading to "chocolate" flavor. Recently, modern molecular biology techniques have been applied to understanding the dynamics of microorganisms during the fermentation process. It is understood that biotechnological manipulation of the component parts of microbial fermentation (microorganisms, amount of pulp, selected strains, etc) can lead to understandable and reasonably predictable effects in chocolate quality. Although much progress has been made in understanding the yeast and bacteria ecology of cocoa fermentation, research of other fermented products suggests that this ecology is more complex than currently appreciated. First, the metabolic diversity of the yeast, LAB and AAB strains during cocoa fermentation can be

interpreted as being a natural consequence of environmental conditions which influence their frequency and selection. The changes in the pH, temperature, sugars concentration, and fermentation products, exert a selection pressure on the already existing natural biotypes, favoring strains that are more adapted to this environment. Analysis of bacteria and yeast strains survival under of these stress conditions could provide useful information about the ability of the yeast and bacteria to start growth and carry out fermentation. In addition, 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. For example, fermentative metabolism of *S. cerevisiae* (main yeast used as starter culture for cocoa fermentation) may be affected by different forms of "yeast-yeast" interactions, as well as – differences in the production of, and sensitivity to, killer-factors; the formation of cell-cell interactions *via* quorum sensing molecules; or the occurrence of spatial phenomena, which are currently subjects of conjecture. Study by Howell et al. (2006) demonstrated the significance yeast-yeast interactions in affecting wine flavour. In addition, a range of "yeast-bacteria" interactions can also affect the growth of yeast and/or bacteria. For instance, the death and autolysis of the yeast cells releases vitamins and other nutrients, and/or, the CO_2 produced during the yeast growth creates micro-aerophilic conditions, it provide a habitat that encourages the growth of the LAB. In wine fermentations some LAB produce substances in addition to lactic acid that inhibit the growth of yeasts and other bacteria. Similar interactions probably occur during cocoa fermentation and the impact of these on the flavour and quality of cocoa beans deserves greater attention.

Another important aspect to be better understood is the true role of the *Bacillus* spp. - being positive, negative or neutral - in the cocoa fermentation process. 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.

Finally, cocoa bean fermentations remains to be done in the field, in an artisan way on small scale or under non-optimal conditions on large scale; the results are very variable in quality. In addition, there might be problems of acidity or lack of cocoa flavor (due to incomplete fermentation) and presence of off-flavors (due to over-fermented beans and spoilage), all of which lead to low crop value for the farmer. Therefore, it would be desirable to change the fermentation from a whole natural and unpredictable process to a controlled process, initiated with an appropriate starter culture, in which fermentation occurs more quickly. However, the fermentation process itself has been largely neglected. To achieve a truly industrialized process further work needs to be done. In particular, production-scale equipment needs to be designed and tested. The demand for more hygienic production practices has made the use of stainless steel containers more widespread in the industrial bioprocesses, such as beer, wine and cider production. These industrial bioprocesses have largely replaced traditional natural fermentations with defined inocula of high-quality raw materials, strict control of the fermentation processes, better treatment of the final products, and diversification of the market. Cocoa fermentations are still at the first stage. The on-fam processing, where the fermentation process is conducted over banana leaves or in wooden boxes, facilitates microbiota natural contamination. This makes impossible the use of starter cultures and generates

cocoa beans with different physicochemical and sensory properties. As cocoa bean fermentation involves a complex microbial succession, the use of a stainless steel tank will is a challenging prospect, and strategies specific to this scenario need to be developed to avoid an unsuccessful campaign of process-validation. Both the biology and chemistry of cocoa bean fermentation are complex and all aspects of the process need to be subjected to comprehensive, simultaneous, dynamic analyses to quantitatively predict the outcomes of defined changes. Understanding the microbial ecology of the fermentation process is the first stage in developing a new procedure and optimising both processing efficiency and the quality of its end products.

REFERENCES

ADDIS, E. et al. The growth, properties and interactions of yeasts and bacteria associated with the maturation of Camembert and blue-veined cheeses. **International Journal of Food Microbiology**, Amsterdam, v. 69, n. 1, p. 25-36, Jan. 2001.

AFOAKWA, E. O. et al. Flavor formation and character in cocoa and chocolate: a critical review. **Critical Reviews in Food Science and Nutrition**, Cleveland, v. 48, n. 9, p. 840-857, Oct. 2008.

_____. Matrix effects on flavor volatiles release in dark chocolates varying in particle size distribution and fat content using GC-mass spectrometry and GC-olfactometry. **Food Chemistry**, London, v. 113, n. 2, p. 208-215, Feb. 2009.

ARDHANA, M. M.; FLEET, G. H. The microbial ecology of cocoa bean fermentations in Indonesia. **International Journal of Food Microbiology**, Amsterdam, v. 86, n. 1, p. 87-99, Jan. 2003.

ANON, C. Genetic resources of cocoa: AGP:IBPGR/80/56. In: WORKING GROUP ON GENETICS RESOURCES OF COCOA, 1., 1981, Rome. **Proceedings…** Rome: IBPGR, 1981. p. 25.

BAILEY, S. D. et al. Studies on the volatile components of different varieties of cocoa beans. **Journal of Food Science**, Chicago, v. 27, p. 165-170, 1962.

BAINBRIDGE, J. A.; DAVIES, S. H. The essential oil of cocoa. **Journal of the Chemical Society**, London, v. 101, p. 2209-2220, 1912.

BAKER, D. M.; TOMLINS, K. I.; GRAY, C. Survey of Ghanaian cocoa farmer fermentation practices and their influence on cocoa flavor. **Food Chemistry**, London, v. 51, n. 4, p. 425-431, Oct. 1994.

BHUMIBHAMON, O.; JINDA, J. Effect of enzymes pectinases on natural cocoa fermentation. **Kasetsart Journal: Nature Science**, Bancoc, v. 31, n. 2, p. 206-212, Apr./June 1997.

BIEHL, B. et al. Chemical and physical changes in the pulp during ripening and post-harvest storage of cocoa pods. **Journal of the Science of Food and Agriculture**, London, v. 208, n. 48, p. 189-208, 1989.

_____. The proteases of ungerminated cocoa seeds and their role in the fermentation process. **Angewandte Botanik**, Berlin, v. 65, n. 6, p. 59-63, Dec. 1993.

BILLON-GRAND, G. A new ascosporogenous yeast genus: *Yamadazyma* gen. nov. **Mycotaxon**, Ithaca, v. 35, n. 2, p. 201-204, Jan. 1989.

BRUYNE, K. et al. *Lactobacillus fabifermentans* sp. nov. and *Lactobacillus cacaonum* sp. nov., isolated from Ghanaian cocoa fermentations. **International Journal of Systematic and Evolutionary Microbiology**, Reading, v. 59, n. 1, p. 7-12, Jan. 2009a.

_____. *Weissella fabaria* sp. nov. from Ghanaian cocoa bean fermentation. **International Journal of Systematic and Evolutionary Microbiology**, Reading, v. 60, n. 9, p. 1999-2005, Oct. 2009b.

_____. *Weissella ghanensis* sp. nov., isolated from a Ghanaian cocoa fermentation. **International Journal of Systematic and Evolutionary Microbiology**, Reading, v. 58, n. 12, p. 2721-2725, Dec. 2008.

BUAMAH, R.; DZOGBEFIA, V. P.; OLDHAM, J. H. Pure yeasts culture fermentation of cocoa (*Theobroma cacao* L.): effect on yield of sweatings and cocoa bean quality. **World Journal of Microbiology and Biotechnology**, Oxford, v. 13, n. 4, p. 457-462, July 1997.

CAMARGO, R. de; LEME, J.; MAGALHÃES FILHO, A. General observations on the microflora of fermenting cocoa beans (*Theobroma cacao*) in Bahia, Brazil. **Food Technology**, Oxford, v. 17, p. 116-118, 1963.

CAMU, N. et al. 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**, Washington, v. 73, n. 6, p. 1809-1824, Mar. 2007.

_____. Fermentation of cocoa beans: influence of microbial activities and polyphenol concentrations on the flavour of chocolate. **Journal of the Science of Food and Agriculture**, London, v. 88, n. 13, p. 2288-2297, Oct. 2008a.

_____. Influence of turning and environmental contamination on the dynamics of populations of lactic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation in Ghana. **Applied and Environmental Microbiology**, Washington, v. 74, n. 1, p. 86-98, Jan. 2008b.

CARR, J. G.; DAVIES, P. A. **Cocoa fermentation in Ghana and Malaysia:** further microbiological methods and results: part 2. Bristol: Long Ashton, 1980. 582 p.

CARR, J. G.; DAVIS, P. A.; DOUGAN, J. **Cocoa fermentation in Ghana and Malaysia II**. Bristol: University of Bristol, 1979. 573 p.

CLAPPERTON, J. F. et al. Effects of planting materiais on flavour. **Cocoa Growers Bulletin**, Bournville, v. 48, n. 1, p. 47-63, 1994.

CLEENWERCK, L. et al. *Acetobacter fabarum* sp. nov., an acetic acid bacterium from a Ghanaian cocoa bean heap fermentation. **International Journal of Systematic and Evolutionary Microbiology**, Reading, v. 58, n. 9, p. 2180-2185, Sept. 2008.

_____. *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**, Reading, v. 57, n. 7, p. 1647-1652, July 2007.

COOK, L. R. **Chocolate production and use**. 3rd ed. New York: Magazines for Industry, 1982. 492 p.

COPETTI, M. V. et al. Mycobiota of cocoa: from farm to chocolate. **Food Microbiology**, London, v. 28, n. 8, p. 1499-1504, Dec. 2011.

DANIEL, H. M. et al. Yeast diversity of Ghanaian cocoa bean heap fermentations. **International Journal of Systematic and Evolutionary Microbiology**, Reading, v. 9, n. 9, p. 774-783, Aug. 2009.

DIRCKS, H. D. **Investigation into the fermentation of Australian cocoa beans and its effect on microbiology, chemistry and flavour**. 2009. 487 p. Thesis (Doctor of Phylosophy) - University of New South Wales, Sidney, 2009.

DUNCAN, R. J. E. et al. Improvement of Malaysian cocoa bean flavor by modification of harvesting, fermentation and drying methods: the Sime-Cadbury process. **The Planter**, Kuala Lumpur, v. 65, n. 2, p. 157-173, Jan. 1989.

DZOGBEFIA, V. P.; BUAMAH, R.; OLDHAM, J. H. The controlled fermentation of cocoa (*Theobroma cacao* L.) using yeasts: enzymatic process and associated physico-chemical changes in cocoa sweatings. **Food Biotechnology**, New York, v. 13, n. 1, p. 1-12, Jan. 1999.

ERCOLINI, D. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. **Journal of Microbiological Methods**, Amsterdam, v. 56, n. 3, p. 297-314, Mar. 2004.

ERCOLINI, D. et al. Changes in the spoilage: related microbiota of beef during refrigerated storage under different packaging conditions. **Applied and Environmental Microbiology**, Washington, v. 72, n. 7, p. 4663-4671, July 2006.

FAPARUSI, S. I. The yeasts associated with cacao (*Theobroma cacao*) pods. **Egyptian Journal of Microbiology**, Alexandria, v. 9, p. 105-115, 1974.

FLEET, G. H. Yeast in foods and beverages: impact on product quality and safety. **Current Opinion in Biotechnology**, London, v. 18, n. 1, p. 170-175, Feb. 2007.

_____. Yeast interactions and wine flavour. **International Journal of Food Microbiology**, Amsterdam, v. 86, n. 1, p. 11-22, Jan. 2003.

FONTANA, C.; COCCONCELLI, P.; VIGNOLO, G. Direct molecular approach to monitoring bacterial colonization on vacuum: packaged beef. **Applied and Environmental Microbiology**, Washington, v. 72, n. 8, p. 5618-5622, Aug. 2006.

FORSYTH, W. G. C.; QUESNEL, V. C. Cacao glycosidase and color changes during fermentation. **Journal of the Science of Food and Agriculture**, London, v. 8, p. 505-509, 1957.

FOWLER, M. F. Cocoa beans: from tree to factory. In: BECKETT, S. T. (Ed.). **Industrial chocolate manufacture and use**. 3rd ed. Oxford: Blackwell Science, 1999. p. 8-35.

FRAUENDORFER, F.; SCHIEBERLE, P. Identification of the key aroma compounds in cocoa powder based on molecular sensory correlations. **Journal of Agricultural and Food Chemistry**, Easton, v. 54, n. 15, p. 5521-5529, July 2006.

GARCIA-ARMISEN, T. et al. Diversity of the total bacterial community associated with Ghanaian and Brazilian cocoa bean fermentation samples as revealed by a 16 S rRNA gene clone library. **Applied Microbiology and Biotechnology**, Berlin, v. 87, n. 6, p. 2281-2292, Aug. 2010.

GASCH, A. P.; WERNER-WASHBURNE, M. The genomics of yeast responses to environmental stress and starvation. **Functional and Integrative Genomics**, Berlin, v. 2, n. 4/5, p. 181-192, Sept. 2002.

GIRAFFA, G.; NEVIANI, E. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. **International Journal of Food Microbiology**, Amsterdam, v. 67, n. 1, p. 19-34, Jan. 2001.

GULLO, M. et al. Characterization of acetic acid bacteria in "traditional balsamic vinegar". **International Journal of Food Microbiology**, Amsterdam, v. 106, n. 2, p. 209-212, Feb. 2006.

HANSEN, B.; HANSEN, A. Volatile compounds in wheat sourdoughs produced by lactic acid bacteria and sourdough yeasts. **Zeitschrift für Lebensmitteluntersuchung und Forschung**, Hamburg, v. 198, n. 3, p. 202-209, Mar. 1994.

HANSEN, C. E.; DEL-OLMO, M.; BURRI, C. Enzyme activities in cocoa beans during fermentation. **Journal of the Science of Food and Agriculture**, London, v. 77, n. 2, p. 273-281, June 1998.

HASHIM, P. et al. Changes in free amino acid peptide-N, sugar and pyrazine concentration during cocoa fermentation. **Journal of the Science of Food and Agriculture**, London, v. 78, n. 4, p. 535-542, Dec. 1998.

HOWAT, G. R.; POWELL, B. D.; WOOD, G. A. R. Experiments on cocoa fermentation in West Africa. **Journal of the Science of Food and Agriculture**, London, v. 8, p. 65-72, 1957.

HOWELL, K. S. et al. Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. **FEMS Yeast Research**, Amsterdam, v. 6, n. 1, p. 91-101, 2006.

JANY, J. L.; BARBIER, G. Culture-independent methods for identifying microbial communities in cheese. **Food Microbiology**, London, v. 25, n. 7, p. 839-848, Oct. 2008.

JESPERSEN, L. et al. Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. **FEMS Yeast Research**, Amsterdam, v. 5, n. 4/5, p. 441-453, Feb. 2005.

JINAP, S.; DIMICK, P. S. Acidic characteristics of fermented and dried cocoa beans from different countries of origin. **Journal of Food Science**, Chicago, v. 55, n. 2, p. 547-550, Mar. 1990.

KIM, H.; KEENEY, P. G. (-)-Epicatechin content in fermented and unfermented cocoa beans. **Journal of Food Science**, Chicago, v. 49, n. 4, p. 1090-1092, July 1984.

KITOS, P. A. et al. Glucose and gluconate dissimilation in Acetobacter oxydans. **Journal of Biological Chemistry**, Bethesda, v. 233, p. 1295-1298, 1958.

KOSTINEK, M. et al. Diversity of predominant lactic acid bacteria associated with cocoa fermentation in Nigeria. **Current Microbiology**, New York, v. 56, n. 4, p. 306-314, Apr. 2008.

KULKA, D.; WALKER, T. K. The ketogenic activities of Acetobacter species in a glucose medium. **Archives of Biochemistry and Biophysics**, New York, v. 50, p. 169-179, 1954.

KURTZMAN, C. P.; ROBNETT, C. J. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. **Antonie van Leeuwenhoek**, Amsterdam, v. 73, n. 4, p. 331-371, May 1998.

KURTZMAN, C. P.; SUZUKI, M. Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces*. **Mycoscience**, Tokyo, v. 51, n. 1, p. 2-14, Jan. 2008.

LAGUNES-GÁLVEZ, S. L. et al. Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. **International Journal of Food Microbiology**, Amsterdam, v. 114, n. 1, p. 124-130, Jan. 2007.

LASS, R. A. Cacao growing and harvesting practices. In: KNIGHT, I. (Ed.). **Chocolate and cocoa:** health and nutrition. Oxford: Blackwell Science, 1999. p. 11-42.

LEAL JÚNIOR, G. A. et al. Fermentation of cacao (*Theobroma cacao* L.) seeds with a hybrid Kluyveromyces marxianus strain improved product quality attributes. **FEMS Yeast Research**, Amsterdam, v. 8, n. 5, p. 788-798, Aug. 2008.

LEFEBER, T. et al. Kinetic analysis of strains of lactic acid bacteria and acetic acid bacteria in cocoa pulp simulation media toward development of a starter culture for cocoa bean fermentation. **Applied and Environmnetal Microbiology**, Washington, v. 76, n. 23, p. 7708-7716, Dec. 2010.

_____. On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. **Food Microbiology**, London, 2011. In press.

LEHRIAN, D. W.; PATTERSON, G. R. Cocoa fermentation. In: REED, G. (Ed.). **Biotechnology, a comprehensive treatise**. Basel: Verlag Chemie, 1983. v. 5, p. 529-575.

LEROY, F.; VUYST, L. Lactic acid bacteria as functional starter cultures for the food industry. **Trends in Food Science and Technology**, Cambridge, v. 5, n. 1, p. 67-78, Jan. 2004.

LIMA, L. J. R. et al. *Theobroma cacao* L., "The Food of the Gods": quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. **Critical Reviews in Food Science and Nutrition**, Cleveland, v. 51, n. 8, p. 731-61, Sept. 2011.

LOPEZ, A.; QUESNEL, V. C. An assessment of some claims relating to the prodution and composition of chocolate aroma. **Revue Internationale de la Chocolaterie**, Paris, v. 26, p. 19-20, 1971.

MAGALHÃES, K. T. et al. Microbial communities and chemical changes during fermentation of sugary Brazilian kefir. **World Journal of Microbiology and Biotechnology**, Oxford, v. 33, n. 1, p. 1-10, Jan. 2010.

MARAVALHAS, N. Mycological deterioration of cocoa beans during fermentation and storage in Bahia. **International Chocolate Review**, New York, v. 21, p. 375-378, 1966.

MATSUSHITA, K.; TOYAMA, H.; ADACHI, O. Respiration chains and bioenergetics of acetic acid bacteria. **Advances in Microbial Physiology**, London, v. 36, n. 3, p. 247-301, May 1994.

MOTAMAYOR, J. C. et al. Cacao domestication I: the origin of the cacao cultivated by the Mayas. **Heredity**, Washington, v. 89, n. 1, p. 380-386, May 2002.

MOUNJOUENPOU, P. et al. Filamentous fungi producing ochratoxin A during cocoa processing in Cameroon. **International Journal of Food Microbiology**, Amsterdam, v. 128, n. 2, p. 234-241, Jan. 2008.

NDOYE, B. et al. *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**, Reading, v. 57, n. 7, p. 1576-1581, July 2007.

NICHOLLS, L. The fermentation of cacao. In: SMITH, H. H. (Ed.). **The fermentation of cacao**. London: J. Bale, 1913. p. 221-351.

NIELSEN, D. S. et al. The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods. **International Journal of Food Microbiology**, Amsterdam, v. 114, n. 1, p. 168-186, Jan. 2007.

_____. Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). **Yeast**, Davis, v. 22, n. 4, p. 271-284, Mar. 2005.

OLIJVE, W.; KOK, J. J. An analysis of the growth of Gluconobacter oxydans in chemostat cultures. **Archives of Microbiology**, New York, v. 121, p. 291-297, 1979.

OSTOVAR, K.; KEENEY, P. G. Isolation and characterization of microorganisms involved in the fermentation of Trinidad's cacao beans. **Journal of Food Science**, Chicago, v. 38, p. 611-617, 1973.

OUATTARA, H. G. et al. Implications of *Bacillus* spp. in the production of pectinolytic enzymes during cocoa fermentation. **World Journal of Microbiology and Biotechnology**, Oxford, v. 24, n. 9, p. 1753-1760, Sept. 2008.

_____. Molecular identification and pectate lyase production by *Bacillus* strains involved in cocoa fermentation. **Food Microbiology**, London, v. 28, n. 1, p. 1-8, Jan. 2011.

PACE, N. R. et al. The analysis of natural microbial-populations by ribosomal-RNA sequences. **Advances in Microbial Ecology**, New York, v. 9, n. 1, p. 1-55, Feb. 1986.

PAPALEXANDRATOU, Z. et al. Comparison of the bacterial species diversity of spontaneous cocoa bean fermentations carried out at selected farms in Ivory Coast and Brazil. **Food Microbiology**, London, v. 28, n. 5, p. 964-973, Aug. 2011.

PAPALEXANDRATOU, Z.; VUYST, L. Assessment of the yeast species composition of cocoa bean fermentations in different cocoa-producing regions using denaturing gradient gel electrophoresis. **FEMS Yeast Research**, Amsterdam, v. 7, p. 564-574, Nov. 2011.

PASSOS, F. M. L. et al. Characterization and distribution of lactic acid bacteria from traditional cocoa bean fermentations in Bahia. **Journal of Food Science**, Chicago, v. 49, n. 1, p. 205-208, Jan. 1984.

QUESNEL, V. C. Curing cocoa in the laboratory. In: COCOA, CHOCOLATE AND CONFECTIONERY ALLIANCE, l., 1957, London. **Proceedings…** London: University of Oxford, 1958. p. 150-155.

QUESNEL, V. C.; LOPEZ, A. A sweat-box for fermenting small samples of cocoa. **Tropical Agriculture**, Surrey, v. 52, p. 309-316, 1975.

RIBEIRO, N. C. A.; BEZERRA, J. L.; LOPEZ, A. Micobiota na fermentação do cacau no estado da Bahia, Brazil. **Revista Theobroma**, Itabuna, v. 16, n. 1, p. 47-55, jan./mar. 1986.

ROELOFSEN, P. A. Fermentation, drying, and storage of cocoa beans. **Advances in Food Research**, San Diego, v. 8, p. 225-296, 1958.

ROELOFSEN, P. A.; GIESBERGER, G. Onderzoekingen over cacaobereidung. **Archiv voor de Koffiecultur in Nederlandisch-Indie**, New Delhi, v. 16, n. 8, p. 1-159, Oct. 1947.

ROHAN, T. A. The precursors of chocolate aroma: a comparative study of fermented and unfermented beans. **Journal of the Science of Food and Agriculture**, London, v. 29, p. 456-459, 1964.

ROMBOUTS, J. E. Observations on the microflora of fermenting cocoa beans in Trinidad. **Society for Applied Bacteriology**, Danvers, v. 15, p. 103-111, 1952.

SAMAH, O. A.; PTIH, M. F.; SELAMAT, J. Biochemical changes during fermentation of cocoa beans inoculated with *Saccharomyces cerevisiae* (wild strain). **Journal of Food Science and Technology**, Trivandrum, v. 29, n. 5, p. 341-343, May 1992.

SANCHEZ, J. et al. A study of the yeast flora and the effect of pure culture seeding during the fermentation of cocoa beans. **Lebensmittel-Wissenschaft und Technologie**, London, v. 18, n. 1, p. 69-76, 1985.

SANCHEZ-HERVAS, M. et al. Mycobiota and mycotoxin producing fungi from cocoa beans. **International Journal of Food Microbiology**, Amsterdam, v. 125, n. 3, p. 336-340, July 2008.

SCHWAN, R. F. Cocoa fermentations conducted with a defined microbial cocktail inoculum. **Applied and Environmental Microbiology**, Washington, v. 64, n. 4, p. 1477-1483, Apr. 1998.

SCHWAN, R. F. et al. Characterization and distribution of aerobic, spore-forming bacteria from cacao fermentations in Bahia. **Journal of Food Science**, Chicago, v. 51, n. 6, p. 1583-1584, Nov. 1986.

_____. Influência da frequência e intervalos de revolvimentos sobre a fermentação de cacau e qualidade do chocolate. **Revista Agrotópica**, Itabuna, v. 2, n. 1, p. 22-31, 1990.

SCHWAN, R. F.; ROSE, A. H.; BOARD, R. G. Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. **Journal of Applied Bacteriology**, Oxford, v. 79, n. 1, p. 96-107, 1995.

SCHWAN, R. F.; WHEALS, A. E. The microbiology of cocoa fermentation and its role in chocolate quality. **Critical Reviews in Food Science and Nutrition**, Cleveland, v. 44, n. 4, p. 205-221, May 2004.

SENGUN, I. Y.; KARABIYIKLI, S. Importance of acetic acid bacteria in food industry. **Food Control**, Guildford, v. 22, n. 5, p. 647-656, May 2011.

SILVA, E. G. et al. Pectinolytic enzymes secreted by yeasts from tropical fruits. **FEMS Yeast Research**, Amsterdam, v. 5, n. 9, p. 859-865, Mar. 2005.

SMITH, H. H. **The fermentation of cocoa**. London: J. Bale, 1913. 665 p.

SNEATH, P. Endospore-forming Gram-positive rods and cocci. In: HOLT, J. G.; SNEATH, P. (Ed.). **Bergey's manual of systematic bacteriology**. Baltimore: Williams and Wilkins, 1986. v. 2, p. 1104-1206.

SOARES, M. M. C. N.; SILVA, R. da; GOMES, E. Screening of bacterial strains for pectinolytic activity: characterization of the polygalacturonase produced by *Bacillus* sp. **Revista de Microbiologia**, São Paulo, v. 30, n. 4, p. 299-230, out./dez. 1999.

SORIANO, M.; DIAZ, P.; JAVIER-PASTOR, F. I. Pectinolytic systems of two aerobic sporogenous bacterial strains with high activity on pectin. **Current Microbiology**, New York, v. 50, n. 1, p. 114-119, Jan. 2005.

STEINKRAUS, K. H. **Industrialization of indigenous fermented foods**. 2nd ed. Boca Raton: CRC, 2004. 600 p.

TAMBURINI, E. et al. Characterization of bacterial pectinolytic strains involved in the water retting process. **Environmental Microbiology**, Netherlands, v. 5, n. 9, p. 730-736, Sept. 2003.

THOMPSON, S. S.; MILLER, K. B.; LOPEZ, A. S. Cocoa and coffee. In: DOYLE, M. J.; BEUCHAT, L. R.; MONTVILLE, T. J. (Ed.). **Food Microbiology:** fundamentals and frontiers. Washington: ASM, 2001. p. 721-733.

_____. 3rd ed. Washington: ASM, 2007. p. 837-850.

TOMLINS, K. I. D.; BAKER, M.; DAPLYN, P. Effect of fermentation and drying practices on the chemical and physical profiles of Ghana cocoa. **Food Chemistry**, London, v. 46, n. 3, p. 257-263, Oct. 1993.

VUYST, L. et al. The functional role of 676 lactic acid bacteria in cocoa bean fermentation. In: MOZZI, F.; RAYA, R. R.; VIGNOLO, G. M. (Ed.). **Biotechnology of lactic acid bacteria:** novel applications. Ames: Wiley, 2010. p. 301-326.

_____. Validation of the (GTG)5-rep-PCR fingerprinting technique for rapid classification and identification of acetic acid bacteria, with a focus on isolates from Ghanaian fermented cocoa beans. **International Journal of Food Microbiology**, Amsterdam, v. 125, n. 1, p. 79-90, Jan. 2008.

WOLLGAST, J.; ANKLAM, E. Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. **Food Research International**, Barking, v. 33, n. 6, p. 423-447, July 2000.

WOOD, B. J. B. **Microbiology of fermented foods**. 2nd ed. London: Thompson Science, 1998. 886 p.

WOOD, G. A. R. **Cocoa**. 3rd ed. Whitstable: D. Rhind, 1975. 292 p.

WOOD, G. A. R.; LASS, R. A. **Cocoa**. 4th ed. London: Longman, 1985. 620 p.

YAMADA, Y.; YUKPHAN, P. Genera and species in acetic acid bacteria. **International Journal of Food Microbiology**, Amsterdam, v. 125, n. 1, p. 96-101, Jan. 2008.

ZAK, D. L.; KEENEY, P. G. Changes in cocoa proteins during ripening of fruit, fermentation, and further processing of cocoa beans. **Journal of Agricultural and Food Chemistry**, Easton, v. 24, p. 483-486, 1976.

SEGUNDA PARTE - ARTIGOS

ARTIGO 1

SPONTANEOUS COCOA BEAN FERMENTATIONS CONDUCTED IN WOODEN BOXES AND STAINLESS STEEL TANK: ANALYSIS OF PHYSICAL-CHEMICAL PROPERTIES AND MICROBIAL COMMUNITY STRUCTURE AND DYNAMICS

(Artigo submetido ao periódico indexado: Applied Microbiology and Biotechnology)

Abstract

Spontaneous cocoa bean fermentations performed in 40-kg stainless steel tank (SST), 40-kg wooden boxes (WB1), and 600-kg wooden boxes (WB2) were studied using a multiphasic approach that entailed culture-dependent and -independent microbiological analyses of fermenting cocoa pulp-bean samples and target metabolite analyses of both cocoa pulp and cotyledons. Both microbiological approaches revealed that the dominant species of major physiological roles were the same for fermentation in SST and boxes. These species consisted of *Saccharomyces cerevisiae* and *Hanseniaspora* sp. in the yeast group; *Lactobacillus fermentum* and *L. plantarum* in the lactic acid bacteria (LAB) group; *Acetobacter tropicalis* belonging to the acetic acid bacteria (AAB) group; and *Bacillus subtilis* in the *Bacillaceae* family. A greater diversity of bacteria and non-*Saccharomyces* yeast was observed in box fermentations. Additionally, a potentially novel AAB belonging to the genus *Asaia* was isolated during fermentation in WB1. Cluster analysis of the rRNA genes-PCR-DGGE profiles revealed a complex picture on the DGGE gels of the box samples, indicating that bacterial and yeast ecology were fermentation-specific processes (wooden boxes *vs.* SST). The profile of carbohydrate consumption and fermentation products in the pulp and beans showed similar trends during both fermentation systems. However, carbohydrate-ethanol-acetic fermentation was achieved with greater efficiency in SST, while temperatures were generally higher during fermentation in wooden boxes. With further refinements, the SST model may be useful in designing novel bioreactors for the optimisation of cocoa fermentation with starter cultures. Some potential industrial applications for SST bioreactors are assessed and discussed.

Keywords: Chocolate, cocoa fermentation, stainless steel fermenter, lactic acid bacteria, acetic acid bacteria, yeast.

1. Introduction

Processing of cocoa beans into cocoa-derived foods, such as cocoa powder, chocolate, and other cocoa-related products, begins with the on-farm fermentation and drying of the beans, followed by roasted during industrial processing (Schwan 1998; Schwan and Wheals 2004; Nielsen et al. 2007). The post-harvest processes are crucial in determining the quality of the finished products, as they initiate the formation of chocolate flavour precursors and the brown colouration of cocoa products (Schwan et al. 1995). Therefore, fermentation of cocoa beans is the first step of the entire chocolate-making process. Prior to exportation, fermentation is performed as a traditional indigenous process in cocoa-producing countries of the equatorial zone, led by the Ivory Coast, Ghana, Nigeria, Cameroon, and Brazil (Camu et al. 2008).

Cocoa bean fermentation provides a man-made ecological niche containing a remarkable diversity of microbial species. Fermentation refers to the microbial activity in the pulp and the metabolites formed therein, which diffuse into the seeds and causes a series of chemical reactions (Lima et al. 2011). During the fermentation process occurs a well-defined microbial succession that is initially dominated by yeasts and subsequently surpassed by LAB; this species then declines after 48 h of fermentation and gives rise to the aggressive development of AAB. Towards the end of the some fermentation process, *Bacillus* spp. predominates over all other microbial groups (Schwan and Wheals 2004). The cocoa pulp is hydrolysed during fermentation; this aids the drying process by allowing the pulp to be drained. Most importantly, fermentation triggers an array of chemical changes within the cocoa bean that are vital to the development of the complex, beloved flavour known as "chocolate".

Cocoa fermentation is an empirical procedure and does not give rise to beans of consistent quality, which requires processors to continuously modify

their blends (Lagunes-Gálvez et al. 2007). Common problems involve levels of acidity, incomplete fermentation, which results in a lack of cocoa flavour, or off-flavours resulting from over-fermentation and spoilage of beans; all of these problems reduce crop value for the farmer (Schwan 1998). While experimental applications of defined starter cultures have produced satisfactory results (Samah et al. 1992; Schwan 1998; Leal et al. 2008), this technique has not been implemented in the field. One limitation to the introduction of starter cultures is that the great majority of cocoa fermentations are conducted over banana leaves or in wooden boxes that facilitate contamination by natural microbiota.

The demand for hygienic production practices has increased the appeal of using stainless steel tanks in industrial bioprocesses (*e.g.*, the production of beer, wine and cider). As cocoa bean fermentation involves a complex microbial succession, the use of a stainless steel tank will be a challenging prospect, and strategies specific to this scenario need to be developed to avoid an unsuccessful campaign of process-validation. Both the biology and chemistry of cocoa bean fermentation are complex and all aspects of the process need to be subjected to comprehensive, simultaneous, dynamic analyses to quantitatively predict the outcomes of defined changes. Understanding the microbial ecology of the fermentation process is the first stage in developing a new procedure and optimising both processing efficiency and the quality of its end products (Schwan 1998).

In the present study, spontaneous cocoa fermentation was performed in a stainless steel tank. In comparison to traditional Brazilian methods of fermentation in wooden boxes, the stainless steel tank was a novel design that was capable of turning a 40 kg load of beans. Validation of the microbial fermentations was based on both culture-dependent and culture-independent approaches, which were applied to assess the dynamics of yeast and bacteria during the fermentation processes. In parallel, a link was established between the

substrates and metabolites found in pulp and beans, and the microbes responsible for these reactions were identified.

2. Materials and Methods

2.1 Fermentation experiments

Fermentation experiments were conducted at a cacao farm in Itajuípe, Bahia State, Brazil, belonging the Marsterfoods Company. The ripe cocoa pods of mixed-hybrids were harvested during the main crop. Cocoa pods were broken open manually with a machete, and the beans were immediately transferred to the fermentation house. Fermentation was performed in 1 m^3 and 0.06 m^3 wooden boxes capable of holding 600 kg and 40 kg of cocoa beans, respectively, or in a 40-kg-capacity stainless steel conical tank (0.05 m^3; noncommercial bioreactors). The stainless steel tank design was as described by Schwan and Wheals (2004). The wooden boxes and the stainless steel tank contained holes at the bottom to allow drainage of liquid sweatings generated during fermentation. The wooden boxes were covered with fresh banana leaves and the stainless steel tank was covered with a polypropylene lid to ensure adequate insulation. All fermentations were turned every 24 h and were performed simultaneously to exclude as many environmental factors as possible (*e.g.*, harvest season and external contamination). Natural fermentation proceeded at ambient temperature for 6 days.

2.2 Sampling

Every 12 hours during the fermentation process, 200 g of each sample was collected randomly, placed in sterile bags and transferred to the laboratory. Samples collected for chemical and culture-independent analyses were sealed in plastic bags and stored in a freezer at -20 °C. Microbiological analyses were performed on the day of sample collection.

2.3 Cultivation-based quantification of microorganisms

Freshly acquired samples were immediately plated in four different culture media following appropriate dilution for enumeration *via* cell counting. For culture-based quantification, 25 g of cocoa beans and adherent pulp were added to 225 ml saline-peptone water [(v/v) (0.1% bacteriological peptone (Himedia), 0.8% NaCl (Merck, Whitehouse Station, USA)], homogenised in a stomacher at normal speed for 5 min (10^{-1} dilution) and diluted serially. Lactic acid bacteria (LAB) were enumerated by pour plate inoculation in MRS agar (Merck) containing 0.2% (v/v) sorbic acid (Merck), 0.1% (v/v) cycloheximide (Merck) to inhibit yeast growth, and 0.1% (v/v) cystein-HCl to maintain anaerobic conditions during incubation. Acetic acid bacteria (AAB) were enumerated by surface inoculation on GYC agar [50 g/l glucose (Merck), 10 g/l yeast extract (Merck), 30 g/l calcium carbonate (Merck) and 20 g/l agar (Merck), pH=5.6] supplemented with 0.1% cycloheximide to inhibit yeast growth and 50 mg/l penicillin (Sigma, St. Louis, USA) to inhibit LAB growth]. Yeast were enumerated by surface inoculation on YEPG agar [1% yeast extract (Merck), 2% peptone (Himedia), 2% glucose (Merck) at pH 5.6] containing 100 mg/l chloramphenicol (Sigma) and 50 mg/l chlortetracycline (Sigma) to inhibit bacterial growth. Nutrient agar (Merck) containing 0.1% cycloheximide (Merck) was used as a general medium for viable mesophilic bacteria populations and *Bacillus* spp. After bacterial spreading, the plates were incubated at 30 °C for 3-4 days for MRS, YEPG and Nutrient agar cultures. GYC agar cultures were incubated at 25 °C for 5-8 days. Following incubation, the number of colony-forming units (CFU) was recorded, and each colony type was morphologically characterised and counted. The square root of the number of colonies counted for each type were re-streaked and purified. Purified isolates of GYC, YEPG, and Nutrient agar were stored at -80 °C in YEPG broth containing 20% glycerol

(w/w). Isolates from MRS agar were stored at -80 °C in MRS broth containing 20% glycerol (w/w).

2.4 Identification of microorganisms

Phenotypic characterisation of bacterial colonies originating from MRS, GYC, and Nutrient agar plates was performed using conventional microbiological methods, including gram staining in conjunction with microscopic examination, determination of catalase and oxidase activity, motility tests and assessment of the following: spore formation; acid and gas production from glucose; acid and gas production from lactate and acetate (only for presumptive AAB isolates from GYC agar plates showing clear zones around the colonies). Yeast colonies were physiologically characterised by determination of their morphology, spore formation, and fermentation of different carbon sources, as described by Kurtzman et al. (2011).

Representative microbial strains were identified by sequence analysis of the full-length 16S rRNA gene or the ITS region for bacteria and yeast, respectively. Bacterial and yeast cultures were grown under appropriate conditions, collected from agar plates with a sterile pipette tip and resuspended in 40 µl of PCR buffer. The suspension was heated for 10 min at 95 °C and 1 µl was used as a DNA template in PCR experiments. For bacterial isolates from MRS and Nutrient agar plates, 16S-rRNA-PCR was performed using primers 27-F and 1512-R (Magalhães et al. 2010). For bacterial isolates from GYC agar plates showing clear zones around the colonies, 16Sd and 16Sr primers were used to amplify the 16S rRNA gene region conserved among AAB according to the method described by Ruiz et al. (2000). For yeast isolates, ITS-PCR was performed with primers ITS1 and ITS4 (Nielsen et al. 2007). Confirmation of isolates as *S. cerevisiae* was accomplished through a species-specific PCR assay with HO gene-derived primers (Pereira et al. 2010). The rRNA gene region was

amplified in a Thermo PCYL220 thermal cycler (Thermo Fisher ScientiWc Inc., Waltham, USA) and the PCR products were sequenced in an ABI3730 XL automatic DNA sequencer. The sequences were aligned using the BioEdit 7.7 sequence alignment editor and were compared to the GenBank database using the BLAST algorithm (National Centre for Biotechnology Information, Maryland, USA).

To differentiate AAB species indicated by their rRNA gene sequences to be closely related, some specific biochemical tests were performed to validate the 16 rRNA sequence data. Growth in 30% D-glucose, 0.3% maltose, 0.3% methanol, and 10% ethanol was examined using basal medium (0.05% yeast extract, 0.3% (w/v) vitamin-free casamino acids, and 2.5% agar) and appropriate concentrations of carbon sources. Medium without a supplemental carbon source was used as a control. The extent of growth in each medium was checked after 7 days of incubation at 28 °C. The utilization of ammonium as the sole nitrogen source in the presence of ethanol as carbon source was tested using Frateur's modified Hoyer ethanol/vitamins medium containing 2.5% agar. The production of acid from D- and L-arabitol was tested in phenol-red broth with the carbon source added to a final concentration of 1 %. The results were assessed with relative to the control medium after incubation at 28 °C for 48 h. All tests were performed as described previously (Joyeux et al. 1984; Ruiz et al. 2000).

2.5. Microbial community analysis through nested PCR–denaturing gradient gel electrophoresis (DGGE)

2.5.1 Total-community DNA isolation

Cocoa beans and pulp were physically separated by adding 100 ml of sterile distilled water to 100 g of beans and adherent pulp in a plastic bag. The beans were homogenised in a stomacher at normal speed for 5 min, and the pulp

fraction was recovered via decantation. The pulp fraction (40 ml) was lyophilised and freeze-dried cocoa pulp was ground thoroughly with a sterile pestle. Freeze-dried pulp (30 mg) was mixture homogenised twice in 1.5 ml of phosphate-buffered saline. The combined fluids were mixed for an additional 10 min and were then centrifuged to remove large particles at 100 × g for 10 min at 4 °C. The supernatant was then centrifuged at 8,000 × g for 20 min at 4 °C to pellet the yeast and bacterial cells, which were subsequently frozen at -20 °C for at least 1 h; this procedure was performed twice. Bacterial cells were lysed using the method described by Pereira et al. (2011). For the lysis of yeast cells, cell pellets were resuspended in 600 µl sorbitol buffer (1 M sorbitol, 100 mM EDTA, and 14 mM β-mercaptoethanol) with 10 mg/ml of lysing enzymes and incubated at 30 °C for 1 h. Spheroplasts were obtained by centrifugation at 5,000 × g for 5 min and were resuspended in 180 µl ATL Buffer (supplied in the QiAamp DNA Mini Kit, Qiagen, Hilden, Germany). After lysis, DNA in the supernatant was purified in accordance with the instructions in step 4 (bacteria procedure) and from step 2 (yeast procedure), which are described in "Protocol: DNA Purification from Tissues" (Qiaamp DNA Mini Kit, Qiagen). The resultant samples were stored at -20 °C for later use.

2.5.2 Nested PCR–DGGE strategy

To increase sensitivity, and to facilitate DGGE by analysing fragments of the same length, a two-step nested PCR technique was utilised. To analyse bacterial diversity, primers 27F and 1512R were used to amplify the near-complete 16S rRNA-encoding gene in the first amplification step, which was performed under conventional PCR conditions (Magalhães et al. 2010). The product of this first PCR reaction was used as a template for a nested PCR reaction that amplified the V3 region of the 16S rRNA gene with GC-338f and 518r primers (Ovreas et al. 1997); this generated a DNA fragment suitable for

DGGE analysis. To analyse yeast diversity, the ITS regions were amplified by PCR with primers ITS1-F and ITS4; the amplification product was then amplified with the nested DGGE primers GC-ITS1-F and ITS2 (White et al. 1990). PCR reactions were performed in a Mastercycler (Eppendorf, Hamburg, Germany). The PCR products were analysed by DGGE using a BioRad DCode universal mutation detection system (BioRad, Richmond, CA, USA). The PCR products of the second step were separated via electrophoresis in 8% (w/v) polyacrylamide gels in running buffer containing 1 x TAE (20 mM Tris, 10 mM acetate, and 0.5 mM EDTA at pH 8.0). Optimal separation for bacterial communities was achieved with a 30–55% urea-formamide denaturing gradient, while a 12–60% gradient yielded optimal separation for yeast communities (100% correspondent to 7 M urea and 40% (v/v) formamide).

To estimate the differences in microbial community structure and dynamics for different fermentation processes, band positions on DGGE patterns were analysed by hierarchical cluster analysis performed with Bionumerics version 6.50 (Applied Maths, Sint-Martens-Latem). Dendrograms were calculated on the basis of Dice's Coefficient of similarity using the unweighted pair group method with the arithmetic averages clustering algorithm (UPGMA).

2.5.3 Sequencing of DGGE bands

DGGE bands of interest were excised from the gel with a sterile scalpel, disrupted in 60 µl of sterile Milli-Q water, and left overnight at 4 °C to allow the DNA to diffuse out of the gel. 10 µl of eluted DNA from each DGGE band was subjected to re-amplification reactions using appropriate primers and the conditions described above. The sequencing products were purified with a QIAquick PCR purification kit (Qiagen) and sequenced in an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). GenBank BLAST

searches were performed to determine the closest known relatives of the partial ribosomal DNA sequences obtained.

2.6 Physical-chemical analysis

For physical-chemical analysis, the beans and pulp were physically separated according to the protocol of Ardhana and Fleet (2003) and Nielsen et al. (2007). To evaluate titrable acid and pH of both pulp and beans, an aliquot was transferred to a beaker and analysed with a pH meter. An additional 25 ml aliquot was titrated to pH 8.1 with 0.1 N NaOH. The values were reported as Eq of sodium hydroxide per 100 g of dry beans. These measurements were performed in triplicate for each fermentation process. The ambient temperature and temperatures of the fermenting cocoa pulp-bean mass were determined every 2 h with a model HD 2105.2 Delta OHM portable datalogger.

To analyse target metabolites, aqueous extracts from fermentation samples were obtained as described previously (Schwan 1998). The presence of alcohols (ethanol and methanol), organic acids (lactic, acetic, and citric acid), and carbohydrates (glucose, sucrose, and fructose) were determined for pulp and bean extracts by high-performance liquid chromatography (HPLC) apparatus (HP series 1200, Hewlett-Packard Company, USA) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, USA) that was connected to a RI detector (HPG1362A, Hewlett-Packard Company). The column was eluted with a degassed mobile phase containing 4 mM H_2SO_4, at 30 °C at a flow rate of 0.6 ml/min.

3 Results

3.1 Cultivation-based quantification of microorganisms

Selective media and/or growth conditions were used to determine the relative abundance of total aerobic bacteria, LAB, AAB and yeasts in each of the fermentation processes (Fig. 1a).

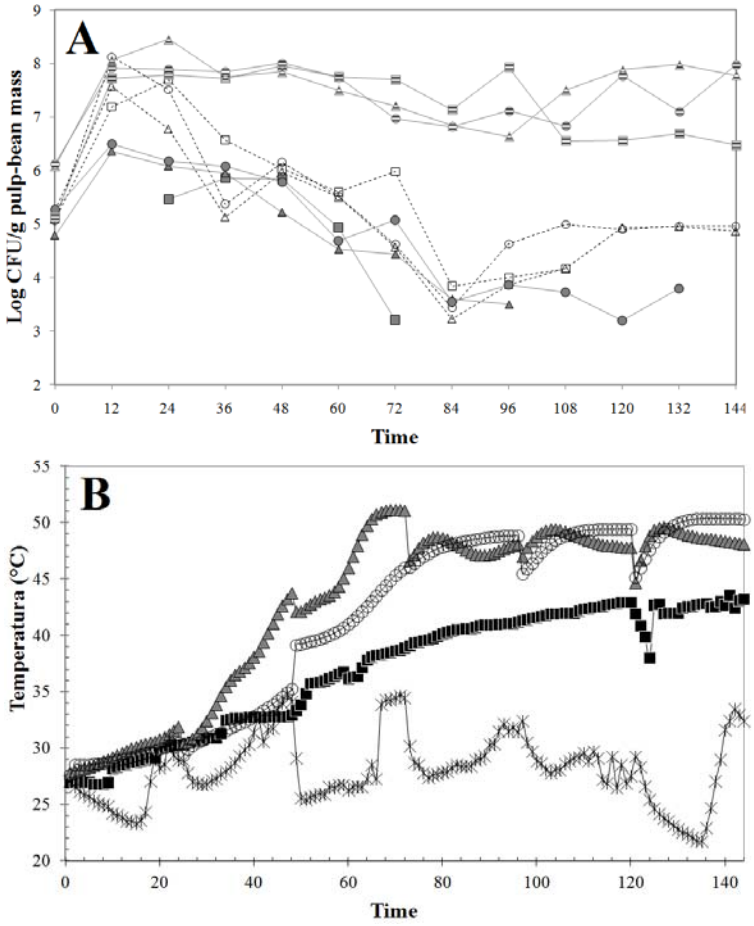

Figure 1 (A) Evolution of LAB in MRS medium (traced symbols), yeast in YEPG medium (open symbols), and AAB in GYC medium (solid symbols). Symbols: ▬, □ and ■ SST; ▲, △ and ▲ WB1; ▬, ○ and ● WB2. (B) Temperature inside the SST ■, WB1▲, and WB2 ○. Ambient temperature ✳

The results of these analyses demonstrated a nearly 2-log increase in the number of LAB, AAB and yeast during a period of 12-24 h in all fermentation processes, with the exception of AAB growth in samples derived from SST fermentation. LAB was the dominant population and showed similar trends in each fermentation process. There was a marked drop in the post-peak LAB phase followed by a slight increase after 84 h of fermentation. Although Nutrient agar medium was used to monitor the growth of aerobic mesophilic bacteria and *Bacillus* spp., several gram-negative and aerobic rods that produced catalase but not formed spores (later identified as AAB) grew on this medium (data not shown).

Yeast demonstrated a typical growth profile in cocoa fermentation that was characterised by a peak between 12 and 24 h, followed by a decrease between 24 and 84 h and a slight increase near the conclusion of the process. However, characteristics of these phases varied considerably for fermentations conducted in SST relative to those conducted in boxes. In both fermentations performed in boxes, yeasts were present throughout the fermentation process and peaked at 12 h (7.57 log CFU g^{-1} and 8.12 log CFU g^{-1} for WB1 and WB2, respectively). In contrast, for fermentation performed in SST, maximum populations were observed at 24 h (7.69 log CFU g^{-1}) and no yeast was found after 108 h. AAB were present throughout WB1 and WB2 fermentations, reaching values between 4.79 log CFU g^{-1} and 6.36 CFU g^{-1}, and between 5.27 log CFU g^{-1} and 6.49 log CFU g^{-1}, respectively. No AAB were detected in samples obtained at the onset of SST fermentation but were subsequently observed at 24 h (5.47 log CFU g^{-1}) and were not present beyond 60 h of fermentation.

3.2 Identification of microorganisms

A total of 1099 isolates were randomly picked up and characterised in terms of cell morphology and biochemical features. Of this total, 315 isolates were identified as LAB, 238 were identified as AAB, and 323 were identified as yeast. The remaining 223 isolates were recovered from Nutrient agar plates and exhibited broad phenotypic diversity. After initial characterisation, the isolates were grouped according to their distributions among different times and fermentation processes, as well as their morphological and physiological characteristics.

Of 323 isolates from YEPG agar, 147 yeast isolates were selected for sequence-based identification (Table 1-3).

Table 1 Species isolated from SST

Closest NCBI match	Accession number	Fermentation time (h)												
		0	12	24	36	48	60	72	84	96	108	120	132	144
Yeast, log(CFU g^{-1})														
S. cerevisiae	AM711362.1	5.09	7.15	7.69	6.47	6.03	5.06	5.98	3.84	4.00	3.98	<1	<1	<1
P. kluyveri	FM199971.1	<1	<1	<1	6.04	<1	<1	<1	<1	<1	<1	<1	<1	<1
H. uvarum	FJ515178.1	<1	<1	<1	<1	<1	4.00	5.08	<1	<1	<1	<1	<1	<1
Wickerhamomyces sp.	FJ873446.1	<1	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
C. orthopsilosis	FM199967.1	<1	<1	<1	5.00	<1	<1	<1	<1	<1	<1	<1	<1	<1
I. orientalis	EU315767.1	<1	<1	<1	5.00	<1	<1	<1	<1	<1	<1	<1	<1	<1
LAB, log(CFU g^{-1})														
L. plantarum	HQ293084.1	5.11	7.54	<10	<10	<10	<10	7.53	7.01	<10	<10	<10	<10	<10
L. fermentum	HQ293040.2	5.01	7.61	7.70	7.72	7.95	7.75	7.04	6.98	7.93	6.55	6.56	6.69	6.48
Weissella fabaria/ghanensis														
AAB, log(CFU g^{-1})														
A. pomorum	AB569643.1	<1	<1	<1	4.00	4.00	4.61	<1	<1	<1	<1	<1	<1	<1
A. malorum	FJ831444.1	<1	<1	5.38	<1	<1	4.04	3.18	<1	<1	<1	<1	<1	<1
A. tropicalis	DQ523494.1	<1	<1	5.04	5.69	5.73	4.34	3.09	<1	<1	<1	<1	<1	<1
Other species, log(CFU g^{-1})														
*Acetobacte*r ssp.	--	<1	<1	6.00	5.98	6.00	<1	<1	<1	<1	<1	<1	<1	<1
B. subtilis	HQ286641.1	4.48	6.42	6.59	6.32	7.03	7.04	7.19	7.11	<1	<1	<1	<1	<1
B. megaterium	*FJ174651.1*	4.56	6.83	7.04	6.97	7.12	7.09	7.02	7.07	<1	<1	<1	<1	<1
Bacillus sp.	GU217692.1	<1	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Mu.luteus	HM449702.1	4.00	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
M. testaceum	HM449703.1	4.00	6.00	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
P. terrea	HM562993.1	4.08	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

Abbreviations: S.: *Saccharomyces*, P.: *Pichia*, H.: *Hanseniaspora*, C.: *Candida*, I.: *Issatchenkia*, L.: *Lactobacillus*, W.:*Weissella*, A.: *Acetobacter*, B.: *Bacillus*, Mu. *Micrococcus*: M.: *Microbacterium*, P. *Pantoea*

Table 2 Species isolated from WB1

		Fermentation time (h)												
Closest NCBI match	Accession number	0	12	24	36	48	60	72	84	96	108	120	132	144
Yeast, log(CFU g-1)														
S. cerevisiae	AM711362.1	5.51	7.57	6.42	5.13	5.97	5.04	<1	<1	3.77	4.09	5.04	4.79	4.68
P. kluyveri	FM199971.1	4.47	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	3.00	3.21
Sz. pombe		4.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
H. uvarum	FJ515178.1	<1	<1	<1	<1	<1	<1	<1	<1	<1	3.00	0.00	3.00	0.00
D. etchellsii	AJ586528.1	<1	<1	<1	<1	<1	5.32	4.57	3.23	<1	<1	<1	<1	<1
C.humilis	AY493349.1	<1	<1	5.60	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
C. inconspicua	EU315757.1	<1	<1	5.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
C. ethanolica	AB534618.1	<1	<1	<1	<1	<1	<1	<1	<1	2.30	<1	<1	<1	<1
LAB, log(CFU g-1)														
L. plantarum	HQ293084.1	6.13	8.06	8.45	7.45	7.56	<10	<10	<10	5.00	<10	<10	7.04	7.17
L. fermentum	HQ293040.2	<10	<10	<10	7.17	7.25	7.50	7.21	6.84	6.48	7.50	7.88	7.67	7.51
AAB, log(CFU g^{-1})														
A. pomorum	AB569643.1	<1	<1	<1	<1	<1	3.00	<1	<1	2.00	<1	<1	<1	<1
A. malorum	FJ831444.1	<1	5.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Acetobacter sp.	DQ887340.1	<1	6.04	<1	<1	<1	<1	<1	<1	2.00	<1	<1	<1	<1
A. tropicalis	DQ523494.1	4.43	6.26	6.04	5.65	5.22	4.47	4.44	3.60	3.26	<1	<1	<1	<1
Asaia sp.	HM051372.1	3.60	5.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Gluconobacter sp.	HM051360.1	<1	<1	<1	<1	4.11	<1	<1	<1	<1	<1	<1	<1	<1
G. oxydans	AB540150.2	<1	<1	5.00	4.85	4.92	<1	<1	<1	<1	<1	<1	<1	<1

Table 2, continuation

Other species, log(CFU g^{-1})														
Acetobacter ssp.	--	<1	<1	7.00	<1	<1	<1	6.00	6.00	6.32	<1	<1	<1	<1
B. subtilis	HQ286641.1	<1	7.04	7.23	6.18	6.47	7.29	6.35	6.30	7.30	<1	<1	<1	6.87
B. flexus	DQ870687.1	<1	<1	<1	<1	<1	<1	<1	6.04	6.57	<1	<1	<1	<1
Paenibacillus sp	FJ899682.1	<1	<1	<1	<1	<1	<1	<1	6.14	<1	<1	<1	<1	<1
Xanthomonas sp.	GQ250436.1	<1	<1	<1	<1	<1	<1	<1	<1	<1	6.00	7.17	6.92	0.00
P. terrea	HM562993.1	4.23	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
T. saanichensis	EU215774.1	4.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
T. ptyseos	EU877958.1	4.00	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Ac. baumannii	DQ379505.1	4.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Brevundimonas sp.	DQ066442.1	4.36	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Ch.letacus	EU121860.1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	6.47
St. aureus	FJ899095.1	4.00	6.14	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
St. xylosus	HM854231.1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	6.32	<1
K. variicola	HQ407252.1	4.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

Abbreviations: Sz. *Schizosaccharomyces*, D.: *Debaromyces*, G.: *Gluconobacter*,T.: *Tatumella*, Ac.: *Acinetobacter*, Ch.: *Chryseobacterium*, St.: *Staphylococcus*, K.: *Klebsiella*. Other abbreviations see Table 1

Table 3 Species isolated from WB2

Closest NCBI match	Accession number	Fermentation time (h)												
		0	12	24	36	48	60	72	84	96	108	120	132	144
Yeast, log(CFUyeast g^{-1})														
S. cerevisiae	AM711362.1	5.06	7.91	7.51	5.17	6.15	5.46	4.62	3.44	4.17	<1	<1	4.19	4.17
P. kluyveri	FM199971.1	<1	7.46	0.00	5.04	<1	<1	<1	<1	4.04	<1	<1	3.00	<1
Sz. pombe	AY046223.1	<1	<1	<1	6.00	<1	6.00	<1	<1	<1	<1	<1	<1	<1
H.uvarum	FJ515178.1	<1	6.00	<1	<1	<1	<1	<1	<1	<1	4.99	4.04	4.86	4.52
C. xylopsoci	FM178339.1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	3.00	3.00	4.06
C. intermedia	EF568011.1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
C. xylopsoci	FM178339.1	<1	<1	<1	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1
Wickerhamomyces sp.	FJ873446.1	<1	<1	<1	<1	<1	<1	<1	<1	3.00	<1	<1	<1	<1
I. orientalis	EU315767.1	0.00	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	3.00
LAB, log(CFULAB/g)														
L. plantarum	HQ293084.1	5.72	7.32	7.59	7.54	7.00	6.87	<10	<10	<10	<10	<10	<10	<10
L. fermentum	HQ293040.2	5.98	7.13	7.24	6.93	8.01	7.76	6.97	6.82	7.12	6.83	7.77	7.10	7.97
AAB, log(CFUAAB/g)														
A. malorum	FJ831444.1	5.18	5.92	6.03	5.87	<1	<1	<1	<1	<1	<1	<1	<1	<1
A. tropicalis	DQ523494.1	5.04	5.76	6.14	6.08	5.92	4.17	4.92	3.23	3.76	3.73	3.20	3.80	<1
A. ghanensis	HM562984.1	<1	<1	<1	<1	<1	4.31	4.06	3.06	<1	<1	<1	<1	<1
A. senegalensis	AM748710.1	<1	<1	<1	<1	4.00	<1	<1	<1	<1	<1	<1	<1	<1
A. orientalis	HM217982.1	4.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
G. oxydans	AB540150.2	5.12	5.32	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

Table 3, continuation

Other species, log(CFU/g)														
Acetobacter ssp.	--	<1	5.00	<1	6.00	6.98	6.43	<1	<1	<1	<1	<1	<1	<1
B. circulans	FN393823.1	5.89	<1	<1	<1	<1	7.21	<1	<1	<1	<1	<1	<1	<1
B. subtilis	HQ286641.1	<1	5.98	6.76	7.06	7.14	7.03	7.25	7.16	7.28	7.08	7.32	7.58	7.15
T. saanichensis	EU215774.1	5.00	5.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
F. aurantia	AB091200.1	5.00	5.23	6.00	<1	6.00	6.00	<1	<1	<1	<1	<1	<1	<1
P. terrea	HM562993.1	5.00	5.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Xanthomonas sp.	GQ250436.1	<1	<1	<1	<1	<1	<1	<1	<1	<1	6.00	6.00	<1	<1
St. pasteuri	HM130543.1	5.00	<1	<1	<1	6.00	6.00	<1	<1	<1	<1	<1	<1	<1
St. xylosus	HM854231.1	<1	<1	<1	<1	7.08	<1	<1	<1	<1	<1	<1	<1	<1
St. saprophyticus	HM130543.1	<1	<1	<1	<1	6.00	<1	<1	<1	<1	<1	<1	<1	<1

Abbreviations: F.: *Frateuria*. Other abbreviations see Table 1 and II

The morphotypes most commonly recovered on YEPG agar plates were shared by a wide variety of species, or even, some species showed different responses in the complex colony morphology (Fig. S1); these species belonged to the *Saccharomyces cerevisiae* (*n*=64), *Hanseniaspora uvarum* (*n*= 31), *Pichia kluyveri* (*n*=17), and *Debaromyces etchellsii* (*n*= 14) (Table 1-3). On the other hand, the isolates that were less recovered showed a species-specific pattern of colony growth and architecture (Fig. S1), and were identified as *C. inconspicua* (*n*=5), *Issatchenkia orientalis* (*n*=4), *C. humilis* (*n*=3), *Schizosachoromyces pombe* (*n*=3), *C. ethanolica* (*n*=2), *C. intermedia* (*n*=2), *C. orthopsilosis* (*n*=1), and *C. xylopsoci* (*n*=1) (Table 1-3).

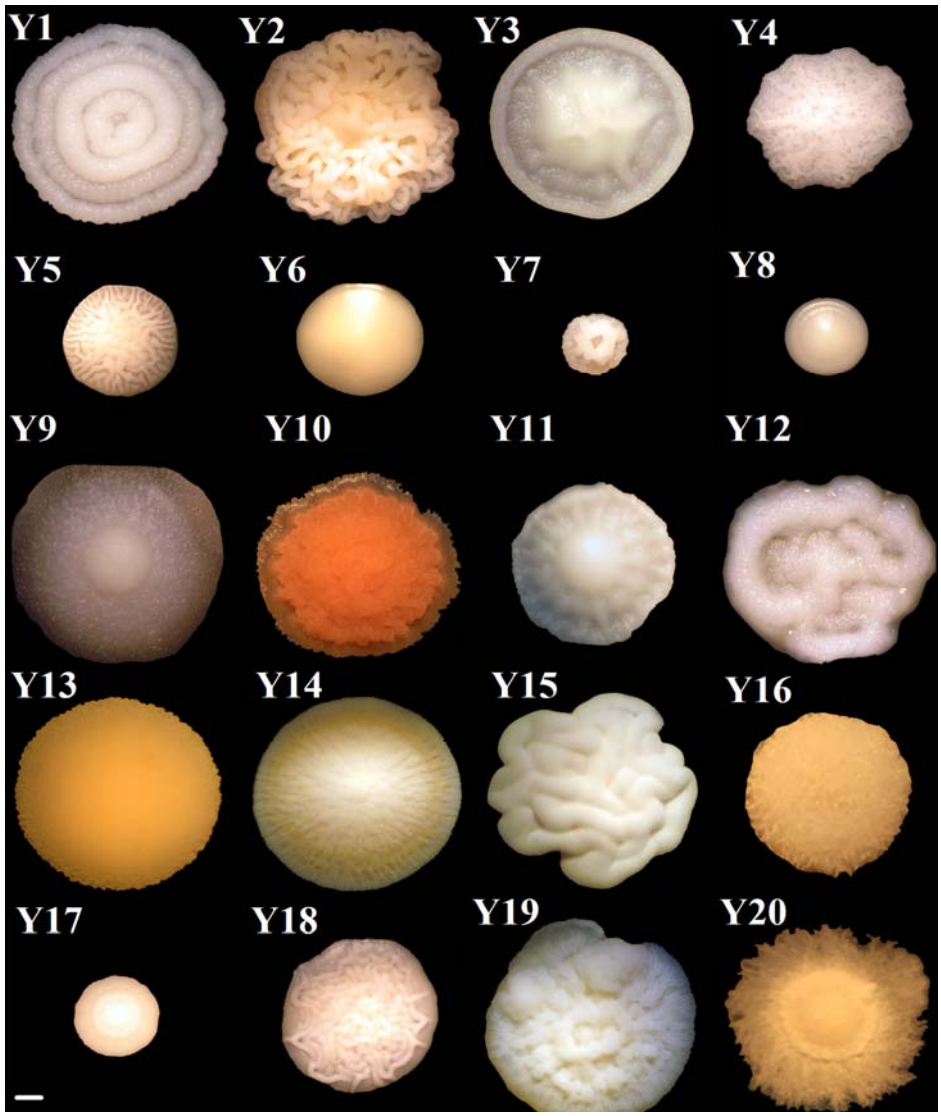

Figure 1 S1. Different colony morphology of the dominant yeast species associated with cocoa beans fermentations performed. Morphotypes Y1, Y2, Y3, Y5, Y7 and Y8 - *S. cerevisiae*; Y4 and Y6 - *S. cerevisiae* or *H.uvarum* or *P. kluyveri* or *D. etchellsii*; Y11- *S. cerevisiae* or *H.uvarum*; Y18 - *S. cerevisiae* or *D. etchellsii*; Y9 - *C. orthopsilosis*; Y10 - *C. ethanolica*; Y12 - *C. inconspicua*; Y13 - *I. orientalis*; Y14 - *C. intermedia*; Y15 - *C.humilis*; Y16 - *P. kluyveri*; Y17 - *Sz. pombe*, Y19 - *Wickerhamomyces sp*; Y20 *C. xylopsoci*. Scale bars is 1 mm

A total of 104 AAB isolates were selected for sequence-based identification. Of these, 89 were assigned to the genus *Acetobacter*, since acetate and lactate were intensely oxidized to CO_2 and H_2O, and 15 isolates were assigned to the genus *Gluconobacter*, since acetate and lactate were not oxidized. Sequence analysis of the 16S rRNA gene confirmed that these isolates belonged to the genera *Acetobacter* and *Gluconobacter*, with the exception of 3 isolates that showed weak oxidation of acetate and lactate; these isolates were assigned to the genus *Asaia* (Table 1-3). Sequence-based identification alone was not sufficient to differentiate some AAB species because 16S rRNA sequences of the type strains exhibited greater than 99% similarity. Thus, the biochemical tests were reliable for allocating isolates at the species level. Using a combination of both approaches, it was determined that the AAB isolates consisted of the following species: *Acetobacter tropicalis* (*n*=65), *A. malorum* (*n*=11), *A. pomorum* (*n*=6), *A. ghanensis* (*n* =5), *A. orientalis* (*n*=1), *A. senegalensis* (*n*=1), *Gluconobacter oxydans* (*n*=10), *Gluconobacter* sp. (*n*=2), and *Asaia* sp. (*n*=3) (Table 1-3).

Of 315 isolates from MRS agar plates, 129 were selected for sequence-based identification. Homofermentative and facultative heterofermentative isolates were observed, and all of these isolates were identified as *Lactobacillus plantarum* (*n*=52). The other 77 isolates were obligate heterofermentatives, and most of them were identified as *L. fermentum* (*n*=72). The remaining obligate heterofermentative isolates were closely related to *Weissella fabaria/ghanensis* (*n*=5) (Table 1-3).

The 16S rRNA gene sequences revealed broad bacterial genotypic diversity among colonies that grew on Nutrient agar plates; these species belonged to the *Proteobacteria* (*Gamma*(γ)-, *Delta*(δ) -, *Alpha*(α)-*Proteobacteria*), *Flavobacteria*, *Firmicutes*, and *Actinobacteria* groups. The *Firmicutes* group included 80 isolates that were affiliated with *Bacillales* and

included endospore-forming bacteria *Bacillus subtilis* (*n*=42), *B. megaterium* (*n*=13), *B. circulans* (*n*=4), *B. flexus* (*n*=4), *Bacillus* sp. (*n*=1) *Paenibacillus* sp. (*n*=3), and the non-spore-forming bacteria *Staphylococcus aureus* (*n*=3), *St. pasteuri* (*n*=4), *St. xylosus* (*n*=3) and *St. saprophyticus* (*n*=3). The other five isolates, consisting of gram-positive bacteria isolated from Nutrient agar plates, exhibited the general characteristics of the *Actinobacteria* class and were identified as *Microbacterium testaceum* (*n*=3) and *Micrococcus luteus* (*n*=2). The γ-*Proteobacteria* group, which was the most diverse among the gram-negative bacteria, included the following species: *Pantoea terrea* (*n*=7), *Klebsiella variicola* (*n*=2), *Tatumella saanichensis* (*n*=2), *T. ptyseos* (*n*=8), *F. aurantia* (*n*=4), *Acinetobacter baumannii* (*n*=1), *Xanthomona*s sp (*n*=5). Some of the isolates were identified as belonging to the α-*Proteobacteria* group (*n*=23), which were related to *A. malorum*/cerevisiae (*n*=12), *A. ghanensis*/*syzygii (n=2), A. pomorum*/*pasteurianus* (*n*=13) and *Brevundimonas* sp (*n*=4). The remaining 3 gram-negative bacterial isolates belonged to the *Flavobacteria* group and consisted of *Ch. letacus* (*n*=3).

3.3 Distribution of microorganisms through cultivation

Independent of the fermentation systems, *S. cerevisiae* was the most prevalent yeast at the start of fermentation and grew to maximum populations of 7.69 log CFU g^{-1} at 24 h, 7.57 log CFU g^{-1} at 12 h and 7.91 log CFU g^{-1} at 12 h in SST, WB1 and WB2, respectively (Table 1-3). There was a marked drop in the post-peak phase during fermentation in wooden boxes in which *S. cerevisiae* was eclipsed by non-*Saccharomyces* yeasts with low-fermentative activity. Conversely, during fermentation conducted in SST, *S. cerevisiae* dominated the whole fermentation process and was the sole yeast observed beyond 72 h. *P. kudriavzevii*, *H.uvarum* and *Wickerhamomyces* sp. were occasionally found in each fermentation condition. The fermentation performed in boxes had higher

diversity of yeast species than SST fermentation, which consisted of *C. ethanolica*, *C. inconspicua*, *C.hum*ilis, *D. etchellsii*, *C. xylopsoci*, *C. intermedia* and *Sz. pombe*. The LAB population was dominated by *L. plantarum* and *L. fementum* (Table 1-3). At the onset of fermentation, *L. plantarum* dominated the LAB population in the box fermentations; however, after 36–48 h, *L. fermentum* became the dominant LAB. In regard to SST fermentation, *L. fermentum* was present initially and dominated the entire fermentation process (maximum population size of 7.93 log CFU g^{-1} at 48 h) — a position challenged only by *L. plantarum* at 72 and 84 h of fermentation (Table 1). Occasionally, *W. fabaria/ghanensis* isolates were found during initial stages of SST fermentation. There was a clear prevalence of *A. tropicalis* among AAB isolates in all fermentation processes (*i.e.*, more than 50% of AAB isolates); this species survived longer during all fermentations with maximum populations sizes of 5.73 log CFU g^{-1} at 48 h, 6.26 log CFU g^{-1} at 12 h and 6.14 log CFU g^{-1} at 24 h in SST, WB1 and WB2, respectively (Table 1-3). A range of other bacterial species grew on Nutrient agar plates throughout each fermentation process, but these populations rarely exceeded 5 log CFU g^{-1}. In this culture medium, *B. subtilis* nearly dominated the box fermentations while *B. megaterium* and *B. subtilis* were equally prevalent during SST fermentation.

3.4 Culture-independent microbiological analysis using a nested PCR–DGGE strategy

3.4.1 Comparative sequence analysis

In addition to enumeration of microbial cultures in culture media, microbial population dynamics were monitored by DGGE. Bacterial and yeast DGGE profiles for each of the three fermentations are shown in Figs. 2 and 3, respectively.

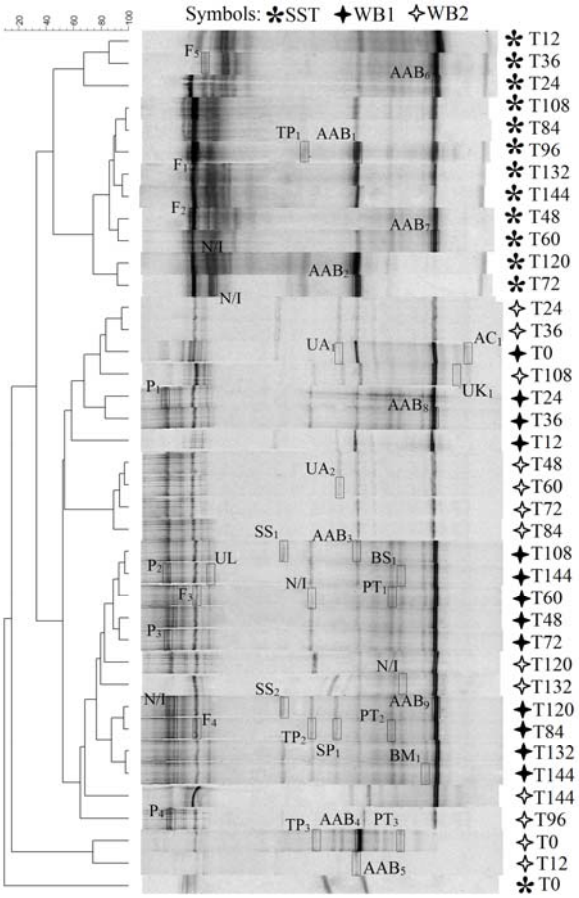

Figure 2 Dendrogram derived from a cluster analysis of the 16S rRNA gene-PCR-DGGE patterns of the bacterial communities associated with cocoa bean fermentation samples from SST, WB1 and WB2, based on the Dice coefficient of similarity (weighted) and generated with the UPGMA clustering algorithm. Prominent bands were excised from the gels, re-amplified, and sequenced. The closest relatives of the sequenced fragments identified through in GenBank searches for sequences sharing greater than 97% similarity: AAB1-AB9 *Acetobacter* sp; F1-F5 *L. fermentum*; P1-P4 *L. plantarum*; TP1-TP3 *T. ptyseos*; PT1-PT3 *P. terrea*; BM1 *B. megaterium*; BS1 *Bacillus* sp. SS1 and SS2 *S. saprophyticus*; SP1 *Staphylococcus* sp; AC1 *Acinetobacter* sp.; UL1 Uncultured *Lactobacillus* sp.; UK1 Uncultured *Klebsiella* sp; UA1 and UA2 uncultured bacterium isolate from DGGE gel band. N/A = not identified (*i.e.*, bands excised from the gel that were not successfully re-amplified)

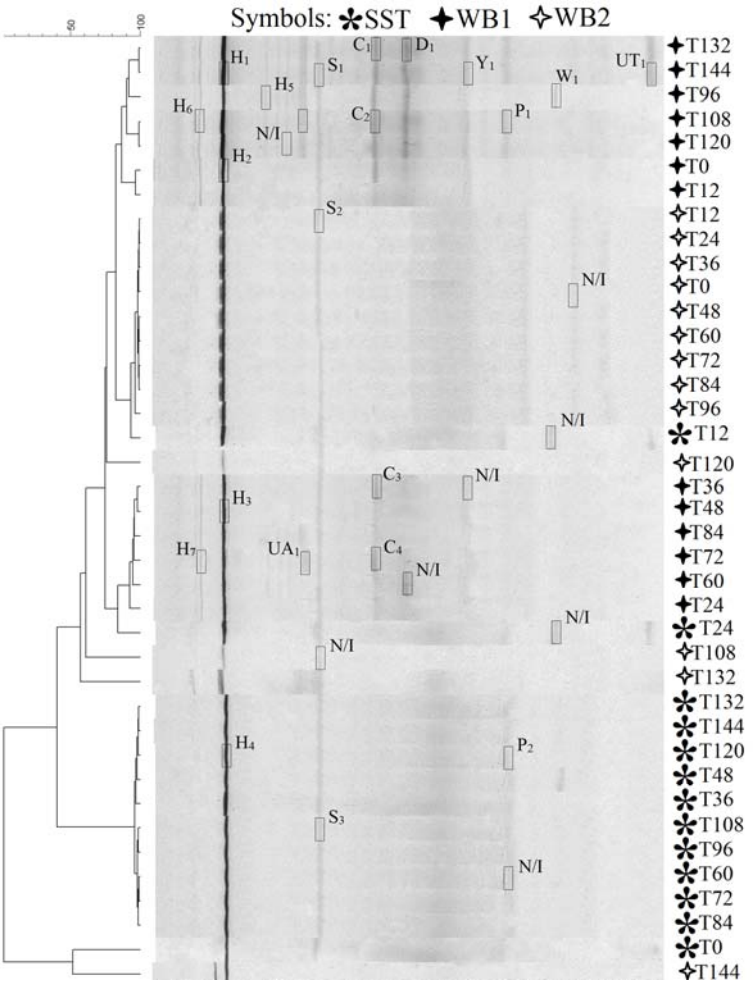

Figure 3 Dendrogram derived from a cluster analysis of the ITS-PCR-DGGE patterns of yeast communities associated with cocoa bean fermentation samples from SST, WB1 and WB2 were based on the Dice coefficient of similarity (weighted) and obtained with the UPGMA clustering algorithm. The closest relatives of the sequenced fragments were determined *via* GenBank searches for sequences with over 97% similarity: H1-H7 *Hanseniaspora* sp; S1-S3 *S. cerevisiae*; C1-C4 *C. humilis*; P1-P2 *P. kluyveri*; D1 *Debaryomyces* sp; Y1 *Y. lipolytica*; UT1 uncultured *Tomentella*; W1 *Wallemia* sp. N/A = not identified (*i.e.*, bands excised from the gel but not successively re-amplified)

In general, bands corresponding to the bacteria species *L. fermentum* and *Acetobacter* sp. (*A. tropicalis* and *A. senegalensis* were the closest relative found by sequence comparison), and to the yeast species *S. cerevisiae* and *Hanseniaspora* sp. (over of 99% identity with *H. opuntiae*, *H. uvarum* and *H. guilliermondii*), were found at all fermentation times in both boxes and SST. This finding supports the results of the cultivation-based method, which establishes these species as dominant throughout fermentation processes. In regard to bacterial ecology, PCR–DGGE revealed a large diversity of bacterial species within fermentations conducted in boxes (Fig. 2). In addition to the dominant species *L. fermentum* and *Acetobacter* sp., members of the *Enterobacteriaceae* family (*T. ptyseos* and *P. terrea*), spore-forming bacteria (*B. megaterium* and *Bacillus* sp.), species of *Staphylococcus* (*S. saprophyticus* and *Staphylococcus* sp.), *L. plantarum* and *Acinetobacter* sp. were also identified. Moreover, three faint bands corresponding to uncultivable bacteria were detected. Conversely, only intense bands corresponding to the dominant species, *L. fermentum* and *Acetobacter* sp., as well as a faint band corresponding to *T. ptyseos*, were successively recovered from the DGGE profile of SST fermentation.

As presented in Fig. 3, DGGE profiles with a *Eukarya*-specific primer were considerably simpler than their bacterial counterparts. *Hanseniaspora* sp. was the most abundant species during fermentation processes, as revealed through sequencing of the robust DGGE band in all fermentation samples. *S. cerevisiae* was also detected in all samples but the density of its corresponding band was usually low. This indicated that a restricted yeast species composition was involved in each cocoa bean fermentation process. An exception was WB1, which that exhibited a significant increase in the number of bands from 96 h; these corresponded to yeast species *C. humilis*, *P. kluyveri*, *Debaryomyces* sp. and *Y. lipolytica*. Moreover, two faint bands corresponding to the fungus

Wallemia sp., as well as one uncultured *Tomentella*, were also present. Additionally, *P. kluyveri* was identified in SST fermentation, and one band corresponding to the genomic DNA of uncultured ascomycota was recovered at 72 h during WB1 fermentation.

3.4.2 Cluster analysis of the DGGE profiles.

Bacterial ecology was found to be a fermentation-specific (wooden boxes *vs.* SST), as samples collected from like fermentations at different time points were usually grouped in the same cluster. Thus, fermentations performed in boxes bore greater similarity to each other than to SST fermentation. In fact, samples from SST fermentation formed a single cluster that was subdivided into two cluster groupings: samples from the start of fermentation (12, 24 and 36 h; similarity of 65%) and samples obtained during the middle and at the end of fermentation (similarity of 75%). Only samples analysed at 0 h were distinct and did not belong to any cluster. No clear distinction regarding bacterial ecology was observed for either box fermentation process, and only samples collected at the start of WB2 fermentation (0 and 12 h) were identified as belonging to an out-group.

In Fig. 3, the cluster analysis of the yeast DGGE profile is presented. On the basis of a coefficient of discrimination of 40%, SST fermentation samples were distinguishable from box fermentations. Only samples analysed at the beginning of SST fermentation and those analysed at 144 h during WB2 fermentation, were distinct from the rest and did not belong to any cluster. WB1 fermentation produced a temporal yeast distribution with samples divided into two major groups. In contrast, WB2 fermentation produced a more stable yeast population throughout the fermentation process.

3.5 pH and temperature measured

The pH and titratable acidity of the pulp and beans were determined during each fermentation and are reported in Table 4.

Table 4 Acidification process (pH and titratable acidity) of the cocoa bean fermentations performed

	Fermentation (h)												
Physicochemical parameters	**0**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**	**108**	**120**	**132**	**144**
SST Fermentation													
pH (pulp)	4.74	4.02	3.87	4.38	3.82	3.52	3.51	3.53	3.31	3.30	3.40	3.95	3.85
pH (bean)	5.71	5.98	6.02	6.06	5.49	4.40	4.35	4.23	4.00	3.85	3.85	4.03	4.14
TTA (pulp)	0.04	0.12	0.08	0.08	0.12	0.16	0.24	0.28	0.28	0.28	0.24	0.24	0.24
TTA (bean)	0.28	0.24	0.24	0.28	0.44	1.20	1.00	1.20	1.20	1.56	1.48	1.04	1.20
WB1 Fermentation													
pH (pulp)	4.64	3.98	3.77	3.76	4.34	4.36	4.25	4.24	4.01	4.32	4.00	4.09	4.13
pH (bean)	5.42	5.47	5.64	5.11	5.22	4.75	4.58	4.50	4.30	4.38	4.37	4.70	4.66
TTA (pulp)	0.12	0.08	0.12	0.20	0.12	0.08	0.12	0.12	0.12	0.12	0.16	0.12	0.08
TTA (bean)	0.28	0.28	0.32	0.48	0.48	1.04	1.08	0.84	1.04	1.28	1.08	0.92	0.88
WB2 Fermentation													
pH (pulp)	4.52	4.15	4.15	3.97	3.92	3.84	3.64	3.77	3.77	3.85	3.82	3.87	3.89
pH (bean)	5.86	5.87	5.48	5.56	5.68	4.93	4.65	4.56	4.45	4.24	4.27	4.30	4.60
TTA (pulp)	0.08	0.08	0.04	0.12	0.04	0.12	0.20	0.24	0.12	0.12	0.20	0.12	0.16
TTA (bean)	0.28	0.28	0.52	0.44	0.48	0.56	0.64	0.72	0.84	1.12	1.00	1.08	1.12

Abbreviation: TTA: Total Titratable Acidity. The values were reported as Eq of sodium hydroxide per 100 g of dry beans

In SST and WB2 fermentations, acidification of the pulp followed a similar trend, in which an initial phase of accelerated acidification occurred from 0 h to 24-36 h and resulted in a pH decrease from 4.74 to 3.87 and 4.63 to 3.76, respectively. Subsequently, a pH increase occurred from 24-36 to 36-48 h to yield values of 4.38 and 4.34. A second phase of acidification occurred thereafter and proceeded until the conclusion of fermentation and producing pH values of 3.85 and 4.13 for SST and WB2, respectively. A different scenario occurred in WB1 fermentation, in which the pH of the pulp decreased more gradually and attained the lowest value of 3.64 at 72 h of fermentation. The pH then gradually increased to a value of 3.89 in the final of fermentation step. Due to the production of organic acids in the pulp and their diffusion into the beans, there was a rise in titratable acidity (TA) concomitant with a sharp drop in pH throughout each of the fermentation processes. In the beans, a continuous and linear increase in TA was observed; this ranged from 0.28 to 1.20, 0.28 to 0.88 and 0.28 to 1.12 mEq of NaoH.100 g^{-1} for SST, WB1 and WB2, respectively. Conversely, only a slight increase in acidity was observed in the pulp that was always statistically lower than the TA in the bean; this most likely resulted from breakdown of citric acid due to microbial activity present only in the pulp. At the end of the 7-day fermentation period, pH and TA values of SST, WB1 and WB2 fermentations were not significantly different.

Variations in ambient temperature correlated with day-time and night-time cycles, ranging from ambient temperatures of 23.93 °C to 32.24 °C and 22.82 °C to 28.27 °C, respectively (Fig. 1b). Box fermentations exceeded 50 °C after 35 hours of fermentation, which was significantly higher than that achieved in SST fermentation. WB1 fermentation reached maximum temperature at 70 h (51.16 °C), followed by WB2 (50.38 °C at 137 h) and SST (43.21 °C at 144 h).

3.6 Sugars and fermentation products

To assess the overall metabolic activity occurring in each fermentation process, the formation of free sugars (*i.e.*, sucrose, glucose and fructose) and fermentation products (*i.e.*, ethanol, methanol and organic acids) was assayed (Fig. 4).

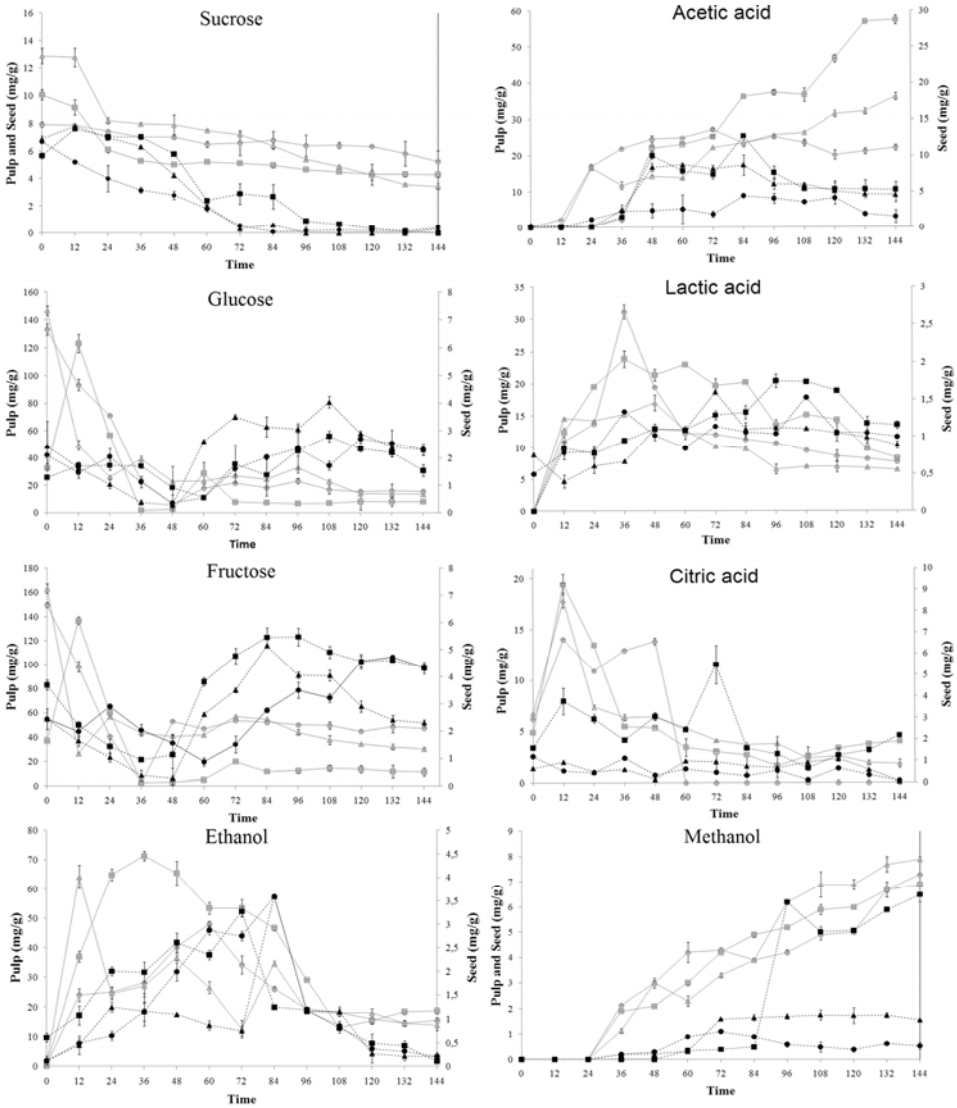

Figure 4 Residual glucose, fructose, and sucrose, as well as production of ethanol, methanol and organic acids in the pulp (traced symbols) and beans (solid symbols) from cocoa bean fermentation samples derived from stainless steel tank (〓 and■), wooden box 1 (▲ and▲), and wooden box 2 (● and〓). Bars represent standard deviation

Sucrose was hydrolysed into glucose and fructose in the pulp, by yeast invertase activity, and in the beans, by diffusion of acetic acid, lactic acid and ethanol into the beans in conjunction with the heat produced therein. Consumption of glucose and fructose in the pulp was similar for both box fermentations. The initial glucose concentrations were rapidly and simultaneously consumed up until 36-48 h; afterward, no significant changes were observed. In contrast, glucose and fructose concentrations in the pulp increased during the first 12 h of SST fermentation. After this initial increase, there was a marked decrease that was followed by another increase in glucose and fructose concentrations throughout the remainder of the fermentation period (Fig. 4). The glucose and fructose profiles inside the beans were similar in all three fermentation processes.

Ethanol was produced and then consumed (Fig. 4), which was similar to the trend observed in dynamic yeast populations (Fig. 1). The maximum ethanol concentration in pulp was achieved during SST fermentation (71.29 mg g^{-1} at 36 h); this was followed by WB1 fermentation (64.25 mg g^{-1} at 12 h) and WB2 fermentation (48.01 mg g^{-1} at 60 h) upon conversion into acetic acid by AAB. The concentration of ethanol in the pulp was higher in the SST fermentation from 12 to 96 h than in either box fermentation, although it reached similar concentrations at the conclusion of fermentation (an average of 15 mg g^{-1}). Ethanol produced in the pulp diffused into the beans where it reached a maximum concentration of 3.27 mg g^{-1} at 72 h of SST fermentation; this concentration was lower for box fermentations at 3.59 mg g^{-1} at 84 h. Methanol produced in the pulp after 24 h of fermentation exhibited a linear increase until the conclusion of fermentation. Diffusion of methanol into the beans began after 36 h and increase significantly after 84 h of SST fermentation. Acetic acid production in pulp was similar for both SST and WB1 fermentations; however, after 24 h, the acetic acid concentrations were higher for the SST process

(maximum concentrations were 57.45 mg g^{-1} and 36.20 mg g^{-1} at 144 h for SST and WB2, respectively) (Fig. 4). In WB2 fermentation, the concentration of acetic acid increased during the first 72 h (27.20 mg g^{-1}), subsequently decreased and then increased again during the last day of fermentation. Prior to a marked decrease, lactic acid increased in the first 36-48 h, reaching maximum concentrations of 23.86 mg g^{-1}, 17.01 mg g^{-1} and 22.13 mg g^{-1} for SST, WB1 and WB2, respectively. Although concentration of lactic acid changed in similar fashion in all three fermentations, its presence in the pulp was higher for SST fermentation from 24 to 84 h. A rapid decrease in citric acid concentration was observed after 12 h for all three fermentations performed (Fig. 4). The amount of citric acid in the beans was stable throughout fermentation and was not detected at the conclusion of the process for either box fermentation; in contrast, 2.18 mg g^{-1} citric acid was observed in SST fermentation.

4. Discussion

Fermentation of cocoa beans continues to be conducted in a traditional manner, yielding great diversity in production methods and organoleptic characteristics of its end products (Camu et al. 2007). For many years, there have been two primary methods of fermentation commonly used throughout the world: the heap and box methods. Box fermentation is conducted on a larger scale, which speeds up bean fermentation and reduces cost relative to heap fermentation. However, box fermentation generally yields a lower quality product because of a lack of homogeneity in the cocoa beans. One promising goal of the chocolate industry is to improve control of the cocoa fermentation process to the point that large-scale fermentation can yield high-quality beans. The development of new methods for fermenting cocoa under controlled conditions will require considerable new research. A promising alternative

strategy to conduct continuous cocoa bean fermentation is to use a sterile stainless steel container in which inoculum, aeration, and turn rate can be controlled (Schwan and Wheals 2004). However, in addition to technology suitable to meet the conditional requirements of cocoa bean fermentation, a thorough understanding of factors that regulate microbial metabolism and stability of the microbiota involved in the process are indispensable; this is especially true because the quality of chocolate is dictated by these parameters. Therefore, we examined the effectiveness stainless steel vessels as an alternative to wooden boxes in cocoa fermentation. Microbiological analyses revealed that the prevailing species credited with major physiological roles were the same in both SST and box fermentations. This finding indicated that the microbial inoculum could be derived from cocoa pod surfaces, and that, even with a low initial population, these species adapted well to the conditions of cocoa fermentation and were able to expand their populations by as much as 7 orders of magnitude.

Consistent with the results of other cocoa fermentation biodiversity studies (Kostinek et al. 2008; Camu et al. 2007; Nielsen et al. 2007), the current investigation found that the complex associations of homo- and heterofermentative species, *L. plantarum* and *L. fermentum*, respectively, were the most prevalent LAB species in Brazilian cocoa fermentations performed. Moreover, *L. fermentum* populations both decreased and increased during fermentation and dominated the fermentation time-course. The technical roles of these groups are not well understood and require further study. The metabolism homolactic of *L. plantarum* has the ability to achieve a high cell density within a reasonable fermentation time, and in contrast to *L. fermentum*, produce high amounts of lactic acid. Conversely, the heterolactic metabolism of *L. fermentum* can rapidly convert citric acid and produce nearly equal masses of lactic acid and acetic acid (Axelsson 2004). In the former case, *L. plantarum* contributed to

increased acidity, while in the latter, *L. fermentum* reduced it. However, according to Carr and Davies (1980), carbohydrate catalysis increases total acidity of the cocoa more so than breakdown of organic acids. Because LAB growth occurred normally in SST fermentation, the dynamics of citric acid consumption and lactic acid production exhibited a profile similar to that of box fermentations.

Although not previously considered to be a prevailing cocoa AAB species, culture-dependent microbiological analyses indicated that *A. tropicalis* may play a significant role during fermentations of Brazilian cocoa beans because of its isolation and metabolic activity during the fermentation process. *A. tropicalis* is primarily associated with fruits and fermented foods and has been selected to produce artisanal vinegar (Ndoye et al. 2006). The dominance of this species during cocoa bean fermentation may be explained by its resistance to acidity and heat (Ndoye et al. 2006). The presence of another representative *Acetobacter* and *Gluconobacter* species, previously reported in cocoa bean fermentation (Schwan and Wheals 2004; Camu et al. 2008; Garcia-Armisen et al. 2010), was confirmed in the present study. In addition, phylogenetic analysis of the 16S rRNA gene of some isolates, which were grown on GYC agar plates during the initial stage of WB1 fermentation, supported that they belonged to the genus *Asaia*; however, based on their sequence divergence from known species, these isolates may represent a potentially novel species. The genus *Asaia* was introduced with a single species, *Asaia bogorensis*, as the fifth genus of the *Acetobacteraceae* family (Yamada et al. 2000). The natural habitats of *Asaia* spp. are reportedly flowers of the orchid tree (*Bauhinia purpurea*), plumbago (*Plumbago auriculate*), and fermented glutinous rice, all of which occur in hot tropical climates; particularly in Indonesia and Thailand (Yamada et al. 2000; Katsura et al. 2001). Interestingly, this AAB group has not been reported by any studies of cocoa bean

fermentation. In contrast to strains of the genera *Acetobacter*, *Asaia* strains are characterised by little or no capacity for oxidation of ethanol into acetic acid, as well as an inability to grow in 0.35% (v/v) acetic acid (Yamada et al. 2000). Their potential significance for cocoa bean fermentation is not known and requires further investigation.

A diverse array of bacterial species, aside from LAB, AAB, and *Bacillus* ssp., was found in box fermentations. The occurrence of these previously unreported bacterial species in box cocoa fermentations demonstrates the importance of monitoring the hygiene of fermentation procedures to ensure that microbial contamination does not spoil the beans (Ardhana and Fleet 2003). These microorganisms could be associated with pod surfaces, banana/plantain leaves and with the material used for fermentation, including the porous wooden structure and farmers' hands. However, these bacteria did not adapt to the matrix during the fermentation process (as indicated by their absence after 12 h; Table 1-3) and are therefore not regarded as important for cocoa fermentation.

This study represents the first assessment of *Bacillus* species from Brazilian cocoa box fermentations using molecular methods. Our results demonstrate that at least four species of *Bacillus* are involved in cocoa fermentation, namely *B. subtilis*, *B. flexus*, *B. circulans*, and *B. megaterium*. The association of *Bacillus* species with cocoa fermentation has been recognised for some time. Using classical microbiological analysis, Ostovar et al. (1973) and Schwan et al. (1986) observed a broad diversity of *Bacillus* species during fermentation in Trinidad and Brazil, respectively. However, recent molecular studies have revealed a much lower diversity, indicating that traditional methods may have misidentified *Bacillus* species and overestimated their abundance in cocoa bean fermentation (Ouattara et al. 2008; this study). The role of *Bacillus* in cocoa fermentation is not well understood, so this bacterium has never been utilised as a starter culture in an attempt to control the fermentation process. The

studies by Zak and Keeney (1976) suggested the involvement of *B. 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 1973; Schwan et al. 1986). Thus, given their abundance in natural cocoa fermentation (Schwan 1998; Ardhana and Fleet 2003; Ouattara et al. 2008), it is tempting to speculate that certain *Bacillus* strains might provide beneficial activity by complementing yeast-mediated pulp depectinisation during advanced stages of cocoa fermentation (Ouattara et al. 2011).

It has been widely reported that *S. cerevisiae* and *Hanseniaspora* spp. are the most abundant species in cocoa fermentation (Schwan et al. 1995; Nielsen et al. 2007). Some strains of this fermentative yeast exhibited positive properties in that they are able to produce many secondary compounds, including organics acids and a larger array of aroma compounds (*e.g.*, fusel alcohols, fatty acids and fatty acid esters) in addition to ethanol production (Schwan and Wheals 2004). It suggests that *S. cerevisiae* and *Hanseniaspora* spp. could be used in cocoa fermentation to enhance the aroma and flavour of chocolate (Schwan 1998; Schwan and Wheals 2004). Interestingly, *S. cerevisiae* yielded only a weak banding in denaturing gels relative to *Hanseniaspora* spp. (Fig. 3), though it represented the most common yeast species isolated by the plating method for all fermentation processes (Table 1-3). A possible explanation for this may be that the fragment of the *S. cerevisiae* ITS region was amplified at lower efficiency using the protocol described here, relative to other yeast species present during cocoa fermentation (Nielsen et al. 2005).

Although it was shown that the dominant species was not replaced in either fermentation method, the SST fermentation process markedly altered the proportion of yeast relative to box fermentations by decreasing their population

size and diversity. Consequently, the presence of a wide variety of low-fermentative-power non-*Saccharomyces* yeasts significantly decreased the ethanol yield in the box conditions, relative to SST; this may have been the result of competition with *S. cerevisiae* strains for available nutrients. Moreover, the fermentative metabolism of *S. cerevisiae* yeast can also be affected by additional forms of yeast-yeast interaction, as well as – differences in the production of, and sensitivity to, killer-factors; the formation of cell-cell interactions *via* quorum sensing molecules; or the occurrence of spatial phenomena, which are currently subjects of conjecture (Fleet 2003). Conversely, the presence of non-*Saccharomyces* species should not be neglected because secondary products of their metabolism (*e.g.*, organic acids, aldehydes, ketones, higher alcohols, and esters) and glycosidase production are likely to be significant and capable of impacting the quality of both beans and chocolate (Ardhana and Fleet 2003). Moreover, some non-*Saccharomyces* yeast, including *Candida* spp. and *Pichia* spp., metabolise citric acid, which causes pH increases in the pulp that permit bacterial growth (Schwan et al. 1995). However, these potentially important influences have been overlooked in previous cocoa fermentation attempts. The use of yeasts as starter cultures for cocoa bean fermentation has been preliminarily examined, particularly with respect to enhancement of pectinolytic activity for the improvement of pulp drainage (Sanchez et al. 1984; Schwan et al. 1997; Leal et al. 2008) and ethanol production (Schwan 1998). Only in two particular cases was *S. cerevisiae* used in combination with LAB and AAB species (Schwan 1998; Lefeber et al. 2011); however, it has never been used in combination with other non-*Saccharomyces* yeasts. For this reason, the reliable identification of yeasts prior to studies of secondary metabolite production during cocoa bean fermentation should be performed to facilitate evaluation of the practical benefits and actual influence of non-*Saccharomyces* species on the final quality of the chocolate. To our

knowledge, this study represents the first assessment of yeast isolates from Brazilian cocoa box fermentations using molecular methods. It is important to stress that, during sampling of the yeast species, we focused on isolating morphologically different colonies, rather than attempting to achieve similar numbers of colonies in all fermentation process. Although applicable to a small number of bacterial species, the use of morphotypes exhibiting marked characteristics may be relevant to the spatial and temporal distribution of some species in the environment of cocoa bean fermentation (Lebaron et al. 2011). Twelve species belonging to the non-*Saccharomyces* genera were identified. *Hanseniaspora*, *Pichia*, *Candida*, *Wickerhamomyces*, *Debaryomyces*, *Issatchenkia*, and *Schizosaccharomyces* have often been detected in spontaneously fermented cocoa beans from other geographical areas (Jespersen et al. 2005; Nielsen et al. 2005; Ardhana and Fleet 2003).

Among the organic acids formed during the fermentation process, acetic acid produced by AAB species is the most important, as its diffusion into the beans initiates the development of chocolate flavour (Schwan and Wheals 2004). However, the amount of acetic acid detected did not correspond to the number of AAB present. Despite a smaller AAB population, acetic acid was more effectively produced in SST relative to each of the box conditions. This indicates that the acetic acid production was more heavily influenced by the yeast-AAB-mediated conversion of carbohydrates into ethanol, and subsequent conversion of ethanol into acetic acid, than by the AAB population size. In addition, heterofermentative LAB can also play an important role in the production of acetic acid during cocoa bean fermentation.

It seems that the high thermal conductivity and geometry of the SST, as well as additional surrounding conditions, resulted in a marked thermal loss that overcame the heat produced by the oxidation process. To avoid heat transfer, a

stainless steel tank surrounded by rubber or an insulating water jacket thermostatically controlled, can be used (RF Schwan, unpublished results).

Biodiversity data obtained through cultivation-dependent isolation and, upon identification, did not fully correspond to the molecular inventory of cocoa samples through DGGE community fingerprinting. Species present in low concentrations could occasionally be detected in agar plates but in many cases did not produce a detectable DGGE band. Thus, it is possible that DGGE fingerprinting might have masked community perturbations of low-abundance organisms in our study. This finding illustrates the intrinsic limitation of DGGE analysis for visualising only the predominant species of a microbial community (Pereira et al. 2011). However, one advantage of DGGE fingerprinting is that it allows the user to compare total microbial communities from different samples using pattern analysis. Computer-aided analysis of the DGGE profiles showed that bacterial and yeast ecology were be fermentation-specific processes (*i.e.*, wooden box *vs*. stainless steel tank), as implied by the complex picture displayed on DGGE gels of each box sample. In addition, bacterial DGGE profiles resolved a group of sequences whose phylogenetic allocation indicated the presence of uncultivable bacteria belonging to the genera *Lactobacillus* and *Klebsiella*. Additionally, DGGE profiles obtained with *Eukarya*-specific primers allowed detection of the yeast *Y. lipolytica*, the fungus *Wallemia* sp., and one uncultured *Tomentella* during fermentation in boxes; these strains were not recovered with cultivation techniques. Yeasts of the species *Yarrowia* (*syn*. *Candid*a, *Saccharomycopsis*, *Endomycopsis*) *lipolytica* are known to produce a variety of organic acids, including TCA cycle intermediates, such as CA and isocitric acid (ICA), from a wide range of carbon sources. Single-cell oil, having a composition similar to cocoa-butter, was produced by the *Y. lipolytica* strain cultivated on mixtures of saturated free fatty acids (Papanikolaou et al. 2003). *Y.*

lipolytica was only isolated during fermentation of cocoa from the Dominican Republic and was able to oxidise citric acid (Lagunes-Gálvez et al. 2008).

In conclusion, this research has contributed to our understanding of the microbial community structure and metabolite production associated with cocoa bean fermentation in boxes and stainless steel tank. The use of cultivation-independent techniques and cultivation-based approaches to the study of bacterial and yeast communities has confirmed that microbial ecosystems in box fermentations of Brazilian cocoa support a wide variety of organisms. In addition, we described the first experimental validation of the stainless steel tank method for cocoa bean fermentation. The use of stainless steel tanks may be of great interest for those who seek improved control over the cocoa fermentation process and/or to optimise cocoa fermentation through the use of starter cultures.

Acknowledgments

The authors wish to thank the technical assistance of Alício Costa, Iara Brito and Ivani Maria Gervásio. GVMP, MGCPM, EGA, and KTM thank the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and CAPES for scholarships. Financial support from the Almirante Cacau Agricola (Masterfoods) is also gratefully acknowledged.

Reference

Axelsson L (2004) Lactic acid bacteria: classification and physiology. In: Salminen S, von Wright A, Ouwehand A (eds) Lactic Acid Bacteria: Microbiological and Functional Aspects, 3rd edn. Marcel Dekker, New York, pp 1–66

Ardhana M, Fleet G (2003) The microbial ecology of cocoa bean fermentations in Indonesia. Int J Food Microbiol 86:87-99.

Camu N, Gonzalez A, De Winter T, Van Schoor A, De Bruyne K, Vandamme P, Takrama JS, Addo SK, De Vuyst L (2008) Influence of turning and environmental contamination on the dynamics of populations of lactic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation in Ghana. Appl Environ Microbiol 74:86–98

Camu N, De Winter T, Verbrugghe K, Cleenwerck I, Vandamme P, Takrama JS, Vancanneyt M, 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. Appl Environ Microbiol 73:1809–1824

Carr JG, Davies PA (1980) Cocoa fermentation in Ghana and Malaysia: Further microbial methods and results. University of Bristol, Bristol

Fleet GH (2003) Yeast interactions and wine flavour. Int J Food Microbiol 86:11-22.

Garcia-Armisen T, Papalexandratou Z, Hendryckx H, Camu N, Vrancken G, De Vuyst L, Cornelis P (2010) Diversity of the total bacterial community associated with Ghanaian and Brazilian cocoa bean fermentation samples as revealed by a 16 S rRNA gene clone library. Appl Microbiol Biotechnol 87:2281-2292

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

Joyeux A, Lafon-Lafourcade S, Ribereau-Gayon P (1984) Evolution of acetic acid bacteria during fermentation and storage of wine. Appl Environ Microbiol 48:153-156

Katsura K, Kawasaki H, Potacharoen W, Saono S, Seki T, Yamada Y, Uchimura T, Komagata K (2001) *Asaia siamensis* sp. nov., an acetic acid bacterium in the a-Proteobacteria. Int J Syst Evol Microbiol 51:559–563.

Kostinek M, Ban-Koffi L, Ottah-Atikpo M, Teniola D, Schillinger U, Holzapfel WH, Franz CMAP (2008) Diversity of predominant lactic acid bacteria associated with cocoa fermentation in Nigeria. Curr Microbiol 56:306–314

Kurtzman CP, Fell JW, Boekhout T (2011) The Yeasts, a Taxonomic Study, 4ed. Elsevier, Amsterdan.

Lagunes-Gálvez S, Loiseau G, Paredes JL, Barel M, Guiraud J-P (2007) Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. Int J Food Microbiol 114:124-130

Leal Jr GA, Gomes LH, Efraim P, Tavares FC, Figueira A (2008) Fermentation of cocoa (*Theobroma cacao* L.) seeds with a hybrid *Kluyveromyces marxianus* strain improved product quality attributes. FEMS Yeast Res 8: 788–798

Lefeber T, Papalexandratou Z, Gobert W, Camu N, De Vuyst L (2011) On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. doi:10.1016/j.fm.2011.12.021

Lebaron P, Ghiglione J-F, Fajon C, Batailler N, Normand P (1998) Phenotypic and genetic diversity within a colony morphotype. FEMS Microbiol Lett 160:137-143

Lopez A, Quesnel VC (1973) Volatile fatty acid production in cacao fermentation and the effect on chocolate flavor. J Sci Food Agr 24:319–326.

Lima LJR, Almeida MH, Rob Nout MJ, Zwietering MH (2011) *Theobroma cacao* L., "The Food of the Gods": Quality Determinants of Commercial Cocoa Beans, with Particular Reference to the Impact of Fermentation. Crit Rev Food Sci Nutr 51(8):731-61.

Magalhães KT, Pereira GVM, Dias DR, Schwan RF (2010) Microbial communities and chemical changes during fermentation of sugary Brazilian kefir. World J Microbiol Biotechnol 26: 1241-1250

Ndoye B, Lebecque S, Dubois-Dauphin R, Tounkara L, Guiro AT, Kere C, Diawara B, Thonart P (2006) Thermoresistant properties of acetic acids bacteria isolated from tropical products of Sub-Saharan Africa and destined to industrial vinegar. Enzyme Microb Tech 39:916–923

Nielsen DS, Honholt S, Tano-Debrah K, Jespersen L (2005) Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). Yeast 22:271–284

Nielsen DS, Teniola OD, Ban-Koffi L, Owusu M, Andersson TS, Holzapfel WH (2007) The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture independent methods. Int J Food Microbiol 114:168–186

Ostovar K, Keeney PG (1973) Isolation and characterization of microorganisms involved in the fermentation of Trinidad's cacao beans. J Food Sci 38:611–617

Ouattara HG, Ban-Koffi L, Karou GT, Sangare A, Niamke SL, Diopoh JK (2008) Implication of *Bacillus* sp. in the production of pectinolytic enzymes during cocoa fermentation. World J Microbiol Biotechnol 24:1753-1760

Ouattara HG, Reverchon S, Niamke SL, Nasser W (2011) Molecular identification and pectate lyase production by Bacillus strains involved in cocoa fermentation. Food Microbiol 28: 1-8.

Ovreas L, Forney L, Daae FL, Torsvik V (1997) Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol 63:3367–3373

Papanikolaou S, Muniglia L, Chevalot I, Aggelis G, Marc I (2003) Accumulation of a cocoa-butter-like lipid by *Yarrowia lipolytica* cultivated on agro-industrial residues. Curr Microbiol 46:124-30

Pereira GVM, Ramos CL, Galvão C, Dias ES, Schwan RF (2010) Use of specific PCR primers to identify three important industrial species of *Saccharomyces* genus: *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces pastorianus*. Lett Appl Microbiol 5:131–137.

Pereira GVM, Magalhães KT, Lorenzetii ER, Souza TP, Schwan RS (2011) Multiphasic Approach for the Identification of Endophytic Bacterial in Strawberry Fruit and their Potential for Plant Growth Promotion. Microb Ecol. doi: 10.1007/s00248-011-9919-3

Ruiz A, Poblet M, Mas A, Guillamón JM (2000) Identification of acetic acid bacteria by RFLP of PCR-amplified 16 rDNA and 16S–23S rDNA intergenic spacer. Int J Syst Evol Microbiol 50:1981–198

Samah OA, Ptih M F, Selamat J (1992) Biochemical changes during fermentation of cocoa beans inoculated with *Saccharomyces cerevisiae* (wild strain). J Food Sci Technol 29:341–343

Sanchez J, Guiraud J P, Galzy P (1984) A study of the polygalacturonase activity of several yeast strains isolated from cocoa. Appl Microbiol Biotechnol 20: 262-267

Schwan R F (1998) Cocoa fermentations conducted with a defined microbial cocktail inoculum. Appl Environ Microbiol 64:1477–1483

Schwan RF, Rose AH, Board RG (1995) Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. J Appl Bacteriol Symp Suppl 79:96S–107S.

Schwan RF, Wheals AE (2004) The microbiology of cocoa fermentation and its role in chocolate quality. Crit Rev Food Sci Nutr 44:205–221

Schwan R F, Cooper RM, Wheals AE (1997) Endopolygalacturonase secretion by *Kluyveromyces marxianus* and other cocoa pulp-degrading yeasts. Enzyme Microb Technol 21:234–244

Schwan RF, Vanetti MCD, Silva DO, Lopez A, Moraes CA (1986) Characterization and distribution of aerobic, spore-forming bacteria from cacao fermentations in Bahia. J Food Sci 51:1583–1584

White T J, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: A guide to methods and applications, Academic Press, San Diego, CA, USA, pp. 315–322

Yamada Y, Katsura K, Kawasaki H, Widyastuti Y, Saono S, Seki T, Uchimura T, Komagata H (2000) *Asaia bogorensis* gen. nov. sp. nov., an unusual acetic acid bacterium in the a-proteobacteria. Int J Syst Evol Microbiol 50:823–829

Zak D L, Keeney PG (1976) Changes in cocoa proteins during ripening of fruit, fermentation, and further processing of cocoa beans. J Agr Food Chem 24:483–486.

ARTIGO 2

MICROBIOLOGICAL AND PHYSICOCHEMICAL CHARACTERIZATION OF SMALL-SCALE COCOA BEAN FERMENTATIONS AND SCREENING OF YEAST AND BACTERIA STRAINS FOR THE DEVELOPMENT OF AN ASSOCIATED STARTER CULTURE

(Artigo preparado segundo normas do periódico indexado: Applied and Environmental Microbiology)

Abstract

Bench- and pilot-scale spontaneous cocoa bean fermentations were studied using an integrated microbiological approach with culture-dependent and culture-independent techniques, as well as the analyses of target metabolites from both cocoa pulp and cotyledons. Both fermentation ecosystems reached equilibrium through a two-phase process, starting with the simultaneous growth of the yeast (*Saccharomyces cerevisiae* as the dominant species) and the lactic acid bacteria (LAB; *Lactobacillus fermentum* and *L. plantarum* as the dominant species), which were gradually replaced by the acetic acid bacteria (AAB; *Acetobacter tropicalis* as the dominant species). A similar sequence of substrate consumption (sucrose, glucose, fructose and citric acid) and metabolite-production kinetics (ethanol, lactic and acetic acid) was observed in both processes compared to previous, larger-scale fermentation experiments. The technological potential of yeast, LAB and AAB isolates was evaluated using a polyphasic study that included the measurement of stress-tolerant growth and fermentation kinetic parameters in cocoa-pulp media. Overall, the strains *L. fermentum* UFLA CHBE8.12 (citric acid fermenting; lactic acid producing; and heat-, acid-, lactic acid- and ethanol-tolerant), *S. cerevisiae* UFLA CHYC7.04 (ethanol producing and acid-, heat- and ethanol-tolerant), and *A. tropicalis* UFLA CHBE16.01 (ethanol and lactic acid oxidizing; acetic acid producing; and acid-, heat-, acetic acid- and ethanol-tolerant) were selected to form a cocktail starter culture that should lead to better-controlled and more reliable cocoa bean fermentation processes.

Keywords: Chocolate, cocoa fermentation, mixed-strain starter cocktail, lactic acid bacteria, acetic acid bacteria, yeast.

1. Introduction

Cocoa fermentation is a key step in the technological transformation of cocoa into chocolate because the highly bitter, astringent unfermented cocoa beans lack the full chocolate flavor. The fermentation of cocoa beans is therefore the first step of the chocolate-making process, which consists of a natural, five- to seven-day microbial fermentation of the pectinaceous pulp surrounding the seeds of the tree *Theobroma cacao* (37, 39). The cocoa pulp is hydrolyzed during fermentation; this aids the drying process by allowing the pulp to be drained. Most importantly, fermentation triggers an array of chemical changes within the cocoa bean that are vital to the development of the complex, beloved flavor of "chocolate".

The fermentation of cocoa beans occurs at two levels: the first involves reactions that take place in the pulp, in the outer part of the beans, and the second involves several hydrolytic reactions that occur within the cotyledons (39). The microbial activity in the cocoa pulp is a well-defined microbial succession led by yeasts, which dominate the total microbial population during the first hours, followed by the LAB, which decline after 48 h of fermentation, and finally the AAB (22, 39). The metabolic diversity of the yeast, LAB, and AAB strains during cocoa fermentation can be interpreted as a natural consequence of the environmental conditions that influence their growth and selection. Changes in the pH, temperature, sugar content and fermentation products exert a selection pressure on the already existing natural biotypes, favoring those strains that are more adapted to this environment. An analysis of the bacteria and yeast strains that survive under these stresses could provide useful information concerning the ability of the yeast and bacteria to initiate growth and complete fermentation. To perform this process, the microbial cells must adapt their own physiology or behavior in response to the changing environmental stresses (11).

Chocolate processors require a constant supply of cocoa beans that must conform to an array of criteria. The industrialization of the cocoa fermentation process may allow greater control over the quality of the cocoa beans and the chocolate derived from them (36). The concept of industrializing traditional fermentation processes to enhance their performance and efficiency is not new. For example, wine, beer, cheese, distilled sugar cane beverages and yogurt were at one time all produced using a traditional process (43). These fermentations have been developed into highly efficient, well-controlled processes in modern fermenter designs often using defined starter cultures (12, 40). However, cocoa fermentation remains an empirical process that does not give rise to beans of consistent quality, which obliges processors to continually alter their formulations (18). The fermentation takes place under uncontrolled environmental conditions that often lead to unsuccessful fermentation, and the variable quality of the product may reflect the vagaries of chance contamination. Although preliminary experiments using defined starter cultures demonstrate satisfying results (8, 19, 35, 36), only one particular study utilized yeast, LAB and AAB simultaneously as a defined microbial cocktail (36) but without any prior study of the stress tolerance and/or the fermentative kinetic parameters of the individual strains.

The first objective of this study was to investigate the physicochemical changes and the dynamics of the microbial community structure during bench- and pilot-scale spontaneous cocoa bean fermentations. Next, the technological potential of the yeast, LAB and AAB isolates was evaluated using a polyphasic screening study that measured the isolates' stress tolerance and fermentation kinetic parameters in a cocoa pulp-simulating medium.

2. Materials and Methods

2.1 Bench and pilot scale cocoa bean fermentations

Freshly harvested cocoa pods, obtained from a cacao farm located in Itajuípe, Bahia State, Brazil, were broken open manually with a machete, and the beans were immediately transferred to the fermentation site. The beans were mixed in a clean vessel to obtain a homogeneous mixture of 20 kg of wet beans and transferred to the laboratory. A total of 500 g of cocoa beans were deposited in plastic containers (PCs) with the dimensions 15 cm x 10 cm x 7 cm. The plastic containers were kept in incubators, and the temperature was adjusted every 12 h to simulate the temperature of large-scale fermentations: 0 h (28°C), 12 h (30°C), 24 h (32°C), 36 h (35°C), 48 h (38°C), 60 h (42°C), 72 h (46°C), 84 h (48°C), 96 h (48°C), 108 h (48°C), 120 h (48°C), 132 h (48°C), 144 h (48°C). To allow sampling every 12 h, 14 individual plastic containers were prepared for each experiment. The pilot-scale fermentation was performed in a 0.015-m^3 double-layer stainless steel conical tank (ST) (10 kg of cocoa beans; noncommercial bioreactors) with temperature control. The stainless steel tank and plastic containers contained holes at the bottom to allow drainage of the sweatings generated during the fermentation. The vessels were partially closed with a steel and plastic lid to ensure adequate insulation. The fermentations were turned every 24 h, and a natural fermentation proceeded for 168 h.

Every 12 hours, 200-g samples were randomly collected in sterile bags. The samples for chemical and culture-independent analyses were sealed in plastic bags and frozen (-20°C). The microbiological analyses were performed on the same day of sampling.

2.2 Culture-dependent microbiological analysis

25 g of cocoa beans and adhering pulp was added to 225 ml saline-peptone water [(v/v) (0.1% bacteriological peptone (Himedia), 0.8% NaCl

(Merck, Whitehouse Station, USA)] and homogenized in a stomacher at normal speed for 5 min (10^{-1} dilution), followed by serial dilutions. LAB were enumerated by pour plate inoculation on MRS agar (Merck) containing 0.2% (v/v) sorbic acid (Merck) and 0.1% (v/v) cycloheximide (Merck) to inhibit yeasts growth, and 0.1% (v/v) cystein-HCl to obtain anaerobic conditions during incubation. AAB were enumerated by surface inoculation on GYC agar [glucose 50 g/l (Merck), Yeast extract 10 g/l (Merck), Calcium carbonate 30 g/l (Merck), agar 20 g/l (Merck), pH=5.6], containing 0.1% cycloheximide to inhibit yeasts growth and 50 mg/l penicillin (Sigma) to inhibit LAB growth. Yeasts were enumerated by surface inoculation on YEPG agar [1% yeast extract (Merck), 2% peptone (Himedia), 2% glucose (Merck)), pH 5.6] containing 100 mg/l chloramphenicol (Sigma, St. Louis, USA) and 50 mg/l chlortetracycline (Sigma) to inhibit bacterial growth. The Nutrient Agar (NA) containing 0.1% cycloheximide (Merck) was used as a general medium for viable mesophilic bacteria population and *Bacillus* spp. After the spreading, the plates were incubated at 30°C for 3–4 days for the growing of cultures on MRS, YEPG, and NA, and at 25°C for 5–8 days for the growing of cultures on GYC agar. Following incubation, the number of colony-forming units (CFU) was recorded, followed by morphological characterization and counts of each colony type obtained. The square-root of the number of colonies of each type were re-streaked and purified. The purified isolates originating from GYC, YEPG and NA were stored at −80 °C in YEPG broth containing 20% glycerol (w/w), and the isolates originating from MRS agar were stored at −80 °C in MRS broth containing 20% glycerol (w/w).

Phenotypic characterization of bacterial colonies originated from MRS, GYC and NA plates was performed by conventional microbiological methods, namely: Gram staining, microscopic examination, catalase and oxidase activity, motility test; spore formation; acid and gas production from glucose; and acid

and gas production from lactate and acetate (only for isolates originated from GYC agar plates showing clear zone around the colony, presumptive AAB). Yeast colonies were physiologically characterized by determining their morphology, spore formation and fermentation of different carbon sources according to Kurtzman and Fell (16).

Molecular identification of representative microbial strains was performed by sequence analysis of the full-length 16S rRNA gene or the ITS region, for bacteria and yeast, respectively. Bacteria or yeast cultures were grown under appropriate conditions and collected from agar plates with a sterile pipette tip and resuspended in 40 µl of PCR buffer. The suspension was heated for 10 min at 95 °C and 1 µl was used as DNA template in PCR experiments. For bacterial isolates originated from MRS and NA, 16S-rRNA-PCR was carried out using primers 27-F and 1512-R (23). For bacterial isolates originated from GYC agar plates showing clear zone around the colony, 16Sd and 16Sr primers was used for the amplification of the 16S rRNA genes region conserved among AAB (34). For yeast isolates, ITS-PCR was carried out using primers ITS1 and ITS4 (26). Isolates identified as *S. cerevisiae* had their identity confirmed through species-specific PCR assay with HO gene-derived primers (32). The rRNA genes region was amplified in a model Thermo PCYL220 thermal cycler (Thermo Fisher ScientiWc Inc., Waltham, USA). The PCR products were sequenced using an ABI3730 XL automatic DNA sequencer. The sequences were aligned using the BioEdit 7.7 sequence alignment editor and compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

For differentiation of AAB species closely related by their sequences of the rRNA gene, some specific-biochemical tests were performed in order to validate the data obtained by 16 rRNA sequencing. Growth on 30% D-glucose, 0.3% maltose, 0.3% methanol and 10% ethanol was examined by using a basal

medium (0.05% yeast extract, 0.3% (w/v) vitamin-free Casamino acids (Difco), and 2.5% agar) and appropriate concentrations of carbon sources. Medium without the carbon source was used as a control. Growth was checked after 7 days incubation at 28 °C. The utilization of ammonium as the sole nitrogen source in the presence of ethanol as carbon source was tested using Frateur's modified Hoyer ethanol/vitamins medium containing 2.5% agar. The acid production from D- and L-arabitol was tested in phenol-red broth with the carbon source added at a final concentration of 1 %. The results were assessed with reference to the control after incubation at 28 °C for 48 h. All the tests were performed as described previously (14, 34).

2.3 Culture-independent analysis through Nested PCR–denaturing gradient gel electrophoresis (DGGE)

2.3.1 Total-Community DNA Isolation

Beans and pulp were physically separated by adding 100 ml of sterile distilled water to 100 g of the cocoa beans and adhering pulp in a plastic bag and homogenized in a stomacher at normal speed for 5 min. The pulp fraction was recovered by decanting. 40 ml of the pulp fraction was lyophilized and the freeze-dried cocoa pulp was ground thoroughly with a sterile pestle. 30 mg freeze-dried pulp was mixture homogenized for two times in 1.5 ml of phosphate-buffered saline. The combined fluids were mixed by vortexing for a further 10 min and subsequently centrifuged at 100 × g at 4°C for 10 min to remove large particles. The supernatant was further centrifuged at 8,000 × g at 4 °C for 20 min to pellet the yeast and bacterial cells, which were subsequently frozen at −20°C for at least 1 h. This procedure was performed twice for the preparation of yeast and bacteria cells separately. The lysis of bacterial cells was performed as described by Pereira et al. (31). For the lysis of yeast cells, the pellet was resuspended in 600 µl sorbitol buffer (1 M sorbitol; 100 mM EDTA;

14 mM β-mercaptoethanol) with 10 mg/ml of Lysing-enzymes, and incubated at 30°C for 1h. Pellet the spheroplasts were obtained by centrifugation for 5 min at 5,000 × g and resuspended in 180 µl Buffer ATL (supplied in the QiAamp DNA Mini Kit). Following of the step lysis, DNA in the supernatant further purified using "Protocol: DNA Purification from Tissues" [(Qiaamp DNA Mini Kit (Qiagen, Hilden, Germany)] from step 4 (bacteria procedure) and from step 2 (yeast procedure), following the instructions of the manufacturer. The final samples were stored at −20°C until further use.

2.3.2 Nested PCR–DGGE strategy

In order to increase the sensitivity and to facilitate the DGGE by analyzing fragments of the same length, a two-step nested-PCR technique was applied. For analysis of bacterial diversity, primers 27F and 1512R were used to amplify the nearly complete 16S rRNA-encoding gene under conventional PCR conditions in the first PCR step (23). Subsequently, the product of this first PCR was used as a template for a nested PCR targeting the V3 region of the 16S rRNA gene using primers GC-338f and 518r (29) to create a DNA fragment suitable for DGGE analysis. For analysis of yeast diversity, PCR amplification of the ITS regions were performed using primers ITS1-F and ITS4 in the first step, followed by nested PCR using the DGGE primers GC-ITS1-F and ITS2 (42). Reactions were performed in a Mastercycler (Eppendorf, Hamburg, Germany). The PCR products were analyzed by DGGE using a BioRad DCode Universal Mutation Detection System (BioRad, Richmond, CA, USA). The PCR products of the second step were loaded onto 8% (w/v) polyacrylamide gels in running buffer containing 1 x TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 8.0). Optimal separation was achieved with a 30–55% urea-formamide denaturing gradient for bacteria community and 12–60% for the yeast community (100% correspondent to 7 M urea and 40% (v/v) formamide).

The DGGE bands of interest were excised from the gel with a sterile scalpel, disrupted in 60 µl of sterile Milli-Q water, and left overnight at 4 °C to let the DNA diffuse out of the bands. 10 µl of the eluted DNA of each DGGE band was re-amplified by using the appropriate primers and the conditions described above. The PCR products for sequencing were purified using the QIAquick PCR purification kit (QIAGEN). The samples were analyzed with an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Searches in GenBank with BLAST were performed to determine the closest known relatives of the partial ribosomal DNA sequences obtained.

2.4 Physical-chemical analysis

For physical-chemical analysis, the beans and pulp were physically separated basically following the protocol of Ardhana and Fleet (2) and Nielsen et al. (26). To evaluate titrable acid and pH of the pulp and the beans, an aliquot was transferred into a beaker and the pH was measured using a pH meter. A further 25-ml aliquot was titrated to an end point pH of 8.1 with 0.1 M NaOH. The values were reported as meq of sodium hydroxide per 100 g dry beans. These measurements were performed in triplicates for each fermentation process. The temperature of the environment and inside the fermenting cocoa pulp-bean mass were measured

For analysis of target metabolite, aqueous extracts from fermentation samples were obtained as described before (36). Alcohols (ethanol and methanol), organic acids (lactic-, acetic-, and citric-acid), and carbohydrates (glucose, sucrose, and fructose) were determined from pulp and bean extracts by high-performance liquid chromatography (HPLC) apparatus (HP series 1200, Hewlett-Packard Company, USA), equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, USA) connected to a RI detector (HPG1362A,

Hewlett-Packard Company). The column was eluted with a degassed mobile phase containing 4 mMH_2SO_4, at 30 °C and at a flow rate of 0.6 ml/min.

2.5 Polyphasic screening study

2.5.1 Screening on agar plates for stress tolerance

For the analysis of ethanol, lactic acid, acetic acid, glucose and fructose tolerance, approximately 10^6 CFU ml^{-1} of the yeast or AAB isolates were plated on basal medium (0.05% yeast extract, 0.3% (w/v) vitamin-free Casamino acids (Difco), and 2.5% agar), and the LAB isolates were plated on MRS agar. The plates were supplemented with 6, 10 or 12% (v/wt) ethanol; 1, 2, 3 or 5% (v/wt) lactic acid; 1, 2, 3 or 5% (v/wt) acetic acid; 5, 15 or 30% (wt/wt) glucose; and 5, 15 or 30% (wt/wt) fructose. Media without a carbon source was used as a control. Growth was observed after seven days at 28°C. To evaluate heat tolerance, the isolates were grown at 30, 37 and 45°C on YEPG agar (the yeast and AAB isolates) or MRS agar (the LAB isolates). To evaluate pH tolerance, each medium was adjusted to pH 2, 3 or 5.

2.5.2 Evaluation of the yeast, LAB and AAB fermentation performance

The LAB and AAB strain fermentations were performed in 2-l Erlenmeyer flasks containing 500 ml of cocoa pulp simulation medium for the LAB (PSM-LAB) (fructose 25 g/l; glucose 25 g/l; citric acid 10 g/l; yeast extract 5 g/l; soya peptone 5 g/l; magnesium sulfate-heptahydrate 0.5 g/l; manganese sulfate-monohydrate 0.2 g/l; Tween 80 1 ml/l), or in cocoa pulp simulation medium for the AAB (PSM-AAB) (calcium lactate-pentahydrate 10 g/l; ethanol 10 ml/l; yeast extract 10 g/l; soya peptone 5 g/l), as described by Lefeber et al. (20). PSM-LAB and PSM-AAB were supplemented with 20% (v/v) fresh cocoa pulp. A cocoa pulp simulation medium for the yeast (PSM-yeast) was formulated contained fructose 25 g/l, glucose 25 g/l, citric acid 10 g/l, yeast extract 5 g/l,

soya peptone 5 g/l and 20% (v/v) fresh cocoa pulp. The pre-cultures were grown in the same substrate at 30 °C for 48 h and then used to inoculate each one of the fermentations (10^6 CFU ml^{-1}). The fermentations were performed in triplicate for each strain at 30 °C for 24 h under static conditions. The alcohol (ethanol and methanol), organic acid (lactic-, acetic- and citric-acid), and carbohydrate (glucose, sucrose and fructose) contents were quantified at 0, 12 and 24 h of fermentation using high-performance liquid chromatography (HPLC) with a model HP series 1200 system (Hewlett-Packard Company, USA) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, USA) connected to an Refractive Index (RI) detector (HPG1362A, Hewlett-Packard Company). The column was eluted with a degassed mobile phase containing 4 mM H_2SO_4 at 30 °C and at a flow rate of 0.6 ml/min.

3 Results

3.1 Microbial counts

The results of the culture-dependent microbiological approach demonstrated that the yeasts, LAB and ABB were able to grow in bench- and pilot-scale cocoa fermentations (Fig. 1a).

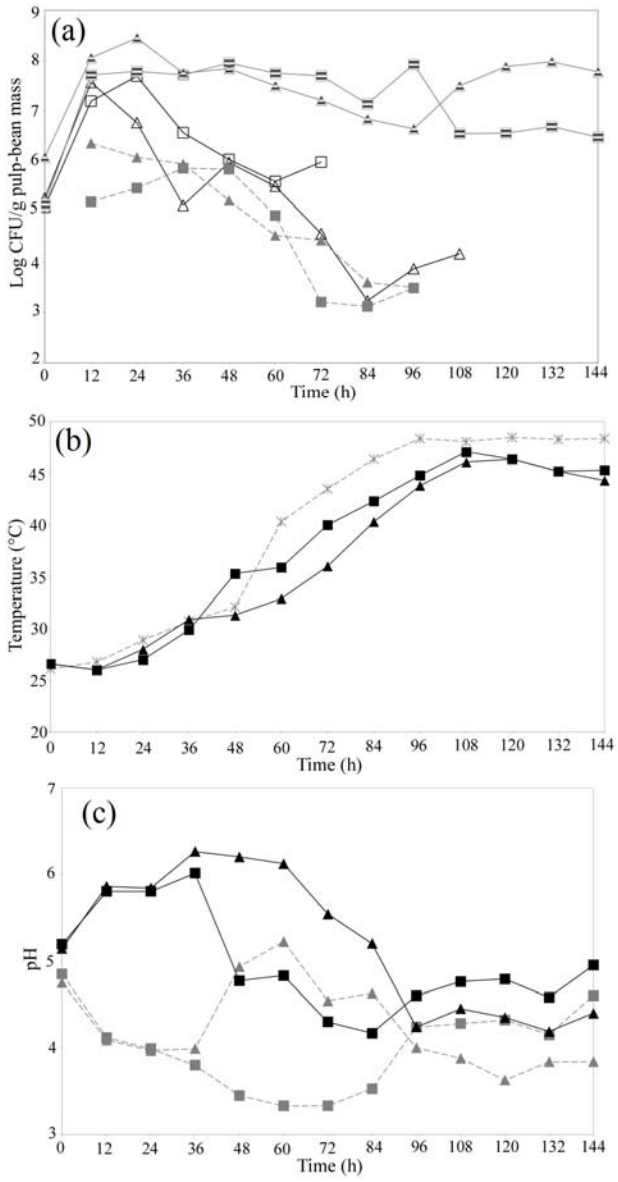

Figure 1 (a) Evolution of LAB in MRS medium (traced symbols), yeast in YEPG medium (open symbols), and AAB in GYC medium (solid symbols). Symbols: ≡, □ and ■ ST; ≜, △ and ▲ PC. (b) Temperature inside the ST ▲ and PC ■, and incubation temperature✕. (c) pH inside the beans (black symbols) and in the pulp (gray symbols). Symbols ▲ and ▲ ST; ■ and ■ PC

The LAB and yeast developed simultaneously and reached a maximum population of 8 log CFU g^{-1} after 12 h of fermentation. The LAB counts remained high throughout the fermentations, while the yeast progressively decreased and became undetectable after 72 and 108 h in the PC and ST fermentations, respectively. The AAB population started to develop after 12 h (5.20 and 6.36 log CFU g^{-1} in the PC and ST, respectively) and was present up to 96 h after the start of fermentation. Although nutrient agar medium was used to monitor the growth of aerobic mesophilic bacteria and *Bacillus* spp., several Gram-negative and aerobic rods that produced catalase and were non-spore forming (later identified as AAB) grew on this medium (data not shown).

3.2 Identification and distribution of the isolated

A total of 96 MRS, 68 GYC, 98 YEPG and 71 NA isolates were randomly picked up at different times during the fermentation processes and identified via biochemical and molecular methods (Table 1 and 2).

Table 1 Species isolated from PC

Closest NCBI match	Accession number	Fermentation time (h)												
		0	12	24	36	48	60	72	84	96	108	120	132	144
Yeast, log(CFU g-1)														
S. cerevisiae	AM711362.1	5.32	7.08	8.29	6.17	5.87	<1	<1	<1	<1	<1	<1	<1	<1
P. kluyveri	FM199971.1	<1	<1	<1	6.09	6.01	<1	<1	<1	<1	<1	<1	<1	<1
H. uvarum	FJ515178.1	<1	<1	<1	<1	6.33	5.93	<1	<1	<1	<1	<1	<1	<1
I. orientalis	EU315767.1	<1	<1	<1	6.13	5.92	<1	<1	<1	<1	<1	<1	<1	<1
LAB, log(CFU g-1)														
L. plantarum	HQ293084.1	4.11	7.23	7.87	6.96	6.84	6.23	6.76	<10	<10	5.98	5.76	5.59	<10
L. fermentum	HQ293040.2	<10	7.01	8.01	7.02	6.92	6.77	6.08	6.60	6.52	6.17	6.02	5.91	6.01
L. vaccinostercus	AB218801.1	<10	<10	7.96	7.11	<10	<10	<10	<10	<10	<10	<10	<10	<10
AAB, log(CFU g-1)														
A. malorum	FJ831444.1	<1	<1	5.23	<1	<1	4.31	3.76	<1	3.49	<1	<1	<1	<1
A. cerevisiae	HM562995.1	<1	4.00	<1	<1	5.00	4.00	<1	<1	<1	<1	<1	<1	<1
A. tropicalis	DQ523494.1	<1	<1	4.00	5.74	5.52	4.74	3.84	3.12	<1	<1	<1	<1	<1
A. ghanensis	HM562984.1	<1	5.10	<1	5.00	<1	<1	3.87	<1	<1	<1	<1	<1	<1
NA, log(CFU g-1)														
B. subtilis	HQ286641.1	5.91	6.73	6.98	5.76	4.00	<1	<1	<1	<1	<1	<1	<1	<1
B. megaterium	FJ174651.1	5.32	5.00	6.32	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
St. pasteuri	HM130543.1	5.17	5.00	6.21	<1	6.00	<1	<1	<1	<1	<1	<1	<1	<1
St. xylosus	HM854231.1	<1	6.19	5.00	<1	6.00	<1	<1	<1	<1	<1	<1	<1	<1
St. saprophyticus	HM130543.1	<1	5.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
T. saanichensis	EU215774.1	5.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

Abbreviations: S.: *Saccharomyces*, P.: *Pichia*, H.: *Hanseniaspora*, I.: *Issatchenkia*, L.: *Lactobacillus*, A.: *Acetobacter*, B.: *Bacillus*, St.: *Staphylococcus*, T.: *Tatumella*. NA.: Species isolated on Nutrient Agar medium

Table 2 Species isolated from ST

Closest NCBI match	Accession number	Fermentation time (h)												
		0	12	24	36	48	60	72	84	96	108	120	132	144
Yeast, log(CFU g-1)														
S. cerevisiae	AM711362.1	5,3	7.14	8.07	6.27	6.45	5.71	5.93	4.45	3.95	<1	<1	<1	<1
P. kluyveri	FM199971.1	<1	6.00	<1	6.34	6.56	<1	<1	<1	<1	<1	<1	<1	<1
H. uvarum	FJ515178.1	<1	<1	<1	<1	5.00	5.65	<1	<1	<1	<1	<1	<1	<1
I. orientalis	EU315767.1	<1	<1	<1	6.56	5.00	<1	<1	<1	<1	<1	<1	<1	<1
D. etchellsii	AJ586528.1	<1	6.00	7.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
K. ohmeri	EF196811.1	<1	6.00	7.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
LAB, log(CFU g-1)														
L. plantarum	HQ293084.1	4.55	7.30	7.97	<10	<10	8.05	8.08	<10	<10	<10	<10	<10	<10
L. fermentum	HQ293040.2	<10	<10	8.01	7.16	7.72	8.09	<10	7.07	7.96	6.48	6.37	6.21	6.09
AAB, log(CFU g-1)														
A. malorum	FJ831444.1	<1	<1	6.08	5.51	<1	4.32	3.00	<1	<1	<1	<1	<1	<1
A. cerevisiae	HM562995.1	<1	5.00	<1	<1	5.00	4.61	<1	<1	<1	<1	<1	<1	<1
A. tropicalis	DQ523494.1	<1	6.22	5.00	5.17	5.10	4.37	3.84	3.61	3.50	<1	<1	<1	<1
A. ghanensis	HM562984.1	<1	<1	<1	<1	5.00	4.00	3.87	<1	<1	<1	<1	<1	<1
NA, log(CFU g-1)														
B. subtilis	HQ286641.1	6.81	6.92	7.42	5.11	4.28	<1	<1	<1	<1	<1	<1	<1	<1
B. megaterium	FJ174651.1	5.00	6.31	7.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
T. saanichensis	EU215774.1	<1	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
St. aureus	FJ899095.1	5.00	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
St. equorum	AM945662.1	<1	<1	<1	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1
Pt. agglomerans	EU596536.1	6.20	<1	7.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Pt. terrea	HM562993.1	<1	6.00	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

Abbreviations: D.: *Debaromyces*, K.: *Kodamaea*, Pt.: *Pantoea*. Other abbreviations see Table 1

The 16S rRNA gene sequence analysis identified all of the homofermentative or facultatively heterofermentative MRS isolates as *L. plantarum* (*n*=39). The other 57 isolates were obligate heterofermentatives, and most of them were identified as *L. fermentum* (*n*=52); the remaining five obligately heterofermentative isolates were closely related to *L. vaccinostercus*. At the onset of fermentation, *L. plantarum* was the dominant species of the LAB with a population of approximately 4 log CFU g^{-1}. After 12-24 h, *L. fermentum* was the dominant species isolated and reached a maximum population of 8.01 log CFU g^{-1} at 24 h and 8.09 log CFU g^{-1} at 60 h in the PC and ST, respectively.

All of the isolates from the GYC agar plates oxidized acetate and lactate to CO_2 and H_2O and were assigned to the genus *Acetobacter*. The sequencing of the 16S rRNA genes and the specific biochemical tests supported the identification of these isolates as four different species of *Acetobacter*, namely *A. tropicalis* (*n*=39), *A. malorum* (*n*=13), *A. cerevisiae* (*n*=9), and *A. ghanensis* (*n*=7). *A. tropicalis* was the dominant species and reached a maximum population after 36 h (5.74 log CFU g^{-1}) and 12 h (6.22 log CFU g^{-1}) in the PC and ST, respectively.

The 16S rRNA gene sequence analysis demonstrated that a range of other bacterial species grew on NA, including members of the Gram-positive order *Bacillales* and the Gram-negative families *Enterobacteriaceae* and *Acetobacteraceae*. The members of the order *Bacillales* were subdivided into the endospore-forming bacteria *B. subtilis* (*n*=29) and *B. megaterium* (*n*=11) and the non-spore forming bacteria *St. pasteuri* (*n*=7), *St. aureus* (*n*=3), *St. saprophyticus* (*n*=3), *St. equorum* (*n*=1) and *St. xylosus* (*n*=1). The *Enterobacteriaceae* family was represented by the species *T. saanichensis* (*n*=3), *P. agglomerans* (*n*=3) and *P. terrea* (*n*=2), while the *Acetobacteraceae* family was represented by the species *A. malorum*/*cerevisiae* (*n*=6) and *A. pomorum*/*pasteurianus* (*n*=2) (data not shown). In this medium, *B. subtilis* was the dominant species with a

maximum population of 6.98 log CFU g^{-1} at 24 h and 6.92 log CFU g^{-1} at 12 h in the PC and ST, respectively.

The yeast isolates from the YEPG agar plates were *S. cerevisiae* (*n*= 63), *P. kluyveri* (*n*=13), *H. uvarum* (*n*=9), *I. orientalis* (*n*=7), *D. etchellsii* (*n*=5) and *K. ohmeri* (*n*=1). *S. cerevisiae* was the most prevalent yeast found at the start of fermentation and grew to a maximum population of 8.29 log CFU g^{-1} and 8.07 log CFU g^{-1} at 12 h in the PC and the ST, respectively. Occasionally, *P. kudriavzevii*, *H. uvarum* and *I. orientalis* were found in both fermentation processes, while *D. etchellsii* and *K. ohmeri* were only isolated at 12 h and 24 h in the ST fermentation.

3.3 Culture-independent microbiological analysis using a nested-PCR–DGGE strategy.

As displayed in Fig. 2, the bacterial and yeast DGGE profiles revealed a stable microbial composition independent of the fermentation time.

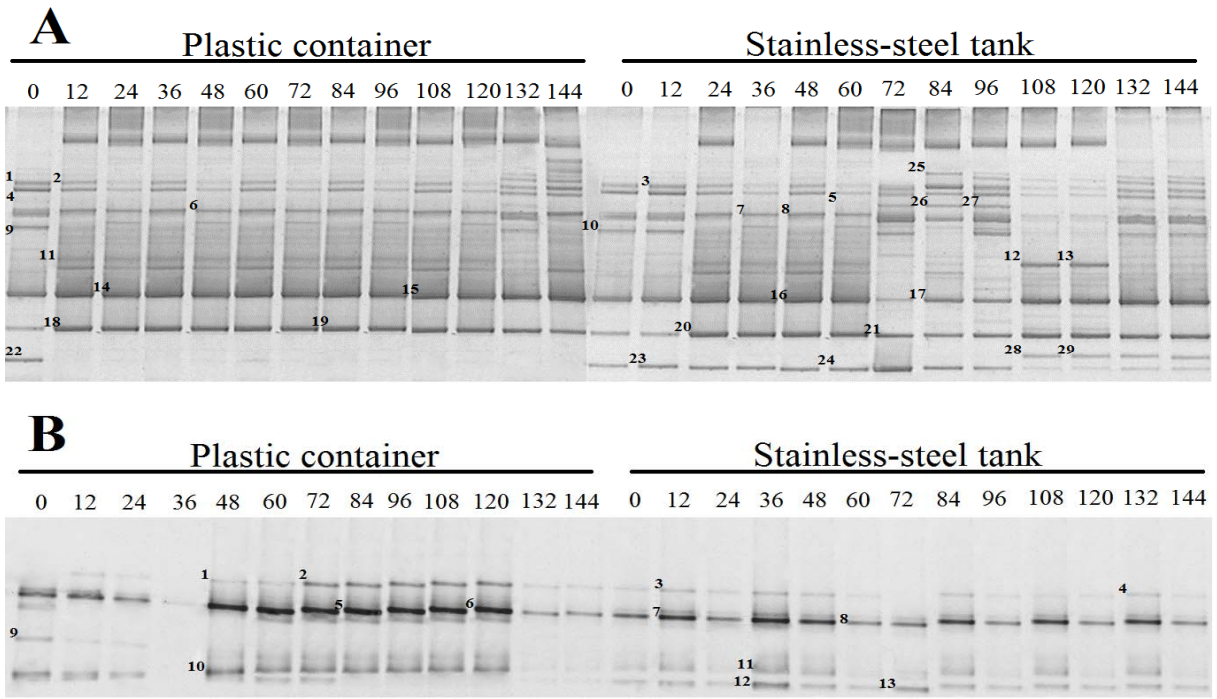

Figure 2 16S rRNA gene-PCR-DGGE patterns of the bacterial communities (A) and ITS-PCR-DGGE patterns of the yeast communities (B) associated with cocoa bean fermentation samples from PC and ST. The closest relatives of the fragments sequenced based on the results of the search for similar sequences in GenBank (over 97% of similarity) were: (A) 1, 2 and 3 *L. plantarum*; 4, 5, 6, 7 and 8 *L. fermentum*; 9 and 10 *P. terrea*; 11 *Staphylococcus* sp.; 12 and 13 *B. subtilis*; 14, 15, 16, 17, 18, 19, 20 and 21 *Acetobacter* sp; 22, 23 and 24 *S. saprophyticus*; 25 Uncultured *Lactobacillus* sp; 26 and 27 *T. ptyseos*; 28 and 29 *Bacillus* sp.; (B) 1, 2, 3, 4, 5, 6. 7 and 8 *Hanseniaspora* sp; 9 *Debaryomyces* sp; 10 and 11 *S. cerevisiae*; 12 and 13 *P. kluyveri*

The PCR–DGGE revealed that *L. fermentum*, *L. plantarum* and *Acetobacter* sp. (*A. tropicalis* and *A. senegalensis* were the closest relatives found using sequence comparisons) were the dominant bacterial species, as revealed through sequencing of the most intense bands (Fig. 2a). Members of the *Enterobacteriaceae* family (*T. ptyseos* and *P. terrea*) and species of *Staphylococcus* (*S. saprophyticus* and *Staphylococcus* sp.) were identified at different times in both fermentation processes, while *B. subtilis* and *Bacillus* sp. were detected at the end of the ST fermentation.

The PCR-DGGE revealed that the yeast population was composed of a few species (Fig. 2b). The most intense bands corresponded to the *Hanseniaspora* species (over 99% identity with *H. opuntiae*, *H. uvarum* and *H. guilliermondii*). *S. cerevisiae* could also be detected in almost all of the samples, but its band density was typically weak. *P. kluyveri* was detected throughout the ST fermentation, while *Debaryomyces* sp. was observed at the beginning of the PC fermentation.

3.4 pH and temperature measured

The initial pH values of the pulp and beans were on average 4.8 and 5.1, respectively (Fig. 1c). In the first 36 h of fermentation, the pH of the pulp decreased to 3.8, while the pH of the beans exceeded 6. Inside the cotyledons, the pH values decreased quickly after 48 h, and the final pH values were 3.84 and 4.4 for the PC and ST fermentations, respectively. The temperature inside the PC and ST fermentations was adjusted from an initial 26.6ºC to a maximum 46-47 ºC obtained at 108 h of fermentation (Fig. 1b). After 108 h of fermentation, a slight decrease to 45-44 ºC was observed.

3.5 *Substrate consumption and fermentation products*

The substrate consumption and the metabolite production of the spontaneous cocoa bean fermentations are shown on Figure 3.

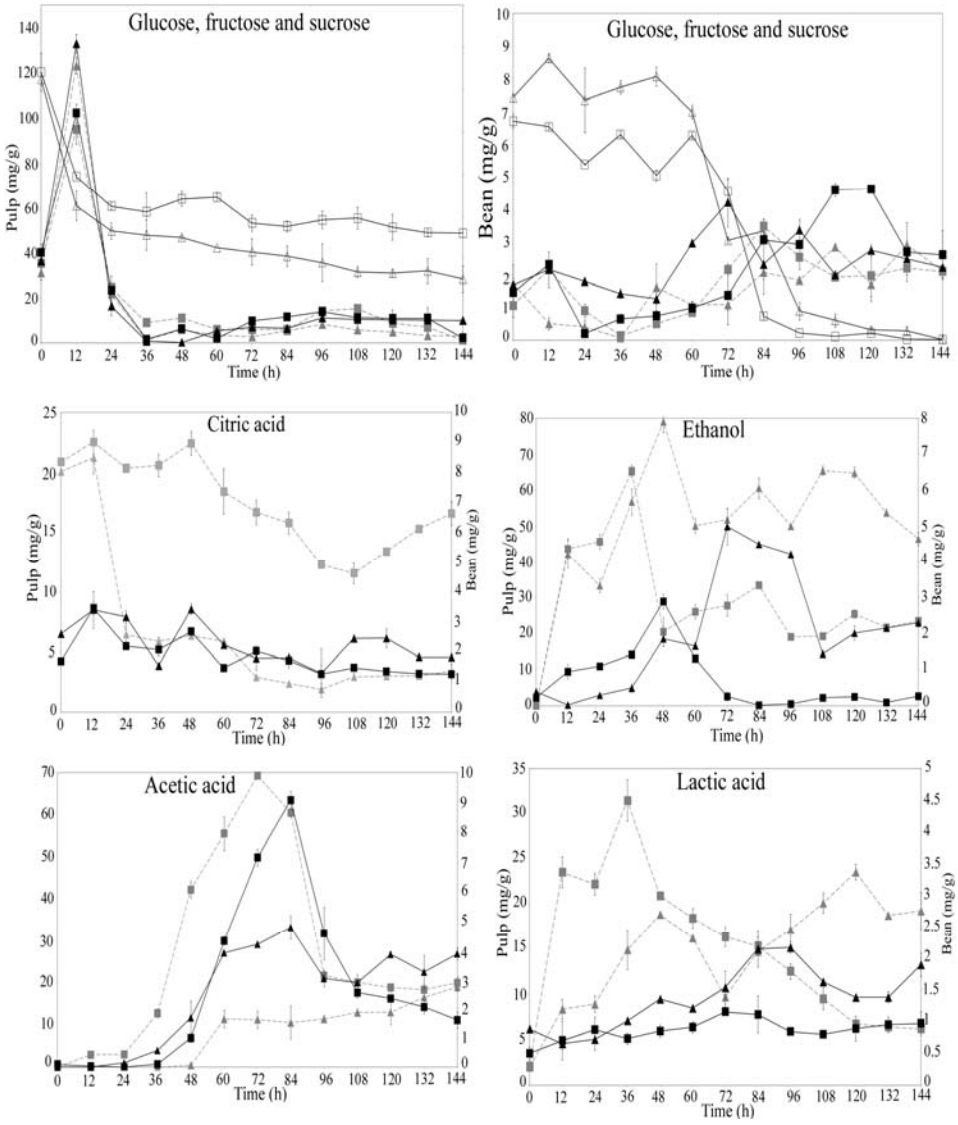

Figure 3 Course of residual glucose, fructose, and sucrose, and production of ethanol, methanol and organic acids during cocoa bean fermentation samples from ST (▲, ▲ and△) and PC (■ , □ and). For ethanol and organic acid in the pulp (gray symbols) and insed the beans (black symbols). Bars represent the standard deviation

During the first 12 h of fermentation, the hydrolysis of nearly 50% of the sucrose increases the level of glucose and fructose to over 90 mg/g. Glucose and fructose were consumed simultaneously and rapidly up to 36-48 h, and afterwards, no significant changes were observed. The major carbohydrate inside the beans was sucrose (6.73 mg/g and 7.44 mg/g in the PC and ST, respectively), which was continuously hydrolyzed into fructose and glucose.

Ethanol was produced and subsequently consumed concomitantly with the growth of the yeast and the AAB (a maximum concentration of 65.37 mg/g at 36 h and 79 mg/g at 48 h in the PC and ST, respectively) (Fig. 3). The ethanol produced in the pulp diffused into the beans and reached a maximum concentration of 2.89 mg/g at 36 h and 4.51 mg/g at 84 h in the PC and ST, respectively, after which it began evaporating.

The ST fermentation exhibited an unexpected profile with little acetic acid production; it was not oxidized during the first 48 h of fermentation, and, with a linear increase, peaked only at the end of the fermentation process (18.86 mg/g). During the PC fermentation, acetic acid quickly appeared within 48 h (from 24 to 72 hours). After 72 h of fermentation, the acetic acid was likely oxidized to CO_2 and H_2O, dropping below 20 mg g^{-1} at the end of the fermentation. A portion of the acetic acid content produced in the pulp diffused into the beans after 36 h of fermentation. The final concentration of acetic acid in the cotyledon was 1.58 mg/g and 3.82 mg/g in the PC and ST, respectively. The concentration of lactic acid in the pulp increased the first 36-48 h, followed by a drop coinciding with the appearance of the AAB; there was again an increase in the concentration during the last three days of the ST fermentation.. The final lactic acid content in the pulp was 6.2 mg/g in the PC fermentation, and the ST concentration was approximately threefold higher (19.22 mg/g). The lactic acid content in the cotyledons was nearly twofold higher at the end of the ST fermentation process (1.89 mg/g and 0.97 mg/g in the ST and PC,

respectively). A rapid decrease in the citric acid content in the pulp was observed after 12 h and 48 h for the ST and PC fermentations, respectively, while inside the beans, the citric acid content was stable during both fermentation processes, with a final concentration of 1.26 and 1.82 in the PC and ST, respectively.

3.6 Poly-phasic selection study

To investigate the physiological adaptation of the yeasts (184 isolates), the LAB (156 isolates), and the AAB (112 isolates) to the cocoa fermentation conditions and to select for strains more adapted to this environment, several growth parameters were evaluated. Initially, the isolates were screened for their abilities to withstand the stressors imposed by cocoa fermentation including variations in the pH, the temperature, and the concentrations of ethanol, lactic acid, acetic acid, glucose and fructose. Isolates from traditional cocoa fermentations performed in wooden boxes in Brazil (G.V.M. Pereira et al., submitted for publication) were also included in this step. The most adapted strains were selected and subjected to additional kinetic analysis performed in cocoa pulp simulation media (Table 3-5).

Table 3 Substrate consumption and metabolite production by selected yeast in the PSM-yeast during 24h of fermentation

Strain	Time (h)	Substrate consumption and metabolite production						
		Glucose	Fructose	Citric acid	Ethanol	Et/Carb (%)**	Lactic acid	Acetic acid
S. cerevisiae UFLA CHYA13.01	0	26.49±1.23	17.39±1.97	11.31 ± 1.37	0.65 ± 0.09	38.89	2.61±0.76	ND
	12	1.68±0.65	4.81±0.26	11.42±0.98	16.71±1.97		2.92±0.43	ND
	24	ND	0.19±0.04	11.07 ± 1.00	18.09 ± 1.42		2.62±0.79	0.63±0.22
S. cerevisiae UFLA CHYA14.08	0	25.53±0.96	17.15±1.04	11.07 ± 0.88	0.61 ± 0.43	39.80	2.56±0.99	ND
	12	3.37±0.72	6.92±0.53	11.44±1.54	14.71±1.56		2.30±0.13	ND
	24	ND	0.22±0.01	11.17 ± 0.70	18.37 ± 1.74		2.00±0.87	0.75±0.04
S. cerevisiae UFLA CHYB14.01	0	25.84±1.11	16.43±0.91	10.53 ± 0.80	1.16 ± 0.57	41.32	2.46±0.03	ND
	12	ND	0.55±±0.03	11.54±0.75	18.97±1.56		2.06±0.05	ND
	24	ND	0.22±0.01	10.98 ± 0.8	19.02 ± 1.78		2.04±0.09	1.01±0.34
S. cerevisiae UFLA CHYB18.03	0	26.22±1.22	15.87±1.06	8.74 ± 0.78	0.61 ± 0.08	49.93	1.48±0.01	ND
	12	13.42±0.76	12.04±1.11	10.33±0.42	7.91±0.67		2.21±0.05	ND
	24	ND	0.92±0.03	11.84 ± 0.94	19.49 ± 1.96		2.55±0.23	0.88±0.19
S. cerevisiae UFLA CHYB4.01	0	26.03±1.87	16.93±0.92	9.46 ± 0.80	0.61 ± 0.13	41.04	1.42±0.05	ND
	12	6.24±0.98	8.48±0.22	9.48±0.24	12.59±1.75		1.07±0.04	ND
	24	ND	0.23±0.01	12.63 ± 0.40	18.62 ± 0.98		2.17±0.11	ND
S. cerevisiae UFLA CHYB4.03	0	21.65±2.97	14.18±1.82	8.6 ± 0.67	0.53 ± 0.08	49.81	1.66±0.34	ND
	12	2.56±0.09	5.60±0.13	11.17±1.65	15.39±1.46		2.18±0.09	ND
	24	ND	0.26±0.03	11.53 ± 0.61	18.77 ± 1.87		2.41±0.23	0.95±0.11
S. cerevisiae UFLA CHYC5.05*	0	27.03±2.65	17.38±1.33	9.67 ± 0.71	0.63 ± 0.18	38.27	1.39±0.05	ND
	12	3.23±0.03	6.59±0.87	10.38±1.36	15.15±1.97		2.21±0.09	ND
	24	ND	0.22±0.01	11.22 ± 1.09	17.99 ± 1.37		2.78±0.45	DN

Table 3, continuation

S. cerevisiae UFLA CHYC7.04*	0	21.74±0.98	13.55±1.87	5.9 ± 0.42	1.36 ± 0.14	49.33	1.27±0.09	ND
	12	11.42±0.57	10.92±1.09	10.34±0.91	9.39±0.85		3.04±0.03	ND
	24	ND	0.35±0.01	11.6 ± 0.53	19.21 ± 0.97		2.86±0.34	ND
S. cerevisiae UFLA CHYE15.07*	0	23.40±1.11	15.41±0.37	9.26 ± 0.79	1.04 ± 0.11	33.03	1.76±0.33	ND
	12	6.66±0.83	8.68±1.11	10.05±1.56	12.61±0.75		1.59±0.43	ND
	24	ND	0.21±0.03	10.51 ± 0.32	14.16 ± 1.87		1.89±0.56	0.45±0.22
D.etchelslsii UFLA CHYB5.56	0	20.55±2.87	13.46±2.21	7.95 ± 0.83	1.42 ± 0.24	40.67	1.43±0.09	ND
	12	ND	0.87±0.09	9.55±0.98	17.83±1.89		1.24±0.23	ND
	24	ND	0.18±0.02	9.55 ± 0.57	15.61 ± 1.56		1.77±0.70	0.48±0.03
C. humilis UFLA CHYD14.32*	0	26.38±2.27	16.74±1.32	9.32 ± 0.50	0.64 ± 0.09	39.09	1.60±0.09	ND
	12	9.65±0.76	10.47±0.93	9.52±0.34	10.08±1.23		1.69±0.04	ND
	24	ND	0.22±0.03	12.16 ± 0.99	17.86 ± 1.14		2.85±0.25	ND
C. orthopsilosis UFLA CHYC5.02*	0	27.98±2.11	16.70±0.84	9.18 ± 0.41	0.59 ± 0.10	8.71	1.49±0.44	ND
	12	25.16±1.41	15.52±0.34	8.77±0.67	0.85±0.09		1.28±0.35	ND
	24	17.01±0.98	12.34±0.12	9.25 ± 0.93	4.56 ± 0.35		1.65±0.09	ND
C. intermedia UFLA CHYE21.01*	0	25.82±1.44	16.49±1.54	9.25 ± 0.57	0.65 ± 0.05	1.04	1.59±0.54	ND
	12	24.35±1.23	16.94±0.34	10.22±1.98	0.68±0.04		2.33±0.45	ND
	24	24.39±1.49	16.07±0.75	8.87 ± 0.80	1.07 ± 0.07		1.44±0.43	ND
I.orientalis UFLA CHYB6.02	0	22.82±1.11	15.25±0.76	9.11 ± 0.70	1.18 ± 0.16	38.21	1.75±0.09	ND
	12	12.35±0.54	9.95±0.16	10.31±0.90	7.69±0.34		2.11±0.13	ND
	24	ND	0.20±0.03	10.35 ± 0.56	16.09 ± 1.65		2.35±0.34	0.43±0.19
I. orientalis UFLA CHYC6.02*	0	20.64±1.11	13.43±0.92	9.6 ± 0.70	1.34 ± 0.27	32.25	1.67±0.09	ND
	12	13.80±0.32	10.40±0.94	9.17±0.87	5.00±0.46		2.61±0.34	ND
	24	3.82±0.08	6.36±0.15	10.67 ± 0.64	12.62 ± 0.87		2.67±0.98	0.29±0.01

Table 3, continuation

P. kluyeri UFLA CHYC2.02*	0	26.08±1.09	16.91±0.94	10.93 ± 0.72	0.62 ± 0.07	36.08	2.55±0.07	ND
	12	2.31±0.04	5.71±0.29	10.94±0.78	15.10±1.34		2.14±0.12	ND
	24	ND	0.20±0.07	11.25 ± 0.88	16.47 ± 0.99		2.40±0.54	ND
P. fermentans UFLA CHYA4.01	0	25.62±1.22	15.45±0.96	9.20 ± 0.64	0.59 ± 0.08	16.69	1.96±0.09	ND
	12	25.54±0.76	15.42±0.82	9.28±0.98	0.03±0.02		2.00±0.86	ND
	24	17.04±1.02	13.17±0.53	10.12 ± 0.77	7.59 ± 0.97		1.35±0.55	0.09±0.01
P. fermentans UFLA CHYD31.07*	0	25.65±1.84	16.08±1.27	8.92 ± 0.21	0.6 ± 0.07	16.11	1.54±0.36	ND
	12	20.01±1.10	13.50±0.83	7.94±0.29	1.60±0.05		1.16±0.31	ND
	24	13.12±0.97	10.96±1.34	10.46 ± 0.32	7.47 ± 1.03		2.06±0.07	ND
K. ohmeri UFLA CHYB2.01	0	29.28±2.56	18.19±2.01	10.04 ± 0.89	0.72 ± 0.11	20.90	1.80±0.05	ND
	12	20.77±1.11	14.14±1.02	10.18±1.23	2.89±0.07		2.18±0.09	ND
	24	8.49±0.76	8.11±0.98	10.9 ± 0.30	10.86 ± 1.03		2.83±0.06	ND
Sz. pombe UFLA CHYE5.39*	0	25.91±1.86	16.28±1.23	9.1 ± 0.61	0.63 ± 0.07	1.07	1.62±0.05	ND
	12	24.23±0.94	15.44±0.23	10.08±1.45	0.81±0.17		2.94±0.08	ND
	24	24.33±1.54	15.76±1.83	12.11 ± 0.41	1.09 ± 0.32		2.48±0.02	ND

Means of duplicated assays are followed by the standard deviation. Microorganism's abbreviations see Table 1 and 2. *strains isolated from traditional cocoa fermentations carried out in wooden boxes in Brazil (G.V.M. Pereira; I.S. Coelho; M.G.C.P. Miguel; K.T. Magalhães; E.G. Almeida, unpublished data). ** Percentage of conversion relative to the theoretical value: Conversion (%) = (product / substrate) x 100

Table 4 Substrate consumption and metabolite production by selected LAB in the PSM-LAB during 24h of fermentation

Strain	Time (h)	Substrate consumption and metabolite production					
		Glucose	Fructose	Citric Acid	Ethanol	Lactic acid	Acetic acid
L. fermentum UFLA CHBE2.02	0	23.43±0.52	13.48±0.33	9.62 ± 0.31	0.82 ± 0.01	2.58 ± 0.13	1.19 ± 0.09
	12	20.71±0.30	13.40±0.13	ND	0.96±0.04	3.59±0.11	4.48±0.21
	24	15.53±1.11	13.40±0.42	ND	0.85 ± 0.01	6.39 ± 0.53	6.15 ± 0.12
L. fermentum UFLA CHBE6.01	0	22.75±1.02	13.31±0.12	9.26 ± 0.82	0.89 ± 0.06	2.58 ± 0.18	1.38 ± 0.07
	12	19.22±0.21	14.23±0.21	ND	0.59±	4.78±0.14	5.21±0.21
	24	7.79±0.78	13.36±0.23	ND	4.3 ± 0.14	6.93 ± 0.35	6.25± 0.16
L. fermentum UFLA CHBE10.02	0	24.27±0.89	14.18±0.19	9.81 ± 1.0	0.87± 0.05	2.35 ± 0.16	0.85 ± 0.05
	12	21.25±0.57	13.83±0.11	ND	1.03±0.02	3.41±0.09	4.16±0.13
	24	14.89±1.35	13.17±0.21	ND	0.59 ± 0.07	6.1 ± 0.18	5.61 ± 0.03
L. fermentum UFLA CHBE8.12	0	23.71±0.40	13.80±0.32	9.65 ± 0.61	0.84 ± 0.02	2.37 ± 0.6	0.82 ± 0.07
	12	22.40±0.30	13.79±0.41	ND	0.69±0.02	4.35±0.21	4.37±0.12
	24	18.14±0.42	12.59±0.71	ND	0.99 ± 0.08	7.72 ± 0.78	5.39 ± 0.14
L. fermentum UFLA CHBC8.15*	0	24.00±0.20	13.97±0.12	9.76 ± 0.60	0.46 ± 0.03	2.34 ± 0.17	0.74 ± 0.04
	12	21.78±0.49	13.92±0.63	ND	0.68±0.04	3.54±0.12	4.48±0.11
	24	15.38±1.21	15.38±0.54	ND	0.6 ± 0.03	6.17 ± 0.15	5.95 ± 0.09
L. fermentum UFLA CHBD8.42*	0	23.51±0.30	13.68±0.71	9.68 ± 0.51	0.8 ± 0.05	2.59 ± 0.18	1.16 ± 0.07
	12	23.09±0.19	13.65±0.62	ND	0.96±0.03	3.03±0.41	4.22±0.21
	24	16.09±0.21	13.53±0.31	ND	0.95 ± 0.07	6.07 ± 0.13	6.2 ± 0.11
W. ghanens UFLA CHBE8.23*	0	23.65±0.21	13.29±0.41	9.74 ± 0.81	0.61 ± 0.05	2.63 ± 0.03	1.47 ± 0.15

Table 4, continuation

	12	22.13±0.17	13.86±0.14	10.18±0.31	0.65±0.02	3.07±0.18	1.10±0.01
	24	17.84±0.4	13.24±0.11	ND	1.00 ± 0.05	3.43 ± 0.15	6.25 ± 0.07
L. plantarum EA7.01	0	23.97±0.66	13.97±0.11	10.68 ± 0.22	0.62 ± 0.02	3.35 ± 0.13	0.88 ± 0.07
	12	23.22±0.21	13.92±0.31	10.96±0.45	0.65±0.01	3.11±0.13	1.20±0.07
	24	23.12±0.52	13.52±0.42	10.96 ± 0.52	0.66 ± 0.03	4.02 ± 0.18	1.36 ± 0.08
L. plantarum UFLA CHBB6.09	0	23.41±0.22	13.61±0.72	9.97± 0.87	0.62 ± 0.03	3.28 ± 0.31	1.32 ± 0.07
	12	23.12±0.12	13.50±0.51	10.39±0.36	0.65±0.01	3.83±0.23	1.33±0.09
	24	23.07±0.31	13.23±0.71	10.77 ± 0.7	0.80 ± 0.02	5.32 ± 0.13	1.38 ± 0.09
L. plantarum UFLA CHBC5.17*	0	23.30±0.23	13.51±0.11	9.96±0.19	0.62±0.01	3.40±0.12	1.11±0.08
	12	23.01±0.41	13.40±0.40	10.07±0.13	0.69±0.07	3.66±0.21	1.13±0.02
	24	22.98±0.21	13.22±0.61	9.02±0.33	0.91±0.02	3.80±0.26	1.22±0.01
L. plantarum UFLA CHBC8.11*	0	24.64±0.43	13.74±0.11	10.18±0.22	0.61±0.02	2.83±0.07	1.55±0.04
	12	20.93±0.32	12.04±0.42	9.75±0.19	1.27±0.12	2.94±0.09	1.61±0.01
	24	19.96±0.24	11.78±0.51	9.66±0.31	0.50±0.01	3.07±0.11	1.74±0.03
L. plantarum UFLA CHBE4.23*	0	24.27±0.14	14.10±0.31	9.72 ± 0.71	0.66 ± 0.05	2.79 ± 0.28	0.56 ± 0.09
	12	24.11±0.19	14.11±0.33	10.48±0.17	0.66±0.04	3.75±0.12	0.65±0.02
	24	23.98±0.31	13.01±0.21	11.28 ± 0.91	0.8 ± 0.03	5.7 ± 0.26	0.72 ± 0.05

Means of duplicated assays are followed by the standard deviation. Microorganism's abbreviations see Table 1 and 2. *strains isolated from traditional cocoa fermentations carried out in wooden boxes in Brazil (G.V.M. Pereira; I.S. Coelho; M.G.C.P. Miguel; K.T. Magalhães; E.G. Almeida, unpublished data)

Table 5 Substrate consumption and metabolite production by selected LAB in the PSM-LAB during 24h of fermentation

Strain	Time	Substrate consumption and metabolite production in PSM-AAB					
		Glucose	Fructose	Citric acid	Ethanol	Lactic acid	Acetic acid
A. tropicalis UFLA CHBB2.10	0	1.14±0.11	0.00	0.75±0.08	9.16±0.32	7.01±0.23	0.29±0.05
	12	1.10±0.10	0.00	0.16±0.04	6.82±0.12	5.22±0.12	2.86±0.55
	24	1.02±0.01	0.00	0.22±0.01	3.78±0.04	4.77±0.22	7.79±0.61
A. tropicalis UFLA CHBE16.01*	0	1.10±0.01	0.00	0.75±0.03	8.91±0.97	7.26±1.02	0.12±0.12
	12	1.07±0.03	0.00	0.04±0.01	7.12±0.02	5.97±0.02	3.01±0.11
	24	0.97±0.01	0.00	0.02±0.00	0.88±0.02	3.01±0.19	14.08±0.12
A. tropicalis UFLA CHBD2.28*	0	1.50±0.01	0.00	0.78±0.03	11.08±1.43	7.02±0.31	0.13±0.01
	12	1.36±0.4	0.00	0.17±0.01	8.12±0.22	5.03±0.09	5.33±0.32
	24	1.17±0.17	0.00	0.50±0.03	0.88±0.02	5.39±0.11	2.22±0.31
A. tropicalis UFLA CHBE6.15	0	1.21±0.01	0.00	0.72±0.01	9.41±0.23	7.10±0.97	0.30±0.01
	12	1.17±0.02	0.00	0.16±0.04	6.92±0.54	5.84±1.09	3.47±0.11
	24	1.06±0.02	0.00	0.13±0.01	4.99±0.08	2.62±0.12	9.56±0.42
A. tropicalis UFLA CHBE9.02*	0	1.44±0.03	0.00	0.77±0.03	9.67±0.54	7.08±0.42	0.25±0.02
	12	1.17±0.04	0.00	0.17±0.01	6.72±0.78	5.09±0.12	4.95±0.11
	24	1.04±0.01	0.00	0.14±0.02	2.51±0.01	3.41±0.145	9.39±0.12
A. ghanensis UFLA CHBA3.01*	0	1.56±0.02	0.00	0.70±0.09	11.06±1.00	7.07±0.21	0.61±0.05
	12	1.49±0.01	0.00	0.46±0.05	8.12±0.01	5.07±0.11	3.63±0.12
	24	0.90±0.10	0.00	0.19±0.02	1.34±0.01	2.75±0.07	13.25±0.97

Table 5, continuation

A. senegalensis UFLA CHBD4.13*	0	1.04±0.02	0.00	0.85±0.04	8.70±0.98	7.61±0.23	0.68±0.21
	12	1.05±0.03	0.00	0.20±0.01	6.55±0.22	5.54±0.19	4.81±0.12
	24	0.87±0.05	0.00	0.12±0.01	3.84±0.02	3.49±0.14	6.76±0.22
A. senegalensis UFLA CHBE6.16*	0	1.10±0.01	0.00	0.74±0.03	9.82±0.43	6.98±0.87	0.29±0.02
	12	0.97±0.02	0.00	0.64±0.02	7.46±0.05	5.09±1.09	3.64±0.22
	24	1.12±0.01	0.00	0.27±0.02	1.88±0.04	3.68±0.12	13.49±0.96
A.malorum UFLA CHBA1.01	0	1.09±0.02	0.00	0.73±0.02	9.86±0.42	7.33±0.23	0.26±0.02
	12	0.98±0.02	0.00	0.11±0.01	6.78±0.11	5.57±0.12	3.23±0.11
	24	0.96±0.11	0.00	0.25±0.03	1.48±0.03	4.26±0.09	11.45±0.45
A.malorum UFLA CHBB1.08	0	1.21±0.12	0.00	0.69±0.10	10.54±1.01	6.82±0.65	0.29±0.02
	12	1.09±0.01	0.00	0.11±0.01	7.24±0.19	4.46±0.12	5.14±0.22
	24	0.36±0.01	0.00	0.17±0.01	4.17±0.07	3.79±0.09	7.28±0.12
A. pasteurianus UFLA CHBC1.16*	0	1.57±0.20	0.00	0.74±0.01	13.90±1.32	7.01±0.52	0.41±0.01
	12	1.38±0.11	0.00	0.17±0.02	8.03±0.11	4.16±0.14	7.29±0.12
	24	1.00±0.01	0.00	0.21±0.01	3.88±0.01	5.35±0.12	9.06±0.22
A. orientales UFLA CHBE2.35*	0	1.29±0.04	0.00	0.75±0.01	11.14±0.21	7.02±0.98	0.27±0.11
	12	1.20±0.01	0.00	0.09±0.01	9.42±0.11	3.35±0.22	4.22±0.12
	24	0.84±0.01	0.00	0.23±0.02	5.25±0.11	3.67±0.23	5.97±0.11
G. frateurii UFLA CHBD4.09*	0	1.10±0.09	0.00	0.68±0.04	8.36±0.17	6.16±0.12	0.21±0.02
	12	0.90±0.02	0.00	0.17±0.03	6.73±0.75	5.26±0.11	2.97±0.55
	24	0.87±0.06	0.00	0.16±0.02	4.35±0.35	3.73±0.09	9.49±0.12

Table 5, continuation

G. oxydans UFLA CHBD7.06*	0	1.21±0.04	0.00	0.76±0.02	9.47±0.21	7.24±0.22	0.31±0.01
	12	0.98±0.01	0.00	0.99±0.03	5.47±0.09	5.02±0.09	4.34±0.12
	24	0.91±0.03	0.00	0.23±0.02	2.28±0.03	3.98±0.02	10.73±0.76

Means of duplicated assays are followed by the standard deviation. Microorganism's abbreviations see Table 1 and 2. *strains isolated from traditional cocoa fermentations carried out in wooden boxes in Brazil (G.V.M. Pereira; I.S. Coelho; M.G.C.P. Miguel; K.T. Magalhães; E.G. Almeida, unpublished data)

3.6.1 The yeast

All of the cocoa yeast isolates tolerated the pH range of a typical cocoa fermentation process (from 2.0 to 5.0). The maximum growth temperature was 30 to 37°C with the exception of four isolates identified as *I. orientalis* and three as *S. cerevisiae*, which were able to grow up to 45°C. Although all of the isolates exhibited a remarkable ability to grow in the presence of 15% glucose and fructose, the best growth was observed when in the presence of 5% carbohydrates. The majority of the yeasts identified as *S. cerevisiae*, *P. kluyveri* and *D. etchellsii* were tolerant of media amended with up to 12 % ethanol, while the other yeast species tolerated only 6% ethanol (except for *P. fermentans* UFLA CHYA4.0 and UFLA CHYD31.07, *I. orientalis* UFLA CHYB6.02 and UFLA CHYC6.02, and *C. humilis* UFLA CHD14.32) (data not shown). From these results, 15 well-adapted isolates of *S. cerevisiae*, *P. fermentans*, *P. kluyveri*, *D. etchellsii* and *I. orientalis* were selected for simulated cocoa fermentation in the PSM-yeast medium. Isolates of *K. ohmeri*, *C. orthopsilosis*, *C. humilis*, *C. intermedia* and *Sz. pombe*, which were not resistant to the stressors employed in this study, were also included in this step. The fermentation kinetics of the pure cultures are reported in Table 3. The strains *S. cerevisiae* UFLA CHYC7.04 and UFLA CHYB4.03 exhibited a higher conversion rate of substrate to ethanol (49.33 and 49.81 % of the theoretical value, respectively). Within the non-*Saccharomyces* species, *D. etchellsii* UFLA CHYB5.56, *C. humilis* UFLA CHYD14.32, *P. kluyveri* UFLA CHYC2.02 and *I. orientalis* UFLA CHYB6.02 and UFLA CHYC6.02 exhibited the highest fermentation efficiencies (over 35% conversion of substrate into ethanol), while *K. ohmeri* BM2.01, *P. fermentans* UFLA CHYA4.01 and UFLA CHYD31.07, *C. orthopsilosis* UFLA CHYC5.02, *C. intermedia* UFLA CHYE21.01 and *Sz. pombe* UFLA CHYE5.39 exhibited the lowest values (less than 20% conversion). None of the organic acid contents changed significantly in the PSM-yeast medium during 24 h of fermentation.

3.6.2 The LAB

The results demonstrated the overall ability of the LAB isolates to grow over a wide range of pH values and lactic acid concentrations. In general, the majority of the *L. plantarum* and *L. fermentum* strains shared the ability to grow at 45 °C and 12% ethanol, while *W. fabaria*/*ghanens*-related isolates did not (data not shown). Six strains of *L. fermentum*, five of *L. plantarum* and one of *W. fabaria/ghanens* were selected on the basis of their growth properties for further kinetic analysis in the PSM-LAB medium (Table 4). The strictly heterofermentative LAB strains (*L. fermentum* and *W. fabaria/ghanens*) fermented glucose but not fructose, converted citric acid to lactic acid, and metabolized lactic acid and acetic acid as the main products. Interestingly, the conversion of citric acid into lactic acid, acetic acid and ethanol by the strictly heterofermentative LAB strains was more efficient than the conversion of carbohydrates (glucose or fructose) into these products. The production of lactic acid was higher for *L. fermentum* UFLA CHBE8.12 (7.72 g l^{-1}), while the production of acetic acid was higher for *W. ghanaians* UFLA CHBE8.23 and *L. fermentum* UFLA CHBE6.01 (6.25 g l^{-1}). All strains of *L. plantarum* tested were not well adapted to the synthetic medium used in this study, and no significant amount of lactic acid or acetic acid was produced by these strains (Table 4).

3.6.3 The AAB

All of the cocoa AAB isolates grew in a basal medium supplemented with up to 5% acetic acid and a pH adjusted to 2.0, 3.0 or 5.0, while these isolates failed to grow at 15 and 30% glucose and fructose concentrations. All of the isolates grew at 30 and 37 °C, as well as in 6% ethanol. Only the isolates of *A. tropicalis* grew at 45 °C and in 12% ethanol (data not shown). Five stress-tolerant *A. tropicalis* strains and representative isolates of the other AAB species were subjected to simulated cocoa fermentation in the medium PSM-AAB

(Table 5). The AAB species oxidized almost all of the ethanol into acetic acid within 24 h of fermentation. These species also oxidized lactic acid during their growth. The production of acetic acid was higher for *A. tropicalis* UFLA CHBE16.01, *A. senegalensis* UFLA CHBE6.16, *A. ghanensis* UFLA CHBA3.01, *A. malorum* UFLA CHBA1.01 and *G. oxydans* UFLA CHBD7.06, which produced 14.08, 13.49, 13.25, 11.45, and 10.73 g/l, respectively, compared to the other isolates, which produced <10 g/l.

4. Discussion

4.1 The microbiological and physicochemical performances of the bench- and pilot-scale cocoa bean fermentations

The microbiological communities in the cocoa bean fermentations performed under l bench and pilot-scale conditions (Fig. 1a; Table 1 and 2) were similar to those observed in previous spontaneous, larger-scale fermentations (2, 13, 15, 18, 25, 27, 30, 39). The most common LAB (*L. fermentum* and *L. plantarum*), AAB (*A. tropicalis*) and yeast (*S. cerevisiae* and *Hanseniaspora* ssp.) cocoa species appear to dominate the cocoa fermentations. The composition and the metabolic activity of these well-adapted cocoa species remained unchanged compared to larger cocoa fermentations once a stable ecosystem was achieved. These similarities suggested that the small-scale fermentations provided a suitable model system for larger-scale fermentations, at least with regards to their microbial ecology. The use of a suitable model system may allow the evaluation of a starter culture under controlled conditions prior to its use in the field.

Several studies have attempted to rapidly and effectively cure small quantities of cacao beans (less than 20 kg) to research aspects of the cocoa fermentations, such as the effects of cultivar, pod storage, pulp removal, and acid

production (5, 10, 33). However, in these studies, the temperature of the cocoa bean mass did not increase normally, often only reaching 35-37°C. Therefore, in the present study, the bench- and pilot-scale fermentations were placed in a temperature-controlled incubator. The program chosen was designed to mimic the increases observed in a traditional fermentation, which allowed the successive growth of the yeast and the LAB (25-32°C; 0-48 h), followed by the AAB (40-48°C; 60-144 h). For both fermentation processes, the temperature of the cocoa bean mass closely followed the temperature of the incubator (Fig. 1b).

The culture-based approach demonstrated that the LAB species *L. fermentum* and *L. plantarum* and the yeast *S. cerevisiae* dominated the early stages of fermentation. The richness in fermentable carbohydrates and the low oxygen content of the cocoa mass favored the growth of these microbial groups, which were hypothesized to rapidly metabolize reducing sugars and citric acid and produce mainly ethanol and lactic acid (Fig. 3). In addition, the simultaneous growth of both the yeast and the LAB can be explained by additional modes of LAB-yeast interaction. For example, the death and autolysis of the yeast cells releases vitamins and other nutrients, and/or the CO_2 produced by the yeast creates microaerophilic conditions, which favors LAB growth (9). Moreover, *L. plantarum* (facultatively heterofermentative) dominated the LAB community, but after 36 h, *L. fermentum* (strictly heterofermentative) became the dominant LAB (Table 1 and 2). A similar growth dynamic between these two LAB species was observed during the large-scale cocoa fermentations (26). This result demonstrated that the yeast were most adaptable when they were associated with the facultatively heterofermentative LAB compared to the strictly heterofermentative LAB, as previously observed during sourdough fermentations (17). Dircks (7) observed that the increased ethanol content during a controlled inoculation of cacao bean fermentations using different yeast species inhibited the growth of *L. fermentum*. Despite the complex microbial

ecology of cocoa bean fermentations, these types of interactions have not been considered.

Higher concentrations of citric and lactic acid were recovered during PC fermentation, which contained higher populations of *L. plantarum* and lower amounts of *L. fermentum* compared to the ST fermentation. The homolactic metabolism of *L. plantarum* allows this species to achieve a high cell density within a reasonable fermentation time and, in contrast to *L. fermentum*, produce high amounts of lactic acid. In contrast, the heterolactic metabolism of *L. fermentum* leads to the rapid conversion of citric acid and the production of almost equal amounts (on a mass basis) of lactic acid and acetic acid (3). This led to higher final concentrations of lactic acid (but not of citric acid) and lower pH values in the cocoa beans from the PC fermentation when compared to the ST fermentation (Fig. 1c; Fig 3). Although the diffusion of lactic acid into the cocoa beans contributes to the breakdown of the seed cell structure, because it is non-volatile, such excess is not reduced during drying, and high concentrations of the residual lactic acid will impart a sour flavor to the chocolate (1).

The kinetics of ethanol production in the pulp of cocoa fermentations performed under bench- and pilot-scale conditions corresponded with the observed growth of the yeast species (specifically the growth of the yeast *S. cerevisiae*; Table 1 and 2) and the utilization of the sugars. However, over time, the ethanol concentration in each fermentation varied, with the lowest content recovered from the PC at 48 h of fermentation. This result may have been due to the higher yeast population observed in the ST fermentation compared to the PC. An additional explanation is that the ST is more enclosed than the PC, and less ethanol may have been lost via evaporation, which instead accumulated inside of the beans.

The metabolic activity of the yeasts and the LAB (temperature increase, pH decrease and ethanol and lactic acid accumulation; Fig 1b and c, Fig. 3)

favored AAB growth (Fig. 1a), which was accompanied by the production of acetic acid and a reduction in the concentration of ethanol (Fig. 3). This finding agrees with the classic description of these bacteria in the literature (22, 39). Both culture-independent and culture-based approaches suggested a major role for the genus *Acetobacter* under bench- and pilot-scale cocoa fermentations. The presence of *Gluconobacter* species, previously reported in larger cocoa fermentations (22, 39), was not confirmed in this study. *A. tropicalis* was the dominant species in both fermentations. The dominance of *A. tropicalis* was previously observed during larger cocoa fermentations in Brazil (G.V.M. Pereira et al., submitted for publication) and Australia (7). The *A. tropicalis* species is mainly associated with fruits and fermented foods and has been selected to produce artisanal vinegar (24). The dominance of this species during cocoa bean fermentation may be explained by its acid and heat resistance (24). The levels of acetic acid produced in the pulp from the PC fermentation were much lower than those produced during ST fermentation. This difference might be due to the low availability of oxygen inside the tank, and consequently, less ethanol may be oxidized to acetic acid.

A range of other bacterial species, such as *Bacillus*, *Staphylococcus* and *Enterobacteria*, were isolated during the last 0-48 h. The PCR–DGGE profile confirmed the presence of these three bacterial groups in both fermentation processes (Fig. 2). The main species isolated were *B. subtilis*, *B. megaterium* and *St. pasteuri*. Their growth appeared to be inhibited by the high population of the LAB and/or yeast, and no growth was observed after 48 h of fermentation (Table 1 and 2). This result was consistent with research that found the LAB inhibited the growth of *Bacillus* ssp. and *Enterobacteria* during other food fermentations (41). The appearance of *Bacillus* spp. during cocoa bean fermentations is less predictable in comparison to other microbial groups. Their role during cocoa fermentation is not well known, and this bacterium has never been involved as a

starter in attempts to control the fermentation process. It is believed that the growth of a low population of certain *Bacillus* strains might have a beneficial action, *e.g.*, by acting as a complementary partner to the yeasts in the pulp depectinization process during the advanced stage of the cocoa fermentation (28). However, a high population may contribute to the acidity and perhaps to the undesirable flavors of fermented cocoa beans (38). In this sense, cocoa fermentations conducted with a defined inoculum of LAB and yeast may prove useful for the control of *Bacillus* spp. during industrialized fermentations. Although the conditions in the cocoa mass do not favor the development of the *Enterobacteria* and *Staphylococcus* species (low pH and high temperatures), their presence was reported using culture-independent methods during Ghanaian, Brazilian and Ivorian cocoa bean fermentations (30). The appearance of these bacterial species underlines the importance of monitoring the hygiene of the fermentation process to ensure that these species do not become dominant and spoil the beans (2). Their presence may indicate human contact with the beans or may also be associated with pod surfaces, banana/plantain leaves and the material used.

The PCR-DGGE method has some potential limitations that made it less suitable for use in this study. Species present in low concentrations could occasionally be grown from agar plates but in many cases did not generate a detectable DGGE band. Thus, it is possible that the DGGE fingerprint might mask the perturbations of low-abundance community members in our study. This finding illustrates the intrinsic limitation of DGGE analysis in visualizing only the predominant species of a microbial community (31). Interestingly, *S. cerevisiae* yielded only a relatively weak band in the denaturing gels compared to *Hanseniaspora* spp. (Fig. 2b), even though *S. cerevisiae* dominated the yeast species isolated using the plating method from both fermentation processes (Table 1 and 2). A possible explanation may be that the *S. cerevisiae* ITS

fragment is less efficiently amplified compared to that of the *Hanseniaspora* species present during the cocoa fermentation using the protocol described here (Nielsen et al. 2005). An accurate assessment of the microbial ecologies using PCR-DGGE requires appropriately designed primers, and the use of poorly targeted primers will skew estimates of the microbial diversity present (4).

4.2 Polyphasic selection study

To our knowledge, this study represents the first assessment of the growth and stress tolerance of yeasts, LAB and AAB under cocoa-fermenting conditions. The criteria used were based on the physical and chemical changes that each microbial group faces in the cocoa pulp substrate when their metabolisms are most active. At the beginning of the process, the yeast and LAB cells are affected by osmotic stress due to the high sugar content in the cocoa pulp (39). As the fermentation progresses, other stressors become relevant as ethanol and lactic acid accumulate and the environment acidifies. After two days of fermentation, the AAB become the dominant group and are faced with an ethanol-rich environment, followed by the accumulation of acetic acid and the increase of temperature to a mean value of 45 °C (39). In the present study, the results indicated that the stressors of the early fermentation phase influenced the prevalence of the most-adapted yeast, *S. cerevisiae*. In addition, some strains of the non-*Saccharomyces* yeasts, such as *P. fermentans*, *P. kluyveri*, *D. etchellsii* and *I. orientalis*, were also well adapted to the conditions imposed. The limited number of yeasts capable of growth at 45°C explains the decreasing yeast population (Fig. 1a) after AAB-facilitated exothermic ethanol oxidation raises the temperature to 48 °C. Similarly, the ability of the *L. fermentum* and *L. plantarum* strains to grow over a wide range of pH values, temperatures, and ethanol and lactic acid concentrations correlated with the stable population of the LAB during the entire fermentation process (Fig. 1a). Finally, the ability of

A. tropicalis isolates to tolerate the stressors imposed during the second stage of the fermentation might be considered an advantage of this species, which led to its dominance in the fermentations performed (Table 1 and 2).

As expected, the fermenting powers of the S. *cerevisiae* strains were generally higher when compared to the non-*Saccharomyces* species in PSM-yeast (Table 3-5). The production of ethanol by the yeasts could affect the course of the cocoa fermentation in several ways, including the inhibition of certain microbial species, the death of the cocoa beans and their de-compartmentalization, and as a substrate for the formation of acetic acid. However, some non-*Saccharomyces* strains (*D. etchellsii* UFLA CHYB5.56, *C. humilis* UFLA CHYD14.32, *P. kluyveri* UFLA CHYC2.02 and *I. orientalis* UFLA CHYB6.02 and UFLA CHYC6.02) also produced significant levels of ethanol. This result, together with the ability of these non-*Saccharomyces* strains to tolerate cocoa-fermenting conditions, indicated that these strains could be used in a controlled cocoa fermentation multi-culture starter with *S. cerevisiae*. The secondary products of their metabolism (e.g., organic acids, aldehydes, ketones, higher alcohols, and esters) and their glycosidase production are likely to be significant and should impact bean and chocolate quality (2). These potentially important influences have been overlooked in previous cocoa fermentation works.

The strictly heterofermentative *L. fermentum* strains were characterized by rapid citric acid conversion, and in the consumption of sugars, glucose was preferentially metabolized over fructose. Citric acid is used for the oxidation of NADH + H^+ to bypass the energy-limiting ethanol pathway and to maximize their growth rate on glucose, thereby producing mannitol and lactic acid plus acetic acid (21). The metabolism of citric acid by cocoa-specific *L. fermentum* has been observed previously (20). The consumption of citric acid resulted in the production of organic acids with a higher pKa value, which increased the pH of

the environment and allowed better bacterial growth and microbiological control of the environment (39).

All of the AAB strains oxidized lactic acid (through lactate dehydrogenase, pyruvate decarboxylase, and acetaldehyde dehydrogenase activities) and ethanol (via the sequential action of pyrroquinoline-quinone-dependent alcohol dehydrogenase and acetaldehyde dehydrogenase activities) into acetic acid in the PSM-AAB. Thus, the simultaneous metabolism of the LAB and yeasts stimulate the growth of the AAB during a natural cocoa fermentation. In agreement with their ability to tolerate cocoa-fermentation stress, the oxidation process of *A. tropicalis* UFLA CBE16.01 was the most effective, followed by *A. senegalensis* UFLA CBE6.16 and *A. ghanensis* UFLA CBA3.01. Interestingly, *A. senegalensis* and *A. ghanensis* species were recently described during Ghanaian cocoa bean fermentation processes (6), which likely explain their adaptation to the cocoa pulp habitat.

5 Conclusions

This study demonstrated that the bench- and pilot-scale cocoa fermentation ecosystems reach equilibrium through a two-phase process, starting with the simultaneous growth of the yeast and LAB, which were gradually replaced by the AAB. Overall, the dominant species largely overlapped with those commonly associated with larger cocoa bean fermentations (*S. cerevisiae* and *Hanseniaspora* sp. in the yeast group, *L. fermentum* and *L. plantarum* in the LAB group, and *A. tropicalis* from the AAB group), proving their adaptation to the cocoa environment under the conditions applied. A similar course of substrate consumption and metabolite production occurred in both processes compared to the spontaneous, larger cocoa bean fermentation processes. However, further studies should evaluate the impact of excessive production of

these metabolites in the pulp on the technological and sensorial quality of the resultant chocolate.

The polyphasic selection study allowed us to construct a better picture of the physiology and ecology of the indigenous yeasts, LAB and AAB strains. As a result, some strains from these three major groups were selected as potential starter cultures. In particular, *L. fermentum* UFLA CBE8.12 (citric acid fermenting; lactic acid producing; and heat-, acid-, lactic acid- and ethanol-tolerant), *S. cerevisiae* UFLA CYC7.04 (ethanol producing and acid-, heat- and ethanol-tolerant) and *A. tropicalis* UFLA CBE16.01 (ethanol and lactic acid oxidizing; acetic acid producing; and acid-, heat-, acetic acid- and ethanol-tolerant) were selected as candidates for a mixed-strain starter cocktail that should lead to better controlled and more reliable cocoa bean fermentation processes. In addition, analysis of the non-*Saccharomyces* strains (*D. etchellsii* UFLA CYB5.56, *C. humilis* UFLA CYD14.32, *P. kluyveri* UFLA CYC2.02 and *I. orientalis* UFLA CYB6.02 and UFLA CYC6.02) indicated that these should be tested in association with *S. cerevisiae* in futures studies as cocoa starter cultures.

Acknowledgments

GVMP, MGCPM, and CLR thank the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico of Brasil (CNPQ), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and CAPES for scholarships. Financial support from the Almirante Cacau Agrícola (Masterfoods) is also gratefully acknowledgments.

References

1. **Afoakwa EO, Paterson A, Fowler M, Ryan A.** 2008. Flavour formation and character in cocoa and chocolate: a critical review. Crit. Rev. Food Sci. Nutr. **48**:840-857
2. **Ardhana M, Fleet G.** 2003. The microbial ecology of cocoa bean fermentations in Indonesia. Int. J. Food Microbiol. **86**:87-99.
3. **Axelsson L**. 2004. Lactic acid bacteria: classification and physiology, p 1-66. *In* Salminen S, von Wright A, Ouwehand A (ed), Lactic Acid Bacteria: Microbiological and Functional Aspects, 3rd edn. Marcel Dekker, New York.
4. **Beh AL, Fleet GH, Prakitchaiwattana C, Heard GM**. 2006. Evaluation of molecular methods for the analysis of yeast in food and beverages. Adv. Exp. Med. Biol. **571**:69-106.
5. **Clapperton, JF, Lockwood G, Yow STK, Um OHK.** 1994. Effects of planting materiais on flavour. Cocoa Growers' Bu. **48**:47-63.
6. **Cleenwerck I, Camu N, Engelbeen K, DeWinter T, Vandemeulebroecke K, De Vos P, De Vuyst L**. 2007. *Acetobacter ghanensis* sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa beans. Int. J. Syst. Evol. Microbiol. **57**:1647–1652.
7. **Dircks HD.** 2009. Ph.D. thesis. University of New South Wales, Sidney, Australia.
8. **Dzogbefia VP, Buamah R, Oldham JH.** 1999. The controlled fermentation of cocoa (*Theobroma cacao* L.) using yeasts: enzymatic process and associated physico-chemical changes in cocoa sweatings. Food Biotechnol. **13**:1-12.
9. **Fleet GH.** 2007. Yeast in foods and beverages: impact on product quality and safety. Curr. Opinion. Biotech. **18**:170-175.
10. **Frederick H.** 1960. Cocoa manual. English ed, Inter-American Institute of Agricultural Sciences. Turrialba, Costa Rica.
11. **Gasch AP, Werner-Washburne M.** 2002. The genomics of yeast responses to environmental stress and starvation. Funct. Integr. Genomics. **2**:181-192.
12. **Holzapfel WH.** 2002. Appropriate stater culture technologies for small scale fermentation in developing countries. Int. J. Food Micro. **75**:197-212.
13. **Jespersen L, Nielsen DS, Hønholt S, Jakobsen M.** 2005. Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. FEMS Yeast Res. **5**: 441–453.
14. **Joyeux A, Lafon-Lafourcade S, Ribereau-Gayon P**. 1984. Evolution of acetic acid bacteria during fermentation and storage of wine. Appl. Environ. Microbiol. **48**:153-156.

15. **Kostinek M, Ban-Koffi L, Ottah-Atikpo M, Teniola D, Schillinger U, Holzapfel WH, Franz CMAP.** 2008. Diversity of predominant lactic acid bacteria associated with cocoa fermentation in Nigeria. Curr. Microbiol. **56**:306–314.
16. **Kurtzman CP, Fell JW, Boekhout T.** 2011. The Yeasts, a Taxonomic Study, 4ed. Elsevier, Amsterdan.
17. **Lacumin L, Cecchini F, Manzano M, Osualdini M, Boscolo D, Orlic S, Comi G.** 2009. Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. Food Microbiol. **26**:128–135.
18. **Lagunes-Gálvez S, Loiseau G, Paredes JL, Barel M, Guiraud J-P.** 2007. Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. Int. J. Food Microbiol. **114**:124-130.
19. **Leal Jr GA, Gomes LH, Efraim P, Tavares FC, Figueira A.** 2008. Fermentation of cocoa (*Theobroma cacao* L.) seeds with a hybrid *Kluyveromyces marxianus* strain improved product quality attributes. FEMS Yeast Res. **8**: 788–798.
20. **Lefeber T, Janssens M, Camu N, De Vuyst L.** 2010. Kinetic analysis of strains of lactic acid bacteria and acetic acid bacteria in cocoa pulp simulation media to compose a starter culture for cocoa bean fermentation. Appl. Environ. Microbiol. **76**:7708-7716.
21. **Li X, Jiang B, Beilei P, Wanmeng M, Zhang T.** 2008. Purification and partial characterization of *Lactobacillus* species SK007 lactate dehydrogenase (LDH) catalyzing phenylpyruvic acid (PPA) conversion into phenyllactic acid (PLA). J. Agric. Food Chem. **56**:2392–2399.
22. **Lima LJR, Almeida MH, Rob Nout MJ, Zwietering MH.** 2011. *Theobroma cacao* L., "The Food of the Gods": Quality Determinants of Commercial Cocoa Beans, with Particular Reference to the Impact of Fermentation. Crit. Rev. Food Sci. Nutr. **51**:731-61.
23. **Magalhães KT, Pereira GVM, Dias DR, Schwan RF.** 2010. Microbial communities and chemical changes during fermentation of sugary Brazilian kefir. World J. Microbiol. Biotechnol. **26**:1241-1250.
24. **Ndoye B, Lebecque S, Dubois-Dauphin R, Tounkara L, Guiro AT, Kere C, Diawara B, Thonart P.** 2006. Thermoresistant properties of acetic acids bacteria isolated from tropical products of Sub-Saharan Africa and destined to industrial vinegar. Enzyme Microb. Tech. **39**:916–923.
25. **Nielsen DS, Honholt S, Tano-Debrah K, Jespersen L.** 2005. Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). Yeast. **22**:271–284.

26. **Nielsen DS, Teniola OD, Ban-Koffi L, Owusu M, Andersson TS, Holzapfel WH**. 2007. The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture independent methods. Int. J. Food Microbiol. **114**:168–186.
27. **Ostovar K, Keeney PG.** 1973. Isolation and characterization of microorganisms involved in the fermentation of Trinidad's cacao beans. J. Food Sci. **38**:611–617.
28. **Ouattara HG, Ban-Koffi L, Karou GT, Sangare A, Niamke SL, Diopoh JK.** 2008. Implication of *Bacillus* sp. in the production of pectinolytic enzymes during cocoa fermentation. World J. Microbiol. Biotechnol. **24**:1753-1760.
29. **Ovreas L, Forney L, Daae FL, Torsvik V.** 1997. Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ. Microbiol. **63**:3367–3373.
30. **Papalexandratou Z. Camu N, Falony G, De Vuyst L.** 2011. Comparison of the bacterial species diversity of spontaneous cocoa bean fermentations carried out at selected farms in Ivory Coast and Brazil. Food Microbiol. **28**:964-973.
31. **Pereira GVM, Magalhães KT, Lorenzetii ER, Souza TP, Schwan RS**. 2011. A Multiphasic Approach for the Identification of Endophytic Bacterial in Strawberry Fruit and their Potential for Plant Growth Promotion. Microb. Ecol. **63**:405-417.
32. **Pereira GVM, Ramos CL, Galvão C, Dias ES, Schwan RF.** 2010. Use of specific PCR primers to identify three important industrial species of Saccharomyces genus: *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces pastorianus*. Lett. Appl. Microbiol. **5**:131–137.
33. **Quesnel VC, Lopez A**. 1975. A sweat-box for fermenting small samples of cocoa. Trop. Agric. **52**:309-316.
34. **Ruiz A, Poblet M, Mas A, Guillamón JM.** 2000. Identification of acetic acid bacteria by RFLP of PCR-amplified 16 rDNA and 16S–23S rDNA intergenic spacer. Int. J. Syst. Evol. Microbiol. **50**:1981–198.
35. **Samah OA, Ptih MF, Selamat J.** 1992. Biochemical changes during fermentation of cocoa beans inoculated with Saccharomyces cerevisiae (wild strain). J. Food Sci. Technol. **29**:341–343.
36. **Schwan RF.** 1998. Cocoa fermentations conducted with a defined microbial cocktail inoculum. Appl. Environ. Microbiol. **64**:1477–1483.
37. **Schwan RF, Rose AH, Board RG**. 1995. Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. J. Appl. Bacteriol. Symp. Suppl. **79**:96S–107S.

38. **Schwan RF, Vanetti MCD, Silva DO, Lopez A, Moraes CA.** 1986. Characterization and distribution of aerobic, spore-forming bacteria from cacao fermentations in Bahia. J. Food Sci. **51**:1583–1584.
39. **Schwan RF, Wheals AE**. 2004. The microbiology of cocoa fermentation and its role in chocolate quality. Crit. Rev. Food Sci. Nutr. **44**:205–221.
40. **Steinkraus KH.** 2004. Industrialization of Indigenous Fermented Foods. Second Edition. CRC Press, Boca Raton.
41. **Vandenbergh PA.** 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiol. Rev. **12**:221-238.
42. **White TJ, Bruns T, Lee S, Taylor J.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p 315-322. *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ (ed), PCR protocols: A guide to methods and applications. Academic Press, San Diego, CA.
43. **Wood BJB**. 1998. Microbiology of fermented foods, 2nd ed. Thompson Science, London, UK.

Annex

Picture showing the bioreactors used in this study. A - 500-g plastic containers; B - 10-Kg stainless steel tank; C - 40-kg stainless steel tank; D - 40-kg wooden box; E- 600-kg wooden box.