IGOR MAGALHÃES DA VEIGA MOREIRA

ANALYTICAL STUDY OF COCOA BEANS AND CHARACTERIZATION OF CHOCOLATES OF DIFFERENT COCOA HYBRIDS DURING THE SPONTANEOUS AND INOCULATED FERMENTATION PROCESSES

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência dos Alimentos, área de concentração em Ciência dos Alimentos, para a obtenção do título de Doutor.

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Orientadora

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ESTUDO ANALÍTICO DAS AMÊNDOAS DE CACAU E CARACTERIZAÇÃO DOS CHOCOLATES DE DIFERENTES HÍBRIDOS DURANTE OS PROCESSOS FERMENTATIVOS ESPONTÂNEO E INOCULADO

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APROVADA em 06 de outubro de 2017.

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LAVRAS – MG

2017

Aos meus pais Maria e Paulo, pela criação e apoio
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RESUMO

Amêndoas de cacau provenientes de diferentes materiais genéticos apresentam dinâmicas de fermentação distintas, consequentemente qualidade instável do chocolate. O processo fermentativo do cacau é espontâneo e empírico, sendo assim, o uso de culturas iniciadoras pode influenciar na padronização e sabor do produto final. O objetivo desse trabalho foi investigar os compostos voláteis e perfil proteico (semi-qualitativo) durante a fermentação espontânea de quatro clones de cacau (CEPEC2004, PH15, PS1319 e SJ02) cultivados no sul da Bahia, e caracterizar sensorialmente os chocolates produzidos a partir dessas variedades clonais. Um segundo aspecto abordado neste estudo, foi o uso de *Saccharomyces cerevisiae* UFLA CCMA 0200 e *Pichia kluyveri* UFLA CCMA 0237 como culturas iniciadoras na fermentação de dois híbridos de cacau (CEPEC2002 e FA13). De acordo com os resultados obtidos, diferentes compostos voláteis foram identificados nas amêndoas fermentadas e nos chocolates. Os grupos de compostos voláteis mais relevantes identificados foram: ácidos, álcoois, aldeídos, cetonas e ésteres. O chocolate produzido a partir do híbrido CEPEC2004 foi o mais aceito pelos provadores e descrito como amargo e doce; este resultado pode ser explicado pela presença de compostos de sabor desejado, como 2,3-butanodiol e 2-metil-1-butanol, encontrados somente nas amostras de chocolate provenientes dessa variedade clonal. Maior presença de ácidos, considerados compostos indesejáveis como, por exemplo, ácido valérico, foi observado em amostras de chocolates do híbrido PS1319, correlacionado com a baixa aprovação no teste de aceitação. A análise de MALDI-TOF (*Matrix Associated Laser Desorption-Ionization - Time of Flight*) mostrou que, durante a fermentação, o perfil proteico foi diferente entre os híbridos. Um total de 80 compostos voláteis foi identificado por HS-SPME GC-MS em amostras de fermentação e chocolate durante os processos fermentativos espontâneos e inoculados dos híbridos CEPEC2002 e FA13. Chocolate produzido a partir do híbrido FA13 inoculado com *S. cerevisiae* apresentou todos os ácidos identificados, e foi considerado mais azedo do que o chocolate produzido a partir da fermentação espontânea. CEPEC2002 inoculado com *S. cerevisiae* em co-cultura com *P. kluyveri* gerou um chocolate menos azedo e mais doce do que a fermentação espontânea. Ambos os chocolates dos ensaios inoculados foram mais aceitos pelos provadores. A análise de MALDI-TOF provou que os perfis de proteína mudaram durante a fermentação e também foram influenciados pelo processo de inoculação. Em estudos futuros, a identificação e sequenciamento dos diferentes peptídeos ajudarão a desvendar quais desses compostos contribuem para a formação do sabor específico ao cacau.

Palavras-chave: Culturas iniciadoras. Fermentação do cacau. Variedades clonais. GC-MS. MALDI-TOF.

ABSTRACT

Cocoa beans from different genetic materials present different fermentation dynamics, consequently an unstable chocolate quality. The cocoa fermentation process is spontaneous and uncontrolled, thus, the use of starter cultures may influence the standardization and flavor of final product. The aim of this study was to investigate the volatile compounds and protein profile (semi-qualitative) during the fermentation of four cocoa hybrids (CEPEC2004, PH15, PS1319 and SJ02) grown in southern Bahia, and sensory characterization of the chocolates produced from these clonal varieties. A second aspect evaluated was the use of *Saccharomyces cerevisiae* and *Pichia kluyveri* as starter cultures on fermentation of two cocoa hybrids (CEPEC2002 and FA13). According to results, different volatile compounds were identified in fermented beans and chocolates samples. The most relevant volatile compounds groups identified were: acids, alcohols, aldehydes, ketones and esters. Chocolate from CEPEC2004 was the most accepted by judges and correlated with sweet and bitter taste, this result can explained by the presence of desired compounds, such as 2,3-butanediol and 2-Methyl-1-butanol in chocolate samples from this hybrid. A higher presence of acids, considered as undesirable compounds, such as valeric acid, was observed in chocolates samples from PS1319 hybrid, which may have generated the low acceptance by judges. MALDI-TOF analysis showed that during fermentation the protein profile was different among the hybrids. A total of 80 volatile compounds were identified by HS-SPME GC-MS in the fermentation and chocolate samples during spontaneous and inoculated processes of the CEPEC2002 and FA13 hybrids. Chocolate FA13 inoculated with *S. cerevisiae* contained all the acids identified, and was considered more sour than chocolate produced from FA13 spontaneous fermentation. CEPEC2002 inoculated with *S. cerevisiae* in co-culture with *P. Kluyveri* generated a chocolate less sour and sweeter chocolate than spontaneous fermentation. Both chocolates from inoculated assays were more accepted by judges. MALDI-TOF analysis proved that protein profiles changed during fermentation and also were influenced by the inoculation process. In future studies, the identification of the different peptides will help unraveling which peptides and free amino acids contribute for formation of cocoa-specific flavor.

Keywords: Starter cultures. Cocoa fermentation. Clonal varieties. GC-MS. MALDI-TOF

LISTAS DE FIGURAS

PRIMEIRA PARTE

SEGUNDA PARTE – ARTIGOS

ARTIGO 1

ARTIGO 2

LISTA DE TABELAS

PRIMEIRA PARTE

SEGUNDA PARTE – ARTIGOS

ARTIGO 1

ARTIGO 2

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A época de ouro para a cultura do cacau no Brasil aconteceu durante a década de 1980. No período de 1979-1989, o país apresentou uma produção anual média de 351.149 toneladas de sementes de cacau (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAOSTAT, 2017). No ano de 1989, a produção brasileira sofreu grande impacto devido à disseminação de uma doença, causada pelo fungo *Moniliophtora perniciosa*, conhecida como "vassoura-de-bruxa" (PEREIRA, 2001). Entre 1989 e 2014, a produção média foi de 250.868 toneladas de amêndoas de cacau por ano. Comparando os dois períodos, houve queda de 28,55% na produtividade nacional (FAOSTAT, 2017).

Entre os países produtores, segundo dados da Organização Internacional do Cacau (INTERNATIONAL COCOA ORGANIZATION - ICCO, 2017), o Brasil ocupa o quinto lugar no ranking com produção de 210.000 toneladas, levando em consideração dados publicados no último boletim (2014) pela ICCO. Como forma de recuperar a lavoura cacaueira, a estratégia mais eficiente é o desenvolvimento de variedades clonais resistentes a doenças (LOPES et al., 2011). O trabalho de melhoramento genético é realizado por agências de pesquisas, como o Centro de Pesquisa do Cacau (CEPEC) e a Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC), em conjunto com os agricultores, com a finalidade de gerar, não só plantas resistentes, mas também com maior capacidade produtiva e qualidade ("cacau fino") (LOPES et al., 2011).

Os programas de melhoramento genético do cacaueiro produzem plantações híbridas que geram frutos com características diferentes, tais como tamanho, cor, quantidade e peso das sementes, quantidade e composição da

polpa e sabor (LOPES; PIRES, 2014). O sabor característico do chocolate é somente desenvolvido a partir de amêndoas de cacau fermentadas (ROHAN, 1964). Grãos de cacau não fermentados são ácidos e adstringentes, além disso, não geram o sabor específico durante a fabricação do chocolate, principalmente nas etapas de torrefação e conchagem (SCHWAN; FLEET, 2014; SCHWAN; WHEALS, 2004). Considerando a variabilidade no conteúdo e composição da polpa, não é surpresa que diferentes híbridos apresentam dinâmicas e requisitos de fermentação distintos, consequentemente, qualidade variável do produto final (MOREIRA et al., 2013; RAMOS et al., 2014).

A fermentação do cacau é espontânea e com intensa atividade microbiana, reações proteolíticas no interior das amêndoas ocorrem e colaboram para o desenvolvimento dos precursores do sabor de chocolate (AFOAKWA et al., 2008; SCHWAN; WHEALS, 2004). Leveduras, bactérias do ácido lático e bactérias do ácido acético são os principais grupos microbianos encontrados durante a fermentação. As leveduras dominam os estágios iniciais e, posteriormente, são superadas pelas bactérias do ácido lático e bactérias do ácido acético (SCHWAN; WHEALS, 2004). A fermentação do cacau ainda é um processo tradicional (espontâneo) e sem controle, dessa forma, a seleção de culturas iniciadoras, para aplicação no processo fermentativo do cacau, é uma alternativa para alcançar matérias primas que contribuem para padronização e qualidade dos chocolates (SCHWAN; PEREIRA; FLEET, 2014).

Diante do exposto, torna-se relevante o estudo da composição química volátil e perfil proteico (semi-qualitativo) das amêndoas fermentadas, bem como avaliação das características sensoriais dos chocolates produzidos a partir de seis variedades clonais cultivadas no sul da Bahia, Brasil. Além disso, experimentos com inoculação de duas espécies de leveduras foram conduzidos a fim de avaliar o efeito na qualidade das amêndoas fermentadas e chocolates.

2 REFERENCIAL TEÓRICO

2.1 *Theobroma cacao* L.: origem e dispersão

O cacaueiro pertence à ordem Malvales, família *Sterculiaceae*, gênero *Theobroma*, espécie *Theobroma cacao* L., as sementes são o principal ingrediente para produção do chocolate (MOREIRA et al., 2013). O cacaueiro (*Theobroma cacao* L.) é uma espécie supostamente originária da América Tropical e domesticado pelos Maias e Astecas aproximadamente a dois mil anos, que utilizavam amêndoas como moeda e para o preparo de uma bebida muito apreciada na época (HURST et al., 2002).

De acordo com Cheesman (1944), o cacaueiro desenvolve-se espontaneamente em florestas tropicais desde o sul do México, até o extremo sul da Amazônia, sendo a distribuição geográfica associada a uma divisão da espécie em dois grandes grupos -*Forastero* e *Criollo*- e um híbrido, entre esses dois grupos, chamado de *Trinitario*. O grupo *Forastero* tem origem na região Amazônica, que posteriormente foi dividido em Alto e Baixo Amazônico. Cacau tipo *Criollo* é originário da América Central e região norte da América do Sul. *Trinitario* é resultado do cruzamento entre o *Criollo* de Trinidad e o tipo *Forastero* (BARTLEY, 2005; LOPES; PIRES, 2014).

Os dois grupos são bastante distintos, sendo que o grupo *Forastero* apresenta tipicamente frutos verdes quando imaturos e sementes marrom escuro. Cacau do tipo *Criollo* apresenta sementes brancas ou rosa-pálido e seus frutos são vermelhos ou verdes quando imaturos. Como o grupo *Trinitario* foi originado do cruzamento dos dois grandes grupos, seus frutos apresentam semelhanças entre o cacau tipo *Forastero* e *Criollo* (LOPES; PIRES, 2014).

Purseglove (1968) relatou que a dispersão do cacau pelo mundo foi feita por Colombo, que levou sementes de cacau para a Europa, como curiosidade, não sendo estas apreciadas a princípio. No entanto, em pouco tempo os

espanhóis desenvolveram um procedimento que as tornavam mais saborosas, através da mistura de grãos torrados e moídos, açúcar e baunilha. No início do século XVIII a bebida passou a ser popular na Itália e França e, logo em seguida, na Holanda, Alemanha e Inglaterra. Em 1828 foi desenvolvido, na Holanda, um procedimento para prensagem e separação da gordura da amêndoa, permitindo a fabricação de chocolate em barras, com posterior incorporação de leite condensado ou em pó, em 1876, na Suíça (PURSEGLOVE, 1968).

Pires (2003) destacou que a crescente demanda do produto induziu a expansão da cultura. A princípio, o México era o único exportador, e no século XVI o cacau já era cultivado para o comércio com a Europa, América Central, América do Sul, Trinidade, Jamaica e Haiti. No século XVII foi introduzido na Venezuela, a partir do México, e, por volta de 1750, este país passa a ser o maior produtor mundial, sendo substituído pelo Equador no século seguinte, que cultivava a variedade local Nacional (DUBLIN, 1984). Em 1746, o cacau foi introduzido na Bahia através de sementes trazidas do Pará, e, entre 1822 e 1878, foi levado à África pelos portugueses, expandindo para Gana, Nigéria, Camarões e Costa do Marfim (PAULIN; ESKES, 1995). Atualmente, o continente Africano produz a maior parte do cacau comercializado no mundo (ICCO, 2017).

2.2 Cacauicultura: Produção mundial e Brasileira

O cacau pode ser considerado como a *commodity* mais representativa de uma agricultura de subsistência para milhares de produtores (AMIN, 2003).

O último boletim (2014) publicado pela Organização Internacional do Cacau (ICCO) destaca que Costa do Marfim lidera a produção mundial de cacau, seguido, na ordem, por Gana, Indonésia, Nigéria, Brasil, Camarões e Equador. Este grupo de sete países concentra mais de 80 % da oferta mundial

(ICCO, 2017). Dados sobre a produção em mil toneladas de amêndoas de cacau estão apresentados na Tabela 1.

Tabela 1 - Produção de amêndoas de cacau (mil toneladas).

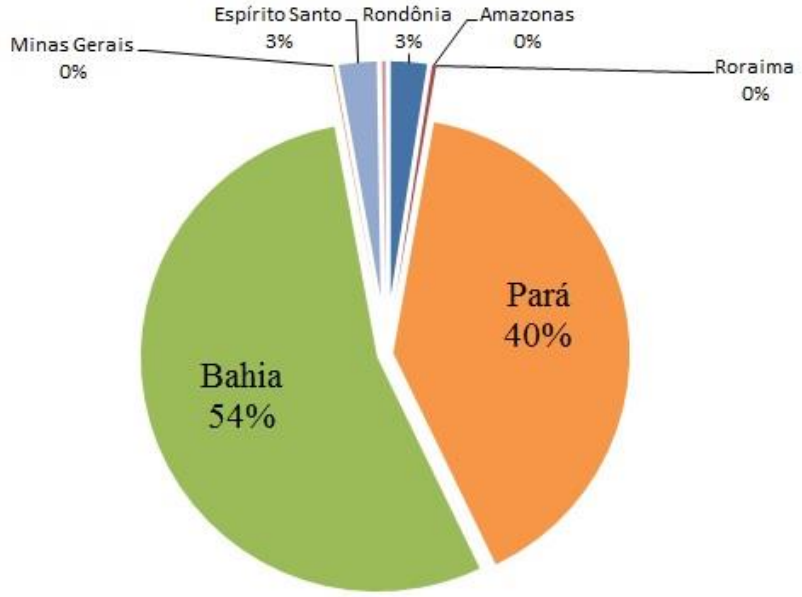
	2011/12		2012/13*		2013/14*	
África	2919	71,5%	2820	71,5%	2942	71,7%
Camarões	207		225		210	
Costa do Marfim	1486		1449		1550	
Gana	879		835		870	
Nigéria	235		225		220	
Outros	113		86		92	
América	655	16,0%	625	15,9%	666	16,2%
Brasil	220		185		200	
Equador	198		192		210	
Outros	237		249		256	
Ásia e Oceania	511	12,5%	496	12,6%	496	12,1%
Indonésia	440		420		410	
Papua Nova Guiné	39		36		40	
Outros	33		40		46	
Total	4085	100%	3942	100%	4104	100%

Fonte: ICCO (2017).
Nota: *Estimativas

A produção nacional de cacau em amêndoas está concentrada no sul da Bahia e no estado do Pará (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE, 2017). Esses dois estados são responsáveis por mais de 90% da produção nacional. Segundo o Instituto Brasileiro de Geografia e Estatística, até 2016 a Bahia era o maior produtor com uma safra de 116.122 t de cacau em amêndoas, porém nas estimativas para safra 2017, o estado do Pará poderá se tornar o maior produtor nacional com 116.450 t, enquanto a Bahia possivelmente sofrerá queda de 10% em relação ao ano anterior (IBGE, 2017).

A Figura 1 mostra a participação na produção nacional de cacau em amêndoas de cada estado produtor durante a safra de 2016.

Figura 1 - Porcentagem de participação na produção nacional de cacau em amêndoas de cada estado produtor.



Fonte: IBGE (2017).

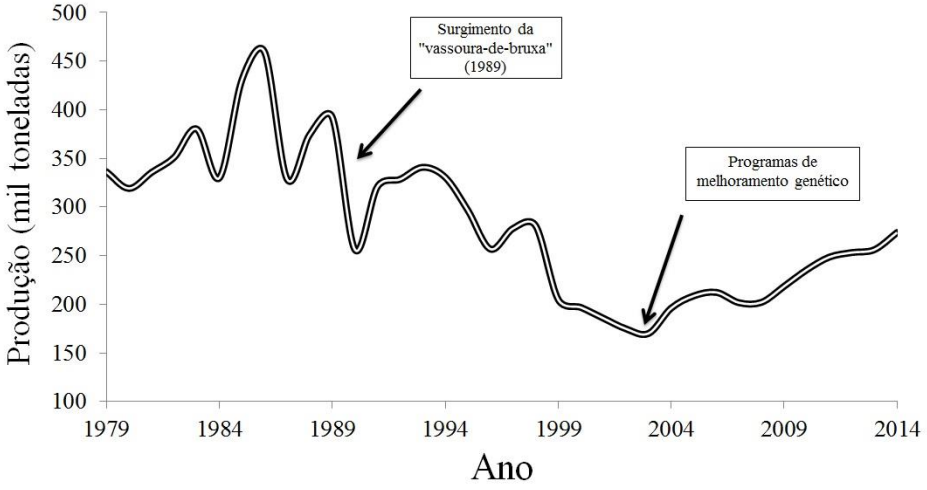
A safra brasileira de 2016 atingiu recebimentos acumulados de 214.964 t, dos quais 54% produzidos pelo estado da Bahia, como mostra a figura 1, ressaltando a importância desse estado para a produção nacional de cacau.

Durante a década de 1980, o Brasil se destacou no mercado mundial de cacau como um grande produtor, atingindo marcas históricas de, por exemplo, 400.000 toneladas de amêndoas secas e produtividade de 750 kg/ha, a maior no contexto mundial da época. A partir da década de 90, devido à disseminação da doença vassoura-de-bruxa (causada pelo fungo *Moniliophtora perniciosa*), houve declínio na produtividade brasileira (EFRAIM et al., 2013).

Alves et al. (2006) ressaltaram que a vassoura-de-bruxa é a doença mais importante da cultura do cacaueiro, nos países onde ela ocorre. Em 1989, foi constatada pela primeira vez a presença do patógeno (*Moniliophtora perniciosa*) na principal região produtora do Brasil, a Bahia. A falta de medidas de controle eficientes resultou, nos últimos anos, em menor produção, mudanças no uso da terra, venda de propriedades, diminuição do nível de emprego e danos ao meio ambiente.

A Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC) aconselha o controle integrado da doença, com a utilização de variedades resistentes, práticas culturais, utilização de defensivos químicos e controle biológico, além de disponibilizar aos agricultores as informações ou materiais relativos a cada um desses métodos de controle (LOPES et al., 2011). Com isso, a produção nacional de cacau vem se recuperando, e o Brasil, de uma baixa produção em 2003 (170.000 t), passou para 220.000 t em 2011/2012, de acordo com os dados da International Cocoa Organization (ICCO, 2017). A figura 2 representa a queda da produção brasileira desde o surgimento da praga e a recuperação da produção desde o início do programa de melhoramento genético, segundo dados fornecidos pela Organização das Nações Unidas para Agricultura e Alimentação (FAOSTAT, 2017).

Figura 2 - Produção brasileira de cacau em amêndoas de 1979-2014.



Fonte: FAOSTAT (2017).

Outra doença que merece destaque é a Monilíase, causada pelo fungo *Moniliophtora roreri*, pode causar grandes perdas de produção. O fungo ataca somente os frutos do cacau, causando danos internos e externos, e consequentemente a perda total do fruto. Em diferentes países da América Latina, as perdas atribuídas à Monilíase foram tão graves que áreas inteiras de plantações de cacau foram abandonadas (EVANS; EVERIS; BETTS, 2004). Como forma de controlar e recuperar plantações de cacau, novas variedades clonais resistentes a doenças foram desenvolvidas.

2.3 Desenvolvimento de novas variedades clonais do cacaueiro

O melhoramento genético com a finalidade de gerar plantas resistentes é sem dúvida a forma mais eficiente e econômica para o controle das doenças que afetam a cultura cacueira. Na Bahia, o cacau foi introduzido em 1746, com poucas sementes provenientes do estado do Pará. A partir do desenvolvimento das plantas dessa primeira introdução, posteriormente outras introdução foram realizadas gerando variedades que são conhecidas como “Comum”. Na década

de 1950, as seleções foram realizadas dentro das variedades “Comuns” pela Estação Experimental de Juçari, Estação Experimental de Uruçuca, na Bahia e a Estação Experimental de Goitacazes, no Espírito Santo, que resultou nas conhecidas seleções SIAL, SIC e EEG, respectivamente (YAMADA et al., 2001).

Yamada et al. (2001) relataram que durante a década de 60, o recém criado Centro de Pesquisa de Cacau (CEPEC) distribuiu grandes quantidades de sementes da variedade conhecida como “Catongo”. Além disso, o CEPEC distribuiu clones de vários outros países entre os agricultores. De 1960 a 1983, a maioria das plantações foi feita com uma mistura de híbridos envolvendo variedades comuns (clones SIC, SIAL e EEG) e variedades introduzidas do Peru (clone IMC e SCA), Trinidad (ICS clones) e Costa Rica (UF clones).

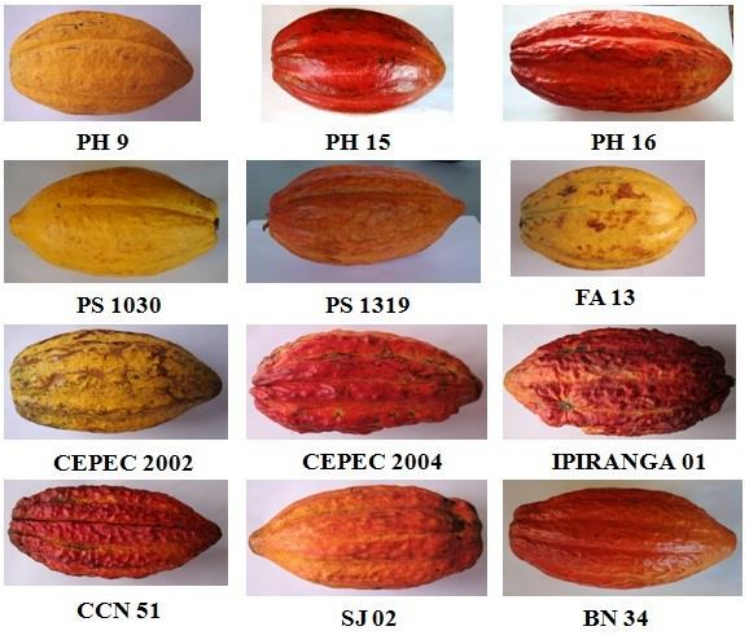
A princípio, a recomendação, por parte dos institutos de pesquisas (CEPEC/CEPLAC), foi gerar misturas de híbridos para aumentar a diversidade genética, garantir boa disseminação da cultura do cacau e aumento de produtividade. Em 1986, a mistura de híbridos foi restrita a cruzamentos entre clones “Comuns” (SIAL e SIC) e a série ICS, mas esses cruzamentos geraram híbridos muito suscetíveis a “vassoura-de-bruxa". Em 1994, essas variedades foram substituídas por uma variedade híbrida chamada Theobahia (SCA 6 x ICS 1), resistente para a “vassoura-de-bruxa” (MONTEIRO; PIRES; PINTO, 1995). Mais tarde, Theobahia I (ICS 6 x SCA 6) e Theobahia II (ICS 8 x SCA 6) foram desenvolvidas e distribuídas. A partir de 1995, o CEPEC adicionou aos clones recomendados derivados do IMC 67, ICS 1 e SCA 6, codificados como TSA, TSH, CEPEC e EET (LOPES; PIRES, 2014).

Com o intuito de recuperação da lavoura cacaueira, o Brasil incentiva a união entre fazendeiros e órgãos de pesquisas, como o Centro de Pesquisa do Cacau – CEPEC e a Comissão Executiva do Plano da Lavoura Cacaueira – CEPLAC. Essa união tem como objetivo desenvolver pesquisas sobre cultivares

de cacau resistentes, produtivos e que originem matérias-primas de qualidade. Sendo considerada uma das formas mais eficientes encontradas para a recuperação da lavoura cacaueira (MOREIRA et al., 2016).

Atualmente, por meio do Instituto Biofabrica de Cacau (Organização social vinculada ao Governo da Bahia), estão sendo distribuídas variedades genéticas de cacau resistentes a "vassoura-de-bruxa" e de alta produtividade. O objetivo básico dessa ação é apoiar o programa de Recuperação da Lavoura Cacaueira Baiana. Alguns destes materiais são: TSH 516, 565, 774, 1188; SJ 02; TSA 654, 656, 792; FA 13; PS 1030, 1319; CCN 51; IPIRANGA 01; BN 34; EET 397; CEPEC 2002, 2003, 2004; PH 9, 15, 16; LCTEEN 37 A (PEREIRA, 2001). A Figura 3 apresenta algumas variedades de cacau cultivadas em solo brasileiro.

Figura 3 - Alguns exemplos de variedades de cacau encontradas em solo brasileiro.



Fonte: Próprio autor (2017).

2.4 Impacto do melhoramento genético na qualidade do cacau

A dificuldade em se avaliar comparativamente as diferenças entre variedades de cacau encontra-se na escassez de trabalhos que tenham utilizado diferentes variedades genéticas submetidas aos mesmos protocolos de fermentação, secagem e torrefação (CROSS, 1999). Mas, algumas pesquisas têm possibilitado um maior conhecimento sobre os fatores que influenciam no sabor de chocolate das novas variedades genéticas (EFRAIM et al., 2013).

Apesar da importância de processos pós-colheita, como a fermentação, secagem e torrefação, no desenvolvimento do sabor de chocolate, alguns estudos mostraram também um forte efeito da variedade de cacau no sabor. Clapperton et al. (1994b) avaliaram sensorialmente mais de quatro mil amostras de liquor de cacau preparado a partir de 107 clones, na Malásia, e relataram um efeito claro no sabor dos diferentes clones avaliados. Clapperton et al. (1994c) avaliaram 64 clones e 12 atributos de sabor, e observaram uma grande diferença entre os clones, especialmente para o sabor do cacau e adstringência.

Efrain et al. (2013) verificou atributos de sabor distintos para o chocolate produzido a partir de nove clones do programa de melhoramento do Brasil, os clones estudados foram: CEPEC 42, EET 397, TSA 654, TSA 656, TSAN 792, TSH 516, TSH 565, TSH 774 e TSH 1188. Os pesquisadores submeteram todas as amostras ao mesmo tipo de processamento, gerando três produtos: massa de cacau, manteiga e chocolate. Avaliando as características desses derivados, constatou-se que a manteiga de cacau obtida de oito das nove variedades mais resistentes à vassoura-de-bruxa apresentava maior dureza em comparação com a extraída do cacau comum cultivado na Bahia, além disso, testes sensoriais comprovaram uma maior preferência pelos produtos gerados a partir de variedades obtidas de misturas de dois grupos de cacaueiros (*Forastero* e *Trinitario*), em comparação com outras variedades descendentes apenas do grupo *Forastero* (EFRAIM et al., 2013).

Sukha et al. (2008) avaliaram o sabor do chocolate produzido a partir de sete clones de cacau fermentados e plantados em quatro regiões de Trinidad e Tobago, e observou que o efeito da variabilidade genética em atributos de sabor foi grande, até mesmo maior do que o efeito do meio ambiente.

O efeito de variabilidade genética na composição química do cotilédone também tem sido alvo de pesquisas. Os conteúdos de flavonoides, cafeína e teobromina em grãos de cacau demonstraram que podem afetar o amargor e adstringência do chocolate, e suas quantidades variam entre os clones de cacau (CAMU et al., 2008; CLAPPERTON et al., 1994a, 1994b; NOOR-SOFFALINA et al., 2009).

Visando contribuir para o aperfeiçoamento do melhoramento do cacaueiro no Brasil, Pires (2003) conduziu estudos sobre a coleção de germoplasma do CEPEC – Centro de Pesquisa do Cacau, em Ilhéus, na Bahia, que abordaram os caracteres de maior importância para a cultura: resistência à vassoura-de-bruxa e podridão parda, produtividade, características físicas de fruto e semente, teor e dureza da gordura e concentração de ácidos graxos e triglicerídeos. Pires (2003) observou que as séries com maiores médias para o teor de gordura são na quase totalidade, originárias da região Amazônica e de material não domesticado, além disso, o genótipo do embrião é fator determinante para o conteúdo de manteiga da semente.

A fim de avaliar o efeito da fermentação sobre o teor de purinas no cacau *Criollo* e um híbrido originado ao Sul do Lago Maracaibo, foi realizado um teste conduzido por Portillo et al. (2011). O teor de teobromina e cafeína diminuiu durante a fermentação para os dois tipos de cacau. O conteúdo teobromina foi menor no cacau *Criollo*. Também a relação de teobromina:cafeína foi menor para os tipos de cacau *Criollo*, indicando que estes são menos amargo.

PH9, PH15 e PH16, híbridos cultivados no sul da Bahia, apresentaram comunidades microbianas e composição química não volátil diferentes durante o processo fermentativo em estudos conduzidos por Moreira et al. (2013). Além disso, Ramos et al. (2014), Visintin et al. (2017) relataram a influência do genótipo em parâmetros como sucessão microbiana envolvida durante o processo fermentativo, compostos voláteis e não voláteis produzidos e características sensoriais dos chocolates produzidos a partir dos híbridos FA13, PH16, PS1330, PS1319 e SJ02.

2.5 O processo fermentativo do cacau

A partir das sementes do cacau é obtido um dos alimentos mais conhecidos e apreciados: o chocolate. Seu sabor é condicionado não apenas a atributos genéticos do cacaueiro (variedade), como também a modificações que ocorrem durante seu beneficiamento. Basicamente, após a colheita do cacau, são efetuadas as operações de abertura dos frutos, fermentação das sementes junto à polpa que as envolve, secagem e torração para obtenção da massa ou liquor de cacau, que será utilizado na obtenção de manteiga e pó de cacau, além de chocolates e produtos derivados (SCHWAN; FLEET, 2014).

Os frutos após a colheita são abertos e levados para os locais de fermentação que variam de acordo com o tipo da região produtora (LEHRIAN; PETTERSON, 1983). No sul da Bahia, a fermentação ocorre em caixas de madeira (cochos de fermentação), durante 6-8 dias, onde o cacau é revolvido de 12 em 12 horas a partir do segundo dia de fermentação. A Figura 4 apresenta as mudanças visíveis que ocorrem na massa de cacau durante os dias de fermentação. No início, a polpa se faz muito presente, com o passar dos dias a atividade microbiana torna–se mais intensa, sendo essa polpa consumida. No final dos dias de fermentação apenas amêndoas prontas para o processo de secagem permanecem no cocho.

Figura 4 - Mudanças visíveis na massa de cacau durante os dias de fermentação.

Fonte: Próprio autor (2017).

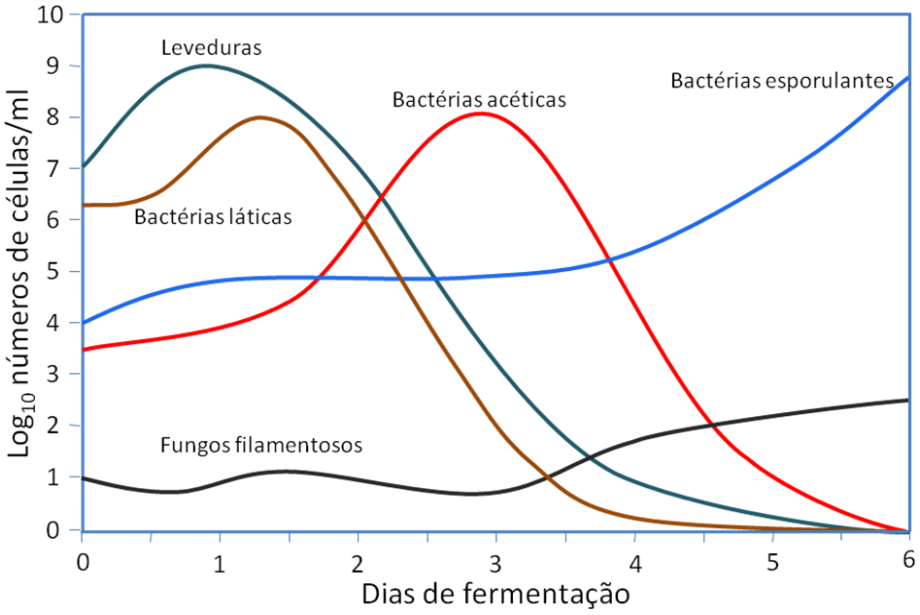
O termo fermentação de cacau implica em um processo microbiológico, de ação enzimática e de produção dos precursores do aroma e sabor de chocolate. Ou seja, é uma mistura de processos microbiológicos externos, caracterizados principalmente pela produção de etanol e ácido acético a partir de carboidratos, e de processos proteolíticos que ocorrem no interior das amêndoas (BIEHL et al., 1989; SCHWAN; WHEALS, 2004).

Esta é uma etapa fundamental do beneficiamento primário do cacau e, juntamente com o processo de secagem define o padrão do cacau comercial, estando as duas etapas comprometidas com a qualidade do produto final. Rohan (1964) provou que o sabor característico do chocolate é somente desenvolvido a partir de amêndoas de cacau fermentadas.

Estudos microbiológicos sobre a fermentação do cacau certificam desde o século passado a presença de várias espécies de microrganismos associados ao processo, como leveduras, bactérias do ácido lático e acético, algumas espécies de *Bacillus*, além de fungos e aeróbios formadores de esporos (JESPERSEN et

al., 2004; LIMA et al., 2011; NIELSEN et al., 2005; SCHWAN; WHEALS, 2004). A Figura 5 representa a sucessão microbiana que ocorre durante o processo fermentativo do cacau.

Figura 5 - Sucessão microbiana durante a fermentação do cacau.



Fonte: Schwan e Wheals (2004).

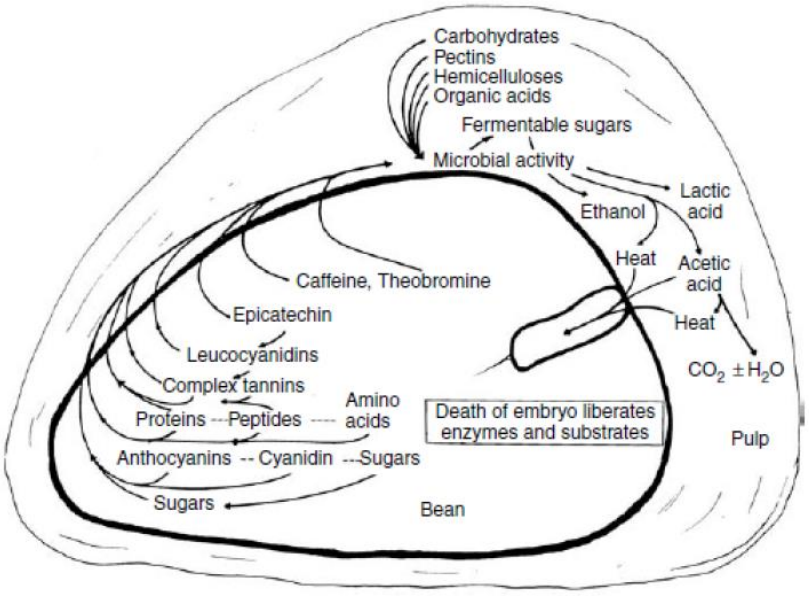
A sucessão microbiana começa quando altas concentrações de açúcares, baixo pH e tensão de oxigênio favorecem o crescimento de leveduras que convertem os carboidratos da polpa em etanol, dominando o processo durante as primeiras 48 horas. As bactérias láticas também fermentam os açúcares e utilizam o ácido cítrico da polpa, seu crescimento é favorecido pela escassez de oxigênio e ligeiras elevações do pH e da temperatura. Estas bactérias pertencem a dois grupos, homo e heterofermentativas, apresentando crescimento máximo

em torno das 16-48h, a partir do inicio da fermentação (ARDHANA; FLEET, 2003; SCHWAN; WHEALS, 2004).

Com a desintegração da polpa que envolve as amêndoas e o revolvimento da massa de cacau, a aeração se torna maior e juntamente com os valores do pH, que aumentam devido à ação das leveduras que consomem o ácido cítrico presente na polpa, o crescimento das bactérias acéticas é favorecido. Tais bactérias promovem a oxidação do etanol, produzido inicialmente pelas leveduras, à ácido acético, em uma reação extremamente exotérmica, elevando a temperatura da massa fermentativa para patamares de 45 a 50ºC. O ácido acético ao penetrar nos tecidos dos cotilédones, juntamente com as altas temperaturas, promove a morte do embrião das sementes (48-72h) desencadeando uma série de processos proteolíticos responsáveis pela liberação dos precursores do aroma de chocolate (BIEHL et al., 1989; SCHWAN, 1998; SCHWAN; ROSE; BOARD, 1995).

Outras espécies de microrganismos como *Bacillus* e fungos filamentosos desenvolvem-se nos estágios finais da fermentação, mas segundo Ardhana e Fleet (2003) o seu papel ainda não está completamente elucidado. A Figura 6 ilustra as alterações químicas que ocorrem durante a fermentação e após a morte embrionária.

Figura 6 - Alterações químicas durante o processo de fermentação.



Fonte: Lopez e Dimick (1995)

Os efeitos favoráveis da fermentação do cacau são a produção de sabores típicos de cacau e chocolate, oxidação e condensação de polifenóis adstringentes em compostos solúveis menos desagradáveis ao paladar, redução na concentração de proteínas que poderiam conferir sabor desagradável após torrefação das amêndoas e a redução na concentração de purinas que são muito amargas. O ritmo do desenvolvimento do sabor e do aroma característicos do cacau e chocolate é função do tempo que o embrião demanda para morrer. Sabe-se que este sabor típico surge após 44-48 horas do início da fermentação (THOMPSON; MILLER; LOPEZ, 2007).

De acordo com Schwan et al. (2014), a técnica de fermentação é variável, dependendo do país, da região e do porte do produtor de cacau (grandes ou pequenas propriedades). Em regiões da América Central, a fermentação, geralmente, é realizada em plataformas de secagem, nas quais as

sementes com polpa são espalhadas durante o dia e reunidas em montes durante a noite como forma de manter o calor. Essa prática mostrava-se eficiente quando a maior parte do cacau produzido na região era do tipo *Criollo*. Porém, atualmente, grande parte do material é constituída por híbridos do grupo *Forastero*, sendo essa prática inadequada para esse tipo de cacau. Em alguns países africanos e esporadicamente na região amazônica brasileira, a fermentação é realizada em montes, sendo que as quantidades podem variar consideravelmente, de 25 a 1.000 Kg de sementes com polpa. Em algumas regiões de Gana, na Nigéria e nas Filipinas, a fermentação é geralmente realizada em cestos. Em fazendas de grande porte, como na Bahia, a fermentação é realizada em caixas de madeira de aproximadamente 1 m^3.

Contudo, a maior parte do cacau no mundo é cultivado em menor escala, em pequenas propriedades com condições precárias de mão-de-obra e mecanização. Nesse caso, a fermentação é realizada de diversas formas, como por exemplo, em montes de sementes com polpa cobertas com folhas de bananeira, os quais permanecem ao lado ou na própria área de cultivo do cacau, em caixas utilizadas no transporte de frutas, em sacos, em plataformas de secagem, entre outros (THOMPSON; MILLER; LOPEZ, 2001).

Embora o crescimento dos microrganismos seja similar nos diversos países produtores de cacau do mundo, acredita-se que cada fermentação tem a sua característica própria com relação a tempo de processo, variações de temperatura e pH, dinâmica de compostos voláteis e não voláteis e microbiota envolvida (COCOLIN; BISSON; MILLS, 2000; SAMAH et al., 1993). Dessa forma, a qualidade do produto final das amêndoas de cacau fermentadas e secas varia de país a país, de fazenda a fazenda e de colheita a colheita (SCHWAN et al., 2014).

2.6 Importância das leveduras para a fermentação de cacau

As leveduras estão largamente distribuídas na natureza e tem capacidades metabólicas extremamente diversas. Estas podem utilizar uma larga cadeia de nutrientes sob variáveis condições ambientais (TORNAI-LEHOCZKI; PÉTER; DLAUCHY, 2003), especialmente em alimentos com baixo pH, alta concentração de açúcar ou de sal, em alimentos contendo sorbato e benzoato como conservantes, bem como na presença de álcool onde muitas espécies de bactérias são inibidas (EVANS; EVERIS; BETTS, 2004; SENSES-ERGUL; OZBAS, 2006).

As condições presentes na fermentação de cacau em respeito à aeração, pH, concentração de etanol e substrato fornece nichos para muitas espécies de leveduras em cada processo fermentativo. Estudos realizados no Brasil identificaram diversas espécies de leveduras associadas à fermentação do cacau, entre elas: *Saccharomyces cerevisiae*, *Candida bombi*, *C. pelliculosa*, *C. rugopelliculosa*, *C. rugosa*, *C. humilis*, *Yarrowia lipolytica*, *Kluveromyces marxianus*, *Kloechera apiculata*, *K. thermotolerans*, *Pichia Kluyveri*, *P. fermentans*, e *Torulaspora pretoriensi* (MOREIRA et al., 2013; PEREIRA et al., 2012; SCHWAN; WHEALS, 2004).

O fato das leveduras serem os agentes principais da fermentação do cacau é explicado pelas seguintes funções:

a) Quebra do ácido cítrico da polpa o que leva a um aumento no pH de 3,5 a 4,2, possibilitando o crescimento de bactérias;
b) Produção de etanol sob condições de baixo oxigênio e altas concentrações de açúcares, os quais são consumidos oxidativamente;

c) Produção de ácidos orgânicos (oxálico, fosfórico, succínico, málico e ácido acético), os quais penetram nas sementes e causam a morte do embrião;
d) Produção de alguns compostos orgânicos voláteis que contribuem tanto para o sabor do chocolate, quanto para os precursores do sabor;
e) E por fim, pela secreção de pectinases, que reduzem a viscosidade da polpa, levando a uma maior aeração da massa fermentativa (SCHWAN, 1998; SCHWAN; WHEALS, 2004).

A importância do metabolismo das leveduras no desenvolvimento do aroma de chocolate foi recentemente esclarecida por Ho, Zhao e Fleet (2014), usando natamicina para inibir o crescimento das leveduras, os autores comprovaram que o cacau fermentado na ausência de leveduras, produziu um chocolate ácido sem os aromas de chocolate. Além de serem responsáveis pela produção de etanol e pela liberação de enzimas, as leveduras são grandes produtores de ésteres e álcoois superiores que contribuem com a complexa mistura de compostos aromáticos voláteis que caracterizam o aroma de chocolate (CRAFACK et al., 2014; SCHWAN; WHEALS, 2004).

A fermentação de cacau, em todos os países produtores, é conduzida de forma espontânea e empírica. A seleção de culturas iniciadoras, para aplicação no processo fermentativo do cacau, é uma alternativa para alcançar matérias primas que contribuem para padronização e qualidade dos chocolates (SCHWAN et al., 2014).

2.7 O uso de culturas iniciadoras na fermentação de cacau

O uso de culturas iniciadoras em processos fermentativos é muito comum para uma série de produtos industrializados, como vinho, cerveja,

queijo, pão, bebidas destiladas, salames e leites fermentados (PEREIRA et al., 2012; WOOD, 1998).

Processos fermentativos controlados, principalmente utilizando culturas iniciadoras, podem gerar produtos padronizados e com alta qualidade. Inoculação na fermentação de cacau não é recente, mas ainda não foi definido e comercializado um *cocktail* de microrganismos adequado para aplicação (SCHWAN et al., 2014). Muitos estudos recentes relataram que diferentes espécies de leveduras influenciam o processo de fermentação, consequentemente o sabor do chocolate.

Ramos et al. (2014) estudaram as comunidades microbianas e o perfil de compostos voláteis de diferentes fermentações, utilizando quatro híbridos de cacau e aplicando *Saccharomyces cerevisiae* como cultura iniciadora. Os autores constataram que a inoculação acelerou o processo de fermentação e causou alterações na dinâmica de compostos voláteis e não voláteis. Moreira et al. (2017) conduziram o processo fermentativo do híbrido PH15 inoculado com um *cocktail* de microrganismos (*S. cerevisiae*, *Lactobacillus plantarum* e *Acetobacter pasteurianus*) e relataram diferenças na composição volátil das amêndoas e no perfil sensorial dos chocolates produzidos a partir das fermentações espontâneas e inoculadas. Visintin et al. (2017) realizaram experimentos de inoculação com duas leveduras (*S. cerevisiae* e *Torulaspora delbrueckii*) durante a fermentação dos híbridos PS1319 e SJ02, os autores concluíram que as culturas utilizadas como iniciadoras alteraram o perfil de compostos voláteis e a percepção sensorial dos chocolates.

Crafack et al. (2014) conduziram fermentações inoculadas de cacau em Ghana, utilizaram como culturas iniciadoras um *cocktail* selecionado de cepas: *P. kluyveri*, *Lactobacillus fermentum*, e *Acetobacter pasteurianus* ou *Kluyveromyces marxianus, L. fermentum* e *A. pasteurianus*. Amêndoas de cacau fermentadas com *P. kluyveri* geraram um chocolate com altas pontuações de

atributos sensoriais (doce, frutado, aroma de chocolate) desejáveis. Chocolates preparados a partir da fermentação inoculada com *K. marxianus* foram considerados amargos, azedos e adstringentes.

Como mencionado em tópicos anteriores, a fermentação é uma etapa importante para o desenvolvimento dos sabores de chocolate, considerando que o uso de culturas iniciadoras podem alterar dinâmicas de fermentação, não é surpresa que o processo de inoculação também afete o desenvolvimentos dos precursores de sabor, e consequentemente o perfil sensorial dos chocolates.

2.8 O desenvolvimento dos precursores de sabor durante a fermentação

Açúcares e polissacarídeos presentes na polpa de cacau são fermentados por microrganismos, produzindo metabólitos e gerando condições que causam a morte do embrião e uma série de reações bioquímicas se desencadeiam dentro da amêndoa, formando assim os precursores do sabor de chocolate (AFOAKWA et al., 2008; SCHWAN; WHEALS, 2004). As fontes destes precursores são certamente numerosas, no entanto, a degradação de proteínas da semente e a liberação de peptídeos e aminoácidos livres estão entre os processos mais importantes para a formação dos precursores de sabor (AFOAKWA et al., 2008).

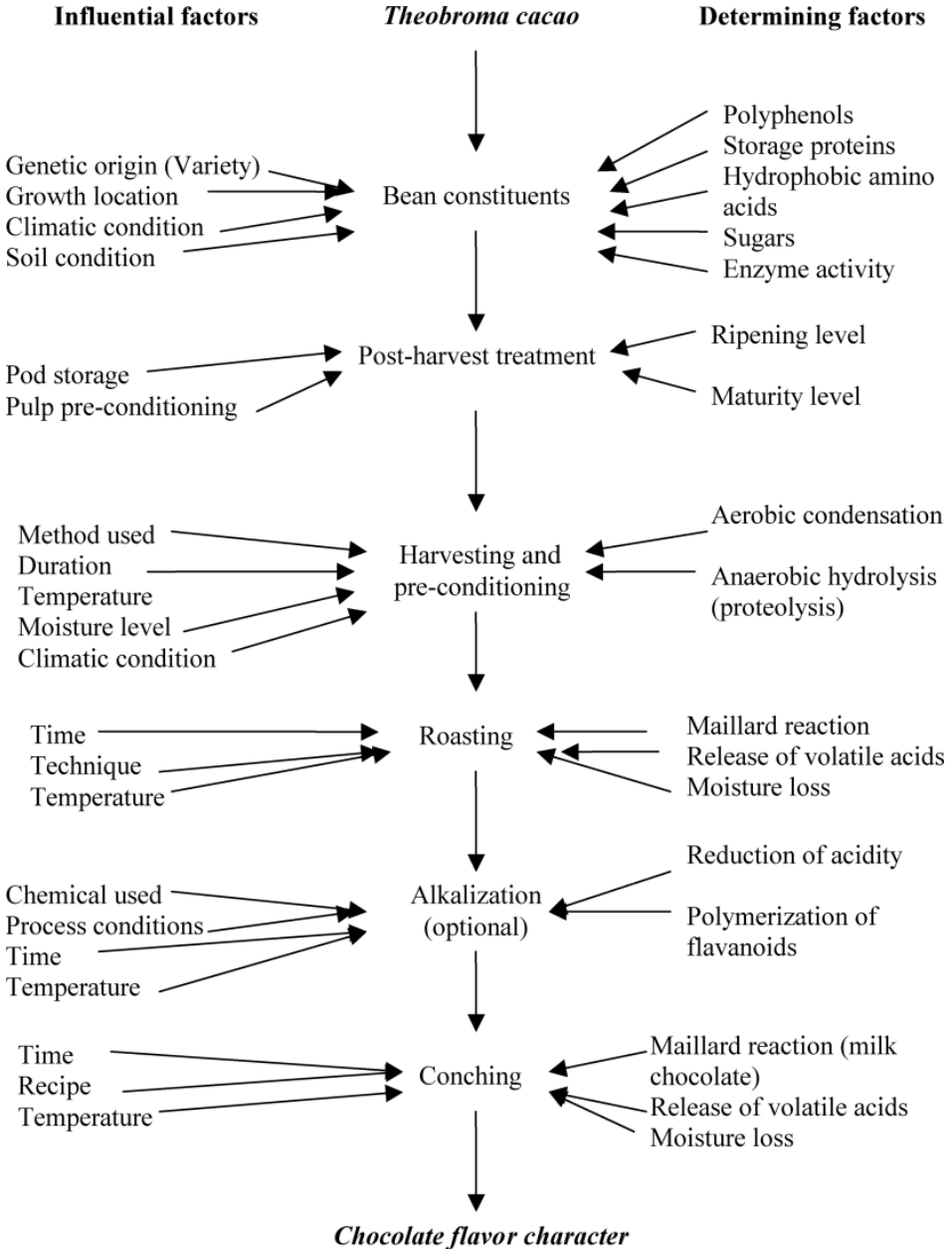
Estudos sobre a atividade das enzimas durante a fermentação por Hansen, Del Olmo e Burri (1998) mostrou que amino peptidase, invertase (na polpa) e polifenol oxidase foram fortemente inativadas durante o processo, enquanto carboxipeptidase foi parcialmente inativada, e endoproteases e glicosidases permaneceram ativas. Proteínas do cacau são clivadas a peptídeos e amino ácidos livres através de autólise por duas enzimas endógenas, endopeptidase aspártica e carboxipeptidase (AMIN et al., 2002; VOIGT et al., 1994). A fermentação do cacau é fundamental para a ativação dessas duas enzimas por metabólitos microbianos (como o ácido acético), já que não há

nenhuma evidência de que enzimas microbianas penetram as amêndoas (SCHWAN; WHEALS, 2004).

As alterações na composição de proteínas das amêndoas de cacau têm sido observadas não só durante a fermentação, mas também como uma consequência do processo de torrefação. Uma diminuição na proteína total, aminoácidos livres e de albumina (35.65% para 18.10%) são vistos durante a torrefação (ABECIA-SORIA; PEZOA-GARCIA; AMAYA-FARFAN, 2005).

A Figura 7 apresenta os mecanismos da formação e desenvolvimento de sabor de chocolate durante o processamento do cacau.

Figura 7 - Mecanismos de formação e desenvolvimento de sabor de chocolate.



Fonte: Afoakwa et al. (2008).

A concentração dos precursores de sabor é dependente de mecanismos enzimáticos. Mudanças de cor também ocorrem com a hidrólise dos componentes fenólicos por glicosidases, influenciando o caráter do sabor final (BIEHL et al., 1990; LOPEZ; QUESNEL, 1973).

Voigt e Lieberei (2014) discutiram que durante o processo de fermentação do cacau, as reações proteolíticas dentro das amêndoas começam após três dias de fermentação e são responsáveis pela liberação de pequenos peptídeos e aminoácidos livres que influenciam o sabor do chocolate gerado nos processos posteriores, como a torração das amêndoas. Conforme relatado recentemente por Hue et al. (2016), de fato, as proteínas são degradadas com um aumento concomitante de conteúdo de aminoácidos durante a fermentação. Voigt e Biehl (1993) ressaltam que o teor de proteínas das amêndoas de cacau é composto principalmente por duas proteínas: albumina e vicilina (globulina da classe 7S). Como mencionado, essas proteínas sofrem ação de uma endoprotease aspártica e uma carboxipeptidase, liberando assim os precursores dos aromas de chocolate.

2.9 Análise de proteínas das amêndoas de cacau fermentadas

A maioria das pesquisas sobre proteômica das amêndoas de cacau buscam identificar e compreender como são formados os precursores do sabor específico de chocolate (VOIGT; LIEBEREI, 2014). Sabe-se que a hidrólise de proteínas no interior das amêndoas de cacau começa após três dias de fermentação, mas são mais notáveis no terceiro dia. A degradação de proteína total durante a fermentação foi estimada em cerca de 57%, mas as duas principais proteínas (albumina e vicilina) parecem se comportar de forma diferente durante a fermentação (AMIN; JINAP; JAMILAH, 1998; LERCETEAU et al., 1999). Albumina apresenta uma degradação limitada de aproximadamente 47%, Voigt et al. (1994) discordam da participação dessa

proteína na formação dos aromas de cacau. Pelo contrário, vicilina é altamente degradada durante a fermentação (88-90% do conteúdo inicial), o que resulta aumento acentuado dos aminoácidos livres e na formação dos precursores de aromas que geram sabor específico de cacau após a etapa de torrefação.

Voigt et al. (2016) desenvolveram um procedimento de fracionamento do extrato de aromas precursores das amêndoas fermentadas por técnicas cromatográficas, e as frações obtidas foram caracterizadas por análises de MALDI-TOF (*Matrix Assisted Lazer Desorption Ionization-Time of flight*). Com os resultados, os autores demonstraram que, além de aminoácidos livres, os peptídeos hidrofílicos derivados da proteína de armazenamento globular da classe vicilina-(7S) são necessários para a geração de notas aromáticas específicas de cacau durante o processo de torrefação. Figura 8 apresenta a sequência do precursor de aroma vicilina, em negrito e/ou sublinhadas são sequências de aminoácidos encontradas nas frações com aromas de cacau (VOIGT et al., 2016).

Figura 8 - Sequência da proteína globular da classe vicilina (7S).

MVISKSPFIV LIFSLLLSFA LLCSGVSAYG RKQYERDPRQ QYEQCQRRCE

SEATEEREQE QCEQRCEREY KEQQRQQEEE LQRQYQQCQG RCQEQQQGQR

EQQQCQRKCW EQYKEQERGE HENYHNHKKN RSEEEEGQ**QR NNPYYFPK**R**R**

SFQTRFRDEE **GNFKILQ**RFA ENSPPL**KGIN** DY**RLA**MFEAN PNT**FILPH**HC

DAEAIYFVTN **GKGTITF**VTH ENKESYNVQ**R GTVVS**VPAGS TVYVVSQDNQ

EKLTIAVLAL **PVNSPGKY**EL FFPAGNN**KPE S**YYGAFSYEV LETVFNTQRE

KLEEILEQR GQKRQQGQQG MFRKAKPEQI RAISQQATSP RHR**GGERLAI**

NLLSQSPVYS NQNGRFFEAC PEDFSQFQNM DVAVS**AFKLN QGAIFVPHYN**

SKATFVVFVT DGYGYAQMAC PHLSRQSQGS QSGRQDRREQ EEESEEETFG

EFQQVKAPLS PGDVF**VAPAG HAVTFF**ASK**D QPLN**AVAFGL NAQ**NNQRIF**L

AGKKNLVRQM DSEAKE**LSFG VPSKL**VDNIF NNPDE**SYF**MS FSQQRQRGDE

RRGNPLASIL DFARLF

Fonte: Voigt et al. (2016)

Análises de MALDI-TOF têm sido utilizadas para avaliar o perfil proteico e identificar quais proteínas e seus derivados influenciam na formação dos precursores do aroma específico de chocolate. Kratzer et al. (2009) utilizaram a técnica de MALDI-TOF para localizarem subunidades da viclina que colaboram para o desenvolvimento do sabor, e identificaram três subunidades com massas moleculares aparentes de 47 kDa, 31 kDa e 15 kDa, que são derivadas de um precursor comum de 66 kDa.

Considerando que diferentes híbridos de cacau apresentem composições químicas (polpa e amêndoa) distintas, o conteúdo proteico pode influenciar no sabor do chocolate produzido, como mencionado acima. Dessa forma, técnicas como MALDI-TOF podem auxiliar na avaliação dos perfis proteicos das variedades clonais e selecionar híbridos de cacau que produzam amêndoas com qualidade industrial para fabricação de chocolate (VOIGT; LIEBEREI, 2014).

REFERÊNCIAS

ABECIA-SORIA, L.; PEZOA-GARCIA, N. H.; AMAYA-FARFAN, J. Soluble albumin and biological value of protein in cocoa (*Theobroma cacao* L.) beans as a function of roasting time. **Journal of Food Science**, Champaign, v. 70, n. 4, p. 294–S298, May 2005.

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

ALVES, S. A. M. et al. Curvas de progresso e gradientes da vassoura-de-bruxa (Crinipellis perniciosa) em cacaueiros enxertados em Uruçuca, Bahia. **Fitopatologia Brasileira**, Brasília, v. 31, n. 5, p. 483-491, set./out. 2006.

AMIN, I. et al. Oligopeptide patterns produced from *Theobroma cacao* L of various genetic origins. **Journal of the Science of Food and Agriculture**, London, v. 82, n. 7, p. 733–737, May 2002.

AMIN, I.; JINAP, S.; JAMILAH, B. Proteolytic activity (aspartic endoproteinase and carboxypeptidase) of cocoa bean during fermentation. **Journal of the Science of Food and Agriculture**, London, v. 76, n. 1, p. 123–128, Jan. 1998.

AMIN, M. M. A ação especulativa dos fundos hedge e de commodities nos mercados futuros de cacau. **Informe GEPEC**, Cascavel, v. 7, n. 1, p. 1-16, 2003.

ARDHANA, M. M.; FLEET, H. G. The microbial ecology of ocoa bean fermentations in Indonesia. **International Journal of Food Microbiology**, Amsterdam, v. 86, n. 1-2, p. 87-99, Sept. 2003.

BARTLEY, B. G. D. **The genetic diversity of cacao and its utilization**. Wallingford: CABI Publishing, 2005. 341 p.

BIEHL, B. et al. Bean spreading: a method for pulp preconditioning to impair strong nib acidification during cocoa fermentation in Malaysia. **Journal of Agriculture and Food Chemistry**, Oxford, v. 51, n. 1, p. 35–45, 1990.

_____. 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. 48, n. 2, p. 189-208, 1989.

CAMU, N. et al. 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. 2008.

CHEESMAN, E. E. Notes on the nomenclature, classification and possible relationship of cocoa populations. **Tropical Agriculture**, Surrey, v. 21, p. 144-159, 1944.

CLAPPERTON, J. et al. Effects of planting materials on flavour. **Cocoa Growers´ Bulletin**, Bournville, n. 48, p. 47-63, 1994b.

_____. Genetic variation in cocoa flavour. In: INTERNATIONAL COCOA RESEARCH CONFERENCE, 11., 1993, Yamoussoukro. **Proceedings...** Lagos: Cocoa Producers' Alliance, 1994c. p. 749-758.

_____. The contribution of genotype to cocoa (*Theobroma cacao* L) flavor. **Tropical Agriculture**, Surrey, v. 71, n. 4, p. 303-308, Oct. 1994a.

COCOLIN, L.; BISSON, L. F.; MILLS, D. A. Direct profiling of the yeast dynamics in wine fermentations. **FEMS Microbiology Letters**, Amsterdam, v. 189, n. 1, p. 81-87, Aug. 2000.

CRAFACK, M. et al. Impact of starter cultures and fermentation techniques on the volatile aroma and sensory profile of chocolate. **Food Research International**, Barking, v. 63, p. 306–316, Sept. 2014.

CROSS, E. Cocoa flavor development. **The Manufacturing Confectioner**, Princeton, v. 79, n. 2, p. 70-77, 1999.

DUBLIN, D. **Handbook of plant cell culture**: techniques for propagation and breeding. New York: McMillan, 1984. v. 3, 620 p.

EFRAIM, P. et al. Characteristics of cocoa butter and chocolates obtained from cocoa varieties grown in Bahia, Brazil. **European Food Research and Technology**, Berlin, v. 237, n. 3, p. 419–428, Sept. 2013.

EVANS, D. G.; EVERIS, L. K.; BETTS, G. D. Use of survival analysis and classification and regression trees to model the growth/no growth boundary of spoilage yeast as affected by alcohol, pH, sucrose, sorbate and temperature. **International Journal of Food Microbiology**, Amsterdam, v. 92, n. 1, p. 55-67, Apr. 2004.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAOSTAT. Disponível em: <http://faostat.fao.org/site>. Acesso em: 15 ago. 2017.

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.

HO, V.; ZHAO, J.; FLEET, G. Yeasts are essential for cocoa bean fermentation. **International Journal of Food Microbiology**, Amsterdam, v. 174, p. 72–87, Mar. 2014.

HUE, C. et al. Impact of fermentation on nitrogenous compounds of cocoa beans (*Theobroma cacao* L.) from various origins. **Food Chemistry**, London, v. 192, p. 958-964, Feb. 2016.

HURST, W. J. et al. Cacao usage by the earliest Maya civilization. **Nature**, London, v. 418, n. 6895, p. 289-290, July 2002.

INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE. Levantamento sistemático da produção agrícola: pesquisa mensal de previsão e acompanhamento das safras agrícolas no ano civil. **Levantamento Sistemático da Produção Agrícola**, Rio de Janeiro, v. 30, n. 7, p. 1-83 jul. 2017.

INTERNATIONAL COCOA ORGANIZATION - ICCO. **ICCO quarterly bulletin of cocoa statistics**. Côte D'Ivoire: ICCO, 2017. Disponível em: <http://www.icco. org/about-us/international-cocoa-agreements/cat_view/30-related-documents/46-statistics-production.html>. Acesso em: 18 ago. 2017.

JESPERSEN, L. et al. Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. **FEMS Yeast Research**, New York, v. 5, n. 4-5, p. 441-453, Feb. 2004.

KRATZER, U. et al. Subunit structure of the vicilin-like globular storage protein of cocoa seeds and the origin of cocoa- and chocolate-specific aroma precursors. **Food Chemistry**, London, v. 113, n. 4, p. 903–913, Apr. 2009.

LEHRIAN, D. W.; PETTERSON, G. R. Cocoa fermentation. **Biotechnology**, Frankfurt, v. 5, p. 12-15, 1983.

LERCETEAU, E. et al. Evolution of cacao bean proteins during fermentation: A study by two-dimensional electrophoresis. **Journal of the Science of Food and Agriculture**, London, v. 79, n. 4, p. 619–625, Mar. 1999.

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**, Boca Raton, v. 51, n. 8, p. 731-761, Sept. 2011.

LOPES, U. V. et al. Cacao breeding in Bahia, Brazil: strategies and results. **Crop Breeding and Applied Biotechnology**, Londrina, v. 11, n. 1, p. 73-81, June 2011.

LOPES, U. V.; PIRES, J. L. Botany and production of cocoa. In: SCHWAN, R. F.; FLEET, G. H. (Ed.). **Cocoa and coffee fermentation.** Boca Raton: CRC Press, 2014. p. 43–71.

LOPEZ, A. S.; DIMICK, P. S. Cocoa fermentation. In: REED, G.; NAGODAWITHANA, T. W. (Ed.). **Biotecnology:** enzymes, biomass, food and feed. 2nd ed. New York: John Wiley & Sons, 1995. p. 561-577.

LOPEZ, A. S.; QUESNEL, V. C. Volatile fatty acid production in cacao fermentation and the effect on chocolate flavour. **Journal of the Science of Food and Agriculture**, London, v. 24, n. 3, p. 319-324, Mar. 1973.

MONTEIRO, W. R.; PIRES, J. L.; PINTO, L. R. M. Variedade Theobahia. **Informação e Difusão,** [S.l.], v. 1, p. 1-2, 1995.

MOREIRA, I. M. V. et al. Impact of a microbial cocktail used as a starter culture on cocoa fermentation and chocolate flavor. **Molecules**, Basel, v. 22, n. 5, p. 766, May 2017.

_____. Influence of cocoa hybrids on volatile compounds of fermented beans, microbial diversity during fermentation and sensory characteristics and acceptance of chocolates. **Journal of Food Quality**, Wastport, v. 39, n. 6, p. 839-849, Dec. 2016.

_____. Microbial succession and the dynamics of metabolites and sugars during the fermentation of three different cocoa (*Theobroma cacao* L.) hybrids. **Food Research International**, Barking, v. 54, n. 1, p. 9–17, Nov. 2013.

NIELSEN, D. S. et al. The microbiology of Ghanaian cocoa fermentations analysed using culturedependent and culture independent methods. **International Journal of Food Microbiology**, Amsterdam, v. 114, n. 2, p. 168-186, Mar. 2007.

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

NOOR-SOFFALINA, S. S. et al. Effect of polyphenol and pH on cocoa Maillard-related flavor precursors in a lipidic model system. **International Journal of Food Science and Technology**, Oxford, v. 44, n. 1, p. 168-180, Jan. 2009.

PAULIN, D.; ESKES, A. B. Le cacayer stratégies de seléction. **Plantations, Recherche, Developpment**, Paris, v. 2, n. 6, p. 5-18, Mar. 1995.

PEREIRA, A. B. Melhoramento clonal. In: DIAS, L. A. S. (Ed.). **Melhoramento genético do cacaueiro**. Viçosa: FUNAPE, 2001. p. 361-384.

PEREIRA, G. V. de M. et al. Bacterial strains to develop a defined fermentations and screening of yeast and characterization of small-scale cocoa microbiological. **Applied and Environmental Microbiology**, Washington, v. 78, n. 78, p. 5395-5405, Aug. 2012.

PIRES, J. L. **Avaliação quantitativa e molecular de germoplasma para o melhoramento de cacaueiro com ênfase na produtividade, qualidade dos frutos e resistência à doenças**. 2003. 226 p. Tese (Doutorado em Genética e Melhoramento) - Universidade Federal de Viçosa, Viçosa, 2003.

PORTILLO, E. et al. Effect of the fermentation on the content of purines in Criollo and hybrid cocoa (Theobroma cocoa L.) in the south of Maracaibo's lake. **Revista de la Facultad de Agronomia**, Maracaibo, v. 28, n. 1, p. 228-237, Dic. 2011.

PURSEGLOVE, J. W. **Tropical crops**: dicotyledons 2. ed. New York: John Wiley & Sons, 1968. 719 p.

RAMOS, C. L. et al. Impact of different cocoa hybrids (*Theobroma cacao* L.) and *S. cerevisiae* UFLA CA11 inoculation on microbial communities and volatile compounds of cocoa fermentation. **Food Research International**, Barking, v. 64, p. 908–918, Oct. 2014.

ROHAN, T. A. The precursors of chocolate aroma: a comparative study of fermented and unfermented cocoa beans. **Journal of the Science of Food and Agriculture**, London, v. 29, n. 4, p. 456-459, July 1964.

SAMAH, A. O. et al. Fermentation studies of stored cocoa beans. **World Journal of Microbiology and Biotechnology**, Oxford, v. 9, n. 5, p. 603-604, Sept. 1993.

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.; FLEET, G. H. **Cocoa and coffee fermentations**. New York: CRC Press, 2014. 633 p.

SCHWAN, R. F.; PEREIRA, G. V. M.; FLEET, G. H. Microbial activities during cocoa fermentation. In: SCHWAN, R. F.; FLEET, G. H. (Ed.). **Cocoa and coffee fermentation**. Boca Raton: CRC Press, 2014. p. 129–190.

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, p. 96-107, 1995. Supplement.

SCHWAN, R. F.; WHEALS, A. E. The microbiology of cocoa fermentation and its role in chocolate quality. **Critical Reviews in Food Science and Nutrition**, Boca Raton, v. 44, n. 4, p. 1-17, May 2004.

SENSES-ERGUL, S.; OZBAS, Z. Y. Characterization of the yeast flora present in some Turkish high-sugar products. **Journal of General and Applied Microbiology**, Tokyo, v. 52, n. 2, p. 99-106, Apr. 2006.

SUKHA, D. A. et al. The use of an optimised organoleptic assessment protocol to describe and quantify different flavour attributes of cocoa liquors made from Ghana and Trinitario beans. **European Food Research and Technology**, Berlin, v. 226, n. 3, p. 405-413, Jan. 2008.

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. 2nd ed. Washington: ASM Press, 2007. p. 837-849.

_____. Cocoa and coffee. In: DOYLE, M. J.; BEUCHAT, L. R.; MONTVILLE, T. J. (Ed.). **Food microbiology:** fundamentals and frontiers. Washington: ASM Press, 2001. p. 721-733.

TORNAI-LEHOCZKI, J.; PÉTER, G.; DLAUCHY, D. M. Agar Candida medium as a practical tool for the differentiation and presumptive identification of yeast species isolated from salads. **International Journal of Food Microbiology**, Amsterdam, v. 86, n. 1-2, p. 189-200, Sept. 2003.

VISINTIN, S. et al. Impact of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* starter cultures on cocoa beans fermentation. **International Journal of Food Microbiology**, Amsterdam, v. 257, p. 31-40, Sept. 2017.

VOIGT, J. et al. Cocoa-specific aroma precursors are generated by proteolytic digestion of the vicilin-like globulin of cocoa seeds. **Food Chemistry**, London, v. 50, n. 2, p. 177-184, 1994.

_____. Partial purification and characterisation of the peptide precursors of the cocoa-specific aroma components. **Food Chemistry**, London, v. 192, p. 706-713, Feb. 2016.

VOIGT, J.; BIEHL, B. The major seed proteins of Theobroma cacao L. **Food Chemistry**, London, v. 47, n. 2, p. 145-151, 1993.

VOIGT, J.; LIEBEREI, R. Biochemistry of cocoa fermentation. In: SCHWAN, R. F.; FLEET, G. H. (Ed.). **Cocoa and coffee fermentation.** Boca Raton: CRC Press, 2014. p. 193–227.

WOOD, B. J. B. **Microbiology of fermented foods**. 2nd ed. London: Thompson Science, 1998. 852 p.

YAMADA, M. M. et al. Genetic variability in cultivated cocoa populations in Bahia, Brazil, detected by isozymes and RAPD markers. **Crop Breeding and Applied Biotechnology**, Londrina, v. 1, n. 4, p. 377-384, Dec. 2001.

SEGUNDA PARTE – ARTIGOS

ARTIGO 1 - ANALYTICAL STUDY OF FERMENTED COCOA BEANS AND CHOCOLATES FROM DIFFERENT HYBRIDS CULTIVATED IN BRAZIL

Artigo formatado nas normas da revista *Food Quality and Preference*

Abstract

Cocoa beans from different geographical and genetic origins show distinct fermentation dynamics which result in different chocolate qualities. In order to understand the effects of genetic improvement of cocoa plants, in this work volatile compounds and proteins profiles of beginning and end of the fermentation from different cocoa hybrids (CEPEC2004, PH15, PS1319, SJ02) were investigated. Moreover, sensorial characterization of chocolate from these hybrids was performed. According to the results obtained, different volatile compounds were identified in fermented beans and in the chocolates. Chocolate from CEPEC2004 was the most accepted by judges and correlated with sweet and bitter taste which can be explained by the presence of desired flavor compounds, such as 2,3-butanediol and 2-methyl-1-butanol. A higher presence of acids (undesirable compounds) was observed in chocolates samples from PS1319 hybrid, which may have generated the low acceptance by judges. In addition, MALDI-TOF MS analysis showed that during fermentation the protein profile was different among the hybrids, which indicates this kind of compounds also contributes to the cocoa-specific flavor.

Keywords: chocolate quality; GC-MS; cocoa fermentation; cocoa varieties; protein profile; MALDI-TOF

1. Introduction

Cocoa is an important commodity for some countries located in the tropic region. Brazil was the major cocoa producer until mid-1980s, when a disease called witches' broom emerged mainly in the southern region of Bahia State (Freire, Schwan, & Mororó, 1999). Witches' broom is a disease caused by the fungus *Moniliophthora perniciosa*, which attacks cocoa trees and causes an excessive budding in the final parts of plants and destroys the plantations (Lopes et al., 2011). In order to recover the cocoa production in Brazil, a fast and efficient strategy for production of disease resistant hybrids was developed (Lopes et al., 2011).

Hybrids cocoa plants lead to a very variable cocoa fruit with different size, color, quantity and weight of the seeds, pulp content, chemical composition and flavor (Lopes & Pires, 2014). Many previous works confirmed the influence of genetic variability on cocoa and chocolate quality (Clapperton, Lockwood, Yow, & Lim, 1994; Efraim et al., 2013; Moreira, Miguel, Duarte, Dias, & Schwan, 2013; Ramos, Miguel, Dias, & Schwan, 2014; Moreira et al., 2016; Moreira et al., 2017).

Fermentation process of cocoa is essential for formation of precursors of chocolate specific flavor (Schwan & Wheals, 2004; Moreira et al., 2017). Voigt and Lieberei (2014) stated in a previous work that during the process of cocoa fermentation proteolytic reactions inside the beans start after 3 days of fermentation. According to these authors, small peptides and free amino acids are released which generate influence on

chocolate flavor in the later process stages, such as beans roasting. As recently reported by Hue et al. (2016), the proteins indeed get degraded with a concomitant increasing in amino acids content during the fermentation. Furthermore, the cocoa samples from different geographical origins presented a kinetic variable of protein degradation which resulted in the formation of a small fraction of peptides (>3 kDa) and a great quantity of amino acids.

According to Crafack et al. (2014), the presence of peptides and reducing sugars in chocolate production promote the Maillard reaction and intermediate compounds produced from this reaction such as furans, aldehydes, ketones, pyrroles and others volatile compounds. These have a great influence on the aroma profile of cocoa and chocolate.

The aim of the present study was to evaluate the volatile compounds and proteins profiles of beginning and end of fermentation from different cocoa hybrids. Moreover, in order to characterize the chocolates sensory analyzes were also assessed.

2. Materials and Methods

2.1. Fermentation experiments and sampling

The fermentation experiments were conducted at the Vale do Juliana cocoa farm in Igrapiúna, Bahia, Brazil. The ripe cocoa pods from five different hybrids (CEPEC2004; PH15; PS1319; SJ02) (Fig. 1) were harvested during the main crop of 2013 (September–December).

The cocoa pods were manually opened with a machete, and the beans were immediately transferred to the fermentation house. The

spontaneous fermentation started approximately 3 h after the breaking of the pods and was performed in 0.06 m^3 wooden boxes (Schwan & Wheals, 2004). Fermentation was evaluated during 6 days (144 h), and the amount used for each one of the hybrids was 60 kg.

The temperatures were evaluated during fermentations by an average of five different points into the fermentation boxes. The samples were taken (every 24 h) approximately 40 cm from the surface of the center of the fermenting cocoa mass and placed in sterile plastic pots. The samples were stored at – 20 ºC.

2.2. Characterization of volatile compounds by Headspace-Solid Phase Microextraction Gas Chromatography-Mass Spectrometry

The volatile compounds from cocoa samples were extracted using the Headspace-Solid Phase Microextraction (HS–SPME) Gas Chromatography-Mass Spectrometry (GC-MS) technique as previously described on the literature (Rodriguez-Campos, Escalona-Buendía, Orozco-Avila, & Lugo-Cervantes, 2011), with modifications. For headspace analysis, cocoa samples (2.0 g) from the beginning (0 h) and end of fermentation (144 h) and chocolate samples (2.0 g) were macerated under liquid nitrogen in a mortar and pestle.

A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 mm SPME fiber (Supelco Co., Bellefonte, PA, USA) was used to extract volatile constituents from the cocoa and chocolate headspace. The fiber was equilibrated for 15 min at 60 ºC and then exposed to the samples (beans and chocolates) for 30 min at the same temperature.

The compounds were analyzed using a Shimadzu GC model QP2010 equipped with a mass spectrometry and a capillary column of silica OV Carbonwax 20M (0.25 □m X 0.25 □m X 30 m). The temperature program began with 5 min at 40 ºC, followed by a gradient of 40 ºC to 200 ºC at 10 ºC/min; the temperature was then maintained at 200 ºC for 20 min. The injector and detector temperatures were maintained at 230 ºC. The carrier gas (He) was used at a flow rate of 1.4 mL/min.

Injections were performed by fiber exposition for 2 min. Volatile compounds were identified by comparing the mass spectra of the compounds in the samples with the database of the National Institute of Standards and Technology (NIST library, Gaithersburg, MD, USA) and the retention time with literature data using the n-Alkane index. All samples were analysed in duplicate.

2.3. Extraction and analysis of protein profile by Matrix Assisted Laser Desorption Ionization Time of flight (MALDI-TOF)

Before protein extraction, all samples, from 0 h and 144 h of fermentation of each hybrid, were subjected to extraction of lipids and polyphenols. For extraction of lipids, 6 g of cocoa beans were macerated using liquid nitrogen, in sequence were placed in papers cartridges for extraction of lipid part. A Soxhlet apparatus was used as proposed by Voigt and Biehl (1993). After 6 h, the solvent [n-hexane (bp 68 ºC)] was evaporated and recovered later in rotary evaporator.

In addition, for extraction of polyphenols acetone was used to prepare a dried defatted cocoa beans powder and prevent influence of polyphenols in analysis of protein profile (Hue et al., 2016). Three g

samples were extracted successively by 70% acetone, 80% acetone and 100% acetone using each time a volume of 60 mL. Acetone solutions were supplemented by 5 mM ascorbic acid. Mixtures were vortexed for 20 min and supernatant was discarded by centrifugation (12.000 rpm for 20 min at 4 ºC).

After the extraction of lipids and polyphenols, 0.5 g of each sample was over again macerated with liquid nitrogen, until obtain very small particles. The particles were transferred to tubes containing beads to help protein extraction and 2 mL of organic solution (water/acetonitrile/trifluoro-acetic acid, 50:47.5:2.5). The tubes were immediate and vigorously vortexed for 10 min. Afterwards, the tubes were centrifuged at 9.000 rpm for 2 min at 4 ºC and the proteins were quantified by Bradford's method.

The protein suspension (1 □L) was transferred into the 96-well MALDI flex target plate (Bruker Daltonics, Bremen, Germany). When the liquid phase was almost evaporated 1 □L matrix solution [saturated solution of a-cyano-4-hydroxy-cinnamic acid (CHCA, Fluka, Buchs, Switzerland) saturated in a solution with 33% ethanol, 33% acetonitrile, 31% H_2O and 3% TFA], was added and gently mixed (Santos et al., 2015; Santos et al., 2016).

Twelve defined ribosomal proteins of *Escherichia coli* strain DH5α cells (4365.4, 5096.8, 5381.4, 6241.4, 6255.4, 6316.2, 6411.6, 6856.1, 7158.8, 7274.5, 7872.1, 9742 and 12227.3 Da) were used as external standard of MALDI-TOF MS equipment (Passarini, Santos, Lima, Berlinck, & Sette, 2013; Lima-Neto et al., 2014). Each analysis was developed in triplicate to evaluate reproducibility. A MALDI-TOF

Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) was used for the spectra acquisition.

Raw spectra data (mzXML format) were preprocessed and analyzed using the software Mass-Up following the protocol presented by López-Fernández et al. (2015). Mass-Up is an open-source mass spectrometry software for proteomics designed to support the preprocessing and analysis of MALDI-TOF MS data. Data treatment involved the following parameters: none for intensity transformation, smoothing by Savitzky-Golay method, baseline correction by Snip method, standardization by TIC (Total Ion Current) method and detection of peaks by Maldiquant method using signal-to-noise ratio (SNR) of 3.

After preprocessing and peak matching operations, analysis of "Peak List Quality Control" and "Biomarker Discovery" were performed to generate a list with m/z values of each sample and thus evaluate presence and absence of each peak in the spectra.

2.4. Sensorial analysis

After the sun drying, an amount dried beans of each hybrid was sent for chocolate production at Sartori & Pedroso Alimentos Ltda. (São Roque, São Paulo, Brazil). The chocolate was prepared with 70% cocoa.

Sensory analyses of chocolates were performed using a consumer acceptance test followed by a check-all-that-apply (CATA) question. The tests were conducted on 71 adults over 18 years old, 41% male and 59% female. All of them were consumers of dark chocolate.

For the acceptance test, the consumers evaluated how much they liked each sample using a 9-point hedonic scale (1 = dislike extremely

and 9 = like extremely) (Stone & Sidel, 1993). For the CATA question, the consumers were asked to evaluate seven sensory attributes (sour, fruity, bitter, astringent, coffee, nut and sweetness) and select those they considered appropriate to describe the chocolate. The tests were performed as previously described elsewhere (Batista, Ramos, Ribeiro, Pinheiro & Schwan, 2015).

2.5. Statistical analysis

Data obtained from acceptance test and proteins quantification were subjected to statistical analysis based on ANOVA. The means were compared using Tukey's test at a 5% level of significance. The analyses were carried out using the SISVAR 5.1 software (Federal University of Lavras, Department of Statistic, Lavras, MG, Brazil).

Agglomerative hierarchical clustering (AHC) was carried out using XLSTAT version 7.5.2. AHC graph was assembled with results of Biomarker Discovery analysis (presence and absence of peaks). Pearson correlation coefficient was used for showing similarities among samples.

3. Results

3.1. General characteristics of cocoa fruit

The cocoa pods of different hybrids are shown in Fig. 1. Characteristics of PH15 and PS1319 hybrids were described previously in other works (Moreira et al., 2013; Ramos et al., 2014).

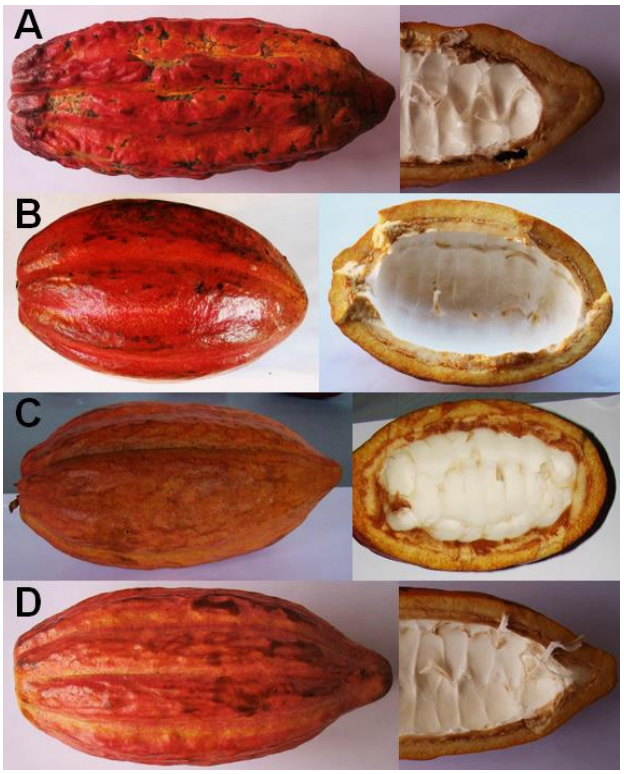


Fig. 1. Pod of four different cocoa hybrids. CEPEC2004 (A), PH15 (B), PS1319 (C) and SJ02 (D).

The CEPEC2004 hybrid (Fig. 1A) is the largest fruit with 21.75 ± 2.17 cm in length and 10.50 ± 0.25 cm in diameter, rind weigh 589.66 ± 120.58 g and contains the highest amount of seeds per fruit (53 ± 4). The PH15 fruit (Fig. 1B) is the smallest among the hybrids (14.45 ± 1.08 cm of length, 8.75 ± 0.61 cm of diameter and 433.16 ± 60.07 g of rind weight) and showed 45 ± 2 seeds per fruit, the least number.

Although rind of PS1319 (Fig. 1C) is the heaviest (781.76 ± 122.68 g), size of the fruit is between the CEPEC2004 and PH15 hybrids, 18.70 ± 1.01 cm of length and 9.65 ± 0.42 cm of diameter, and 49 ± 3

seeds per fruit. The SJ02 hybrid (Fig. 1D) produces fruits measuring 20.00 ± 1.29 cm and 9.00 ± 0.41 cm in length and diameter, respectively, has a rind weight of 505.95 ± 78.95 g and 47 ± 5 seeds per fruit.

3.2. Physical-chemical changes

Temperature was measured during 144 h of fermentation and is presented in Fig. 2. Temperatures changed according to the time for all fermentations, which ranged from around 29 °C at 0 h to 48 °C at 144 h.

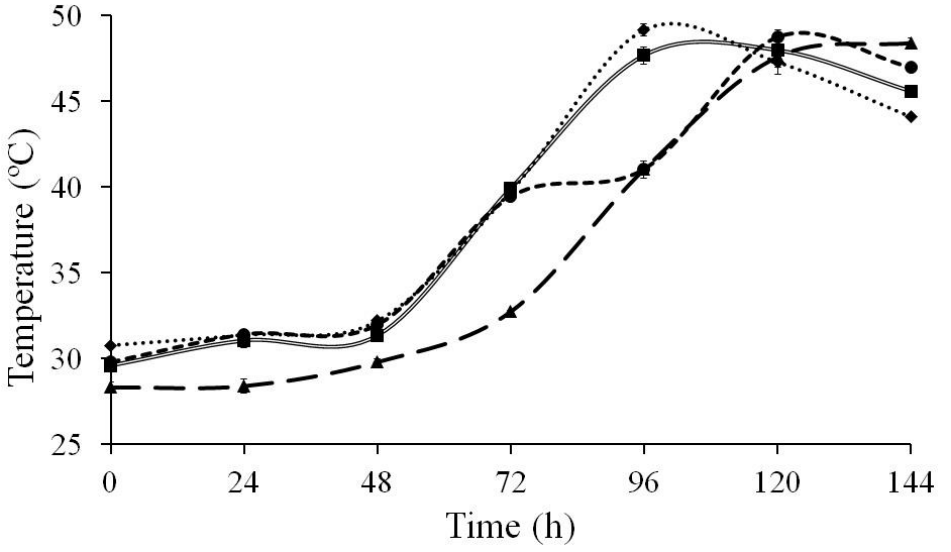


Fig. 2. Measurements of the mass temperature during the 144 h of fermentation for the different hybrids. CEPEC2004 (–●–), PH15 (···◆···), PS1319 (–■–) and SJ02 (–▲–). Mixing of the mass was performed at 48 and 72 h of fermentation process.

The maximum temperatures for each fermentation were: 48.8 ºC (at 120 h) for CEPEC2004; 49.18 ºC at (96h) for PH15, 48.00 ºC (at 120 h) PS1319 and 48.42 ºC (at 144 h) for SJ02. Lower temperatures (28.32

ºC, 28.40 ºC and 29.80 ºC) during fermentation were observed for SJ02 hybrid; however, at the end of process (144 h) higher temperature (48.42 ºC) was observed for the fermentation of this hybrid, as presented above. The rate of temperature increasing was higher in the fermentations of the hybrids PH15 and PS139, approximately 0.35 ºC per hour between fermentation times 48 and 96h.

3.2.1. Volatile compounds

All the compounds identified by HS–SPME GC–MS during fermentation and chocolate samples of four hybrids (CEPEC2004, PH15, PS1319 and SJ02) are shown in Table 1. Third-six volatile compounds were detected by HS–SPME GC–MS at the beginning (0 h) of CEPEC2004 fermentation; while 41 volatile compounds were found at 144 h of its fermentation. For fermentation of PH15 hybrid, 35 compounds were identified at 0 h and 42 at the 144 h. The highest amounts of volatile compounds were found in the fermentation of PS1319 hybrid, 38 and 46 at 0 h and 144 h, respectively. Concerning the fermentation of SJ02 hybrid, 31 volatile compounds were detected at 0 h and 42 at the end of process.

Table 1. Volatile compounds identified by Headspace—Solid Phase Microextraction—Gas Chromatography Mass Spectrometry (HS–SPME GC–MS) during fermentation times (0h and 144h) and in chocolate samples, and the reference odor of each compound.

Compounds	Odor Description[a]	Fermentation time		Chocolate
		0h	144h	
Acids				
Acetic acid	Sour, astringent	CEPEC2004, PH15, PS1319	-	All hybrids
Caprinic acid		-	-	All hybrids
Caproic acid	Sweat, pungent	CEPEC2004, PH15, PS1319	All hybrids	All hybrids
Caprylic acid	Sweaty, fatty	-	All hybrids	All hybrids
Isobutyric acid	Rancid, butter, cheese	PH15, PS1319	All hybrids	All hybrids
Isovaleric acid	Sweat, rancid	PH15, PS1319	All hybrids	All hybrids
Phenylacetic acid		-	-	All hybrids
Propanoic acid		PS1319	All hybrids	All hybrids
Valeric acid	Sweat, rancid	-	PS1319	PS1319
Alcohols				
1-Butanol		PH15	-	-
1-Hexadecanol	Fruity, green	-	-	All hybrids
1-Pentanol		CEPEC2004, PH15, PS1319	CEPEC2004, PS1319, SJ02	-
1-Phenylethanol		All hybrids	All hybrids	-
2,3-Butanediol	Cocoa butter	CEPEC2004	CEPEC2004	CEPEC2004
2-Ethyl-1-hexanol		All hybrids	All hybrids	-
2-Heptanol	Sweet, citrusy	All hybrids	All hybrids	-
2-Hexanol	Fruity, green	All hybrids	All hybrids	-
2-Methyl-1-butanol	Malty, chocolate	All hybrids	All hybrids	CEPEC2004
2-Methyl-3-buten-2-ol		All hybrids	All hybrids	-
2-Nonanol		PS1319	PS1319	-
2-Octanol		PS1319	PS1319	-
2-Pentanol		All hybrids	All hybrids	-
2-Phenylethanol		All hybrids	All hybrids	-

Compounds	Odor Description[a]	Fermentation time		Chocolate
		0h	144h	
3-Methyl-1-butanol		All hybrids	All hybrids	PH15, SJ02, CEPEC2004
4-Methyl-1-pentanol		CEPEC2004, PS1319	CEPEC2004, PS1319	-
Benzyl alcohol	Sweet, flower	All hybrids	All hybrids	-
Ethyl alcohol		All hybrids	All hybrids	All hybrids
Furfuryl alcohol		-	-	All hybrids
Guaiacol	Smoke, sweet	-	PH15	-
Isobutyl alcohol		All hybrids	All hybrids	-
Phenethyl alcohol	Honey, rose, caramel	-	-	All hybrids
α-Terpineol		-	PS1319	-
Aldehydes and Ketones				
(E)-2-butenal		CEPEC2004, PS1319	-	-
1-phenylethanone	Honey	All hybrids	All hybrids	-
2,3-Butanedione	Butter	PS1319	All hybrids	-
2-Furaldehyde		-	-	All hybrids
2-Heptadecanone		-	-	All hybrids
2-Heptanone		All hybrids	PH15, PS1319, SJ02	-
2-Hexanone		SJ02	-	-
2-Octanone		PS1319	PS1319	-
2-Pentanone	Fruit	All hybrids	All hybrids	-
2-Propanone		All hybrids	All hybrids	-
2-Pyrrolidone		-	-	All hybrids
2-Vinylfuran		All hybrids	All hybrids	-
3-Methyl-2(5H)-furanone		SJ02	-	-
3-Methylbutanal	Chocolate	All hybrids	All hybrids	-
5-Methyl-2-hexanone		All hybrids	PH15, PS1319, SJ02	-
5-Methyl-2-phenyl-2-hexenal		-	CEPEC2004, SJ02	-

Compounds	Odor Description[a]	Fermentation time		Chocolate
		0h	144h	
6,10,14-Trimethyl-2-pentadecanone		PS1319	-	-
Acetaldehyde	Sherry, nutty, apple	All hybrids	All hybrids	-
Acetoin	Butter, cream	CEPEC2004, PH15, SJ02	All hybrids	All hybrids
Benzaldehyde	Bitter	All hybrids	All hybrids	All hybrids
Benzeneacetaldehyde		All hybrids	All hybrids	-
Butyrolactone		-	-	All hybrids
Hexanal	Green, grass	CEPEC2004, PS1319	-	-
Pentanal		PH15, PS1319	All hybrids	-
Esters				
2-Pentyl acetate	Fruity	-	PH15, SJ02	-
2-phenylethyl isobutyrate		CEPEC2004, PH15, SJ02	All hybrids	-
Allyl acetate	Fruity, banana	-	All hybrids	-
Amyl phthalate		-	-	All hybrids
Dibutyl phthalate		-	-	All hybrids
Diisobutyl phthalate		-	-	All hybrids
Ehtyl acetate		PS1319	-	-
Ethyl benzoate		SJ02	PH15, SJ02	-
Ethyl caprate	Pear, grape	-	-	All hybrids
Ethyl caprylate	Fruity, flowery	-	All hybrids	-
Ethyl laurate	Fruity, floral	-	-	All hybrids
Ethyl myristate	Waxy, soapy	FA13, PH15	All hybrids	All hybrids
Ethyl palmitate	Waxy, green	-	-	All hybrids
Ethyl phenylacetate		CEPEC2004, PH15	All hybrids	-
Ethyl pyruvate		CEPEC2004, PH15, SJ02	All hybrids	All hybrids
Isoamyl acetate		-	All hybrids	-
Methyl palmitate		-	-	All hybrids
Phenylethyl acetate	fruity, sweet	-	-	All hybrids
Others*				

Compounds	Odor Description[a]	Fermentation time		Chocolate
		0h	144h	
2,3,5,6-Tetramethylpyrazine	Chocolate, coffee	-	-	All hybrids
2,3,5-Trimethylpyrazine	Cocoa, rusted nuts		-	All hybrids
2-acetyl Pyrrole	Chocolate, hazelnut	-	-	All hybrids
Linalool	Flower, lavender	CEPEC2004, PH15, SJ02	All hybrids	-

*Inclued: Pyrazines, pyrroles and terpenes
[a]Obtained from literature

Between hybrids, regarding to chocolates samples, many common compounds were detected. A total of 32 volatile compounds were detected on chocolates samples of CEPEC2004 and PS1319 hybrids, while 33 for PH15 and SJ02 chocolates. In all fermentations and chocolate samples, the following groups of compounds were found: organic acids, alcohols, aldehydes and ketones, esters and others (pyrazines, pyrroles and terpenoids). Considering the sequence, first 0 h of fermentation (Fig. 3A), second 144 h of fermentation (Fig. 3B) and finally chocolate samples (Fig. 3C), percentages of volatile compounds of the acids and esters groups increased, while amount of alcohols and aldehyde and ketones decreased for all samples. Alcohols, aldehydes and ketones were most found at the beginning (Fig. 3A) of fermentation process, while acids and esters were most predominant in the chocolate samples (Fig. 3C).

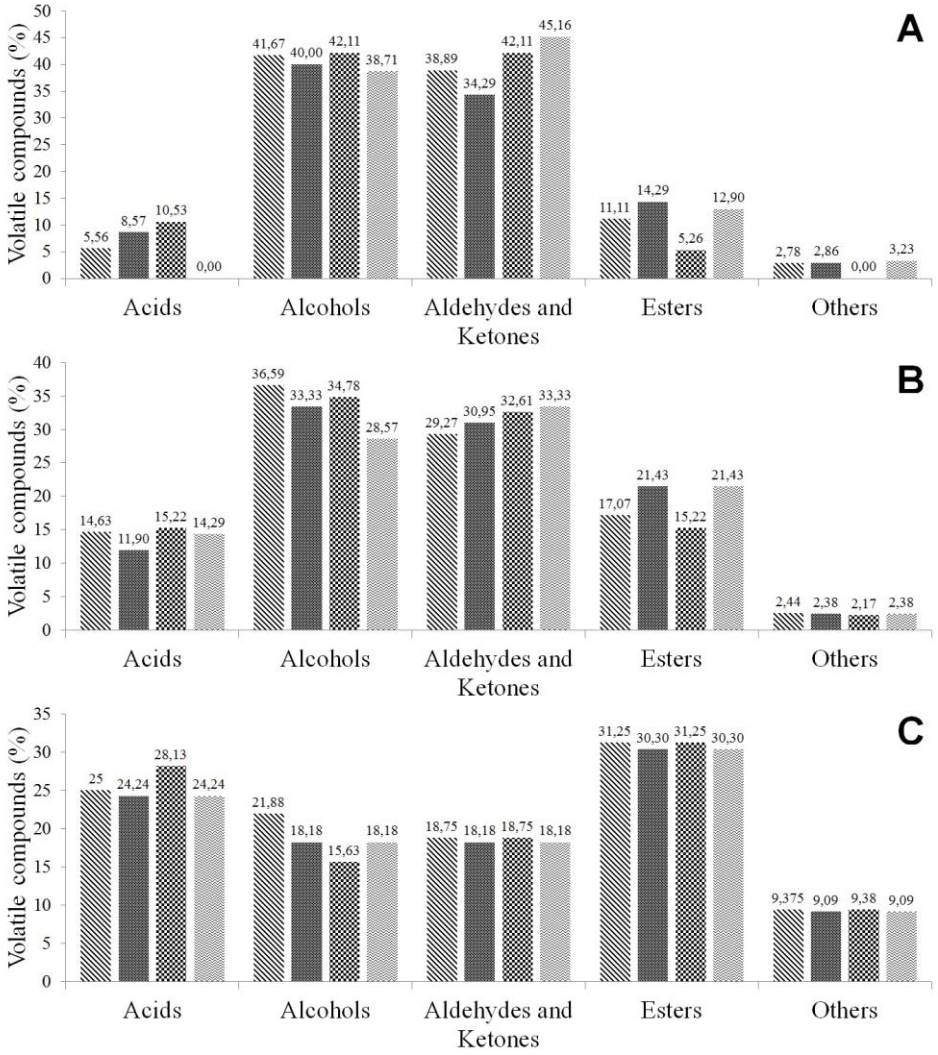


Fig. 3. Profile of volatile compounds identified by HS-SPME GC-MS during fermentation of CEPEC2004 (▨), PH15 (▩), PS1319 (▩), and SJ02 (▨), and in the chocolate samples. Fermentation times: 0h (A) and 144h (B). Chocolate samples (C). Total of compounds: CEPEC2004 0h (36),CEPEC2004 144h (41),CEPEC2004 Ch (32), PH15 0h (35), PH15 144h (42), PH15 Ch (33), PS1319 0h (38), PS1319 144h (46), PS1319 Ch (32), SJ02 0h (31), SJ02 144h (42), SJ02 Ch (33).

3.2.2. Protein degradation kinetics and clustering by MALDI-TOF analysis results

After extraction, proteins were quantified by Bradford's method and results are presented in Table 2. The amount of proteins decreased during the fermentation time for all hybrids studied. The highest difference was observed for PS1319 hybrid, i.e. 0.49 mg of protein per mL of extract, while lowest was 0.21 mg/mL of extract to the SJ02 hybrid.

Table 2. Results of proteins quantification by the Bradford's method of each hybrid (0h and 144h of fermentation).

Samples	mg/mL of extract
CEPEC2004 0h	0.80±0.02
CEPEC2004 144h	0.41±0.08
PH15 0h	0.70±0.05
PH15 144h	0.37±0.05
PS1319 0h	0.61±0.01
PS1319 144h	0.12±0.01
SJ02 0h	0.58±0.07
SJ02 144h	0.37±0.02

Analyze of Peak List Quality Control, performed in Mass-Up software, allowed identify a minimum molecular mass (m/z) and a maximum molecular mass (m/z) for each sample, and moreover shows the total of peaks after treatment of the spectra obtained by MALDI-TOF MS analysis. Results are presented in the Table 3.

Table 3. Minimum and maximum molecular masses, and number of mass peaks found in each hybrid at beginning and end of fermentation.

Samples	Min. Mol. Mass (m/z)		Max. Mol. Mass (m/z)		N.º of mass peaks	
	0 h	144 h	0 h	144 h	0 h	144 h
CEPEC2004	1968.749	1980.906	9697.598	12078.339	41	50
PH15	1970.005	1980.906	9864.243	11864.830	52	54
PS1319	1968.330	1980.067	13242.174	11598.089	62	59
SJ02	1968.749	1980.067	11517.646	12076.257	63	59

A total of 144 distinct molecular mass peaks (m/z) were observed in the different MALDI-TOF mass spectra. Four common peaks were found in all spectra of fermentations samples, m/z 2515.385, 2570.638, 4194.994, 9537.834. Sample from SJ02 at the beginning of fermentation showed the highest number of peaks (63), while the sample CEPEC2004 at 0 h showed the lowest number of peaks (41) (Table 3). Peaks with molecular mass values of m/z 4635.140 and 5821.460 were found only at the beginning of all fermentations (CEPEC2004, PH15, PS1319 and SJ02), while mass values m/z 2818.639 and 9698.426 appeared only at the end of fermentation process for all studied hybrids.

The presence and absence of peaks indicate variability between the two times of fermentation process and among the different hybrids. Figure 4 shows the clustering by similarity of the samples in case.

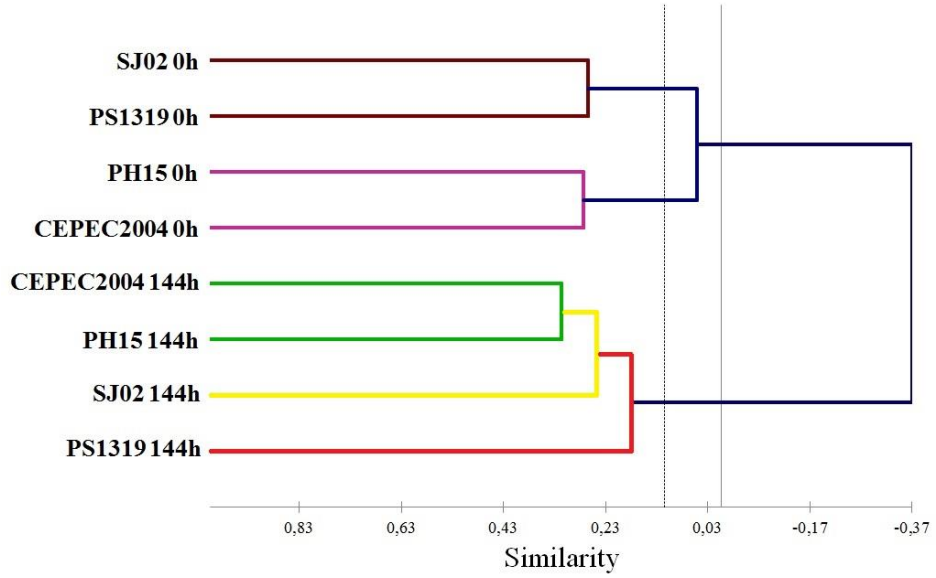


Fig. 4 Agglomerative hierarchical clustering by presence and absence of peaks with differents m/z values.

3.3. Sensorial analysis

The results of sensory analyzes of the chocolates produced from each hybrid are shown in Table 4 and Fig. 5. There were significant differences ($p < 0.5$) in acceptance between the four samples of chocolate. Chocolate from CEPEC2004 hybrid was the most appreciated (score 7.29), while chocolate from PS1319 hybrid was the less appreciated (score 6.55) by consumers.

Table 4. Results of the acceptance test for the chocolate samples of each cocoa hybrid.

Chocolates samples	Acceptance test
CEPEC2004	7.29[a]
PH15	6.90[b]
PS1319	6.55[c]
SJ02	7.06[b]

Values followed by the same letter in the same row are not different at the 5% level of significance by Tukey's test.

Answers to the CATA questions highlighted that the main parameters used to describe chocolates were bitter, sweetness, coffee and nutty (Fig. 5). Chocolates from CEPEC2004 and SJ02 hybrids were described with a bitter taste, although chocolate of CEPEC2004 was sweeter than SJ02 chocolate. Fermentation of PH15 hybrid resulted in a chocolate with higher values of sweet and nut tastes. Chocolates produced by fermentation of PS1319 hybrid were related to coffee and fruit taste, however, a sour taste was reported by the consumers (Fig. 5).

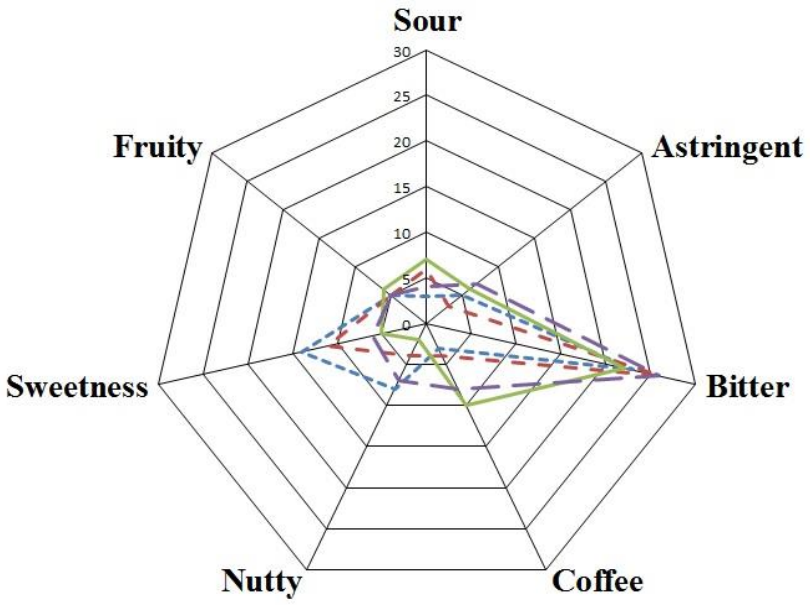


Fig. 5. Flavor profiles of the chocolates produced from four different hybrids [CEPEC 2004 (— —), PH15 (- - -), PS1319 (——), and SJ02 (—·)]. The center of the diagram corresponds to the lowest flavor intensity and the perimeter to the highest flavor intensity.

4. Discussion

As a way to recover the Brazilian cocoa productivity, disease-resistant hybrids has been developed by farmers and research centers over the last years (Moreira et al., 2016). In order to overcome the lower quality of chocolate produced with fruits of hybrids cocoa plants a better knowledge about fermentation process and chocolates production from these new hybrids is essential. In the present study fruits of four different hybrids cocoa (CEPEC 2004, PH15, PS1319 and SJ02) were selected and evaluated according to the volatile compounds present in both a) during the cocoa fermentation and b) on final chocolates samples. Moreover,

qualitative protein profile during cocoa fermentation and sensorial characterization of chocolate produced from the fermented beans were assessed.

According to the CATA results, chocolate from CEPEC2004 hybrid was correlated with sweet and bitter taste, furthermore it was the most accepted by judges (score 7,29) (Fig. 5 and Table 4). These characteristics coincide with results for volatile compounds analysis presented in Table 1 and Fig. 3. Samples of CEPEC2004 fermentation showed a greater number of alcohols compounds which confers desirable flavor, such as 2,3-butanediol (cocoa butter flavor) and 2-methyl-1-butanol (malty and chocolate flavor) found only in this hybrid (Table 1).

Esters are correlated to fruity flavors, while acids are considered undesirable compounds which confer unpleasant odors (e.g. sweat, pungent, fatty and rancid flavors) (Luna, Crouzillat, Cirou, & Bucheli, 2002; Frauendorfer & Schieberle, 2008). Figure 3 shows that in the fermentation process the amount of esters was greater than organic acids. An exception for this was observed on the fermentation of PS1319 hybrid, which presented more acids than esters at the beginning and equal values of these compounds at the end of fermentation. Furthermore, valeric acid, which is correlated with odors description of sweat and rancid, was only found in PS1319 samples (144 h and chocolate) (Table 1).

Regarding to chocolates produced from fermented beans of the PS1319 hybrid, amounts of organic acids were greater than alcohols (Fig. 3C), this fact explains both the lower score (6.55) in acceptance test

(Table 4) and sour taste reported by judges in CATA question analysis (Fig. 5).

The formation of chocolate flavor is complex, more than 500 nonvolatile and volatile compounds contributing to chocolate characteristic flavors (Serra-Bonvehí, 2005; Afoakwa, Paterson, Fowler, & Ryan, 2008; Rodriguez-Campos et al., 2012; Ho, Zhao, & Fleet, 2014). It is not surprise that different hybrids have a distinct fermentation dynamic (e.g. microbial diversity and volatile and non-volatile compounds profiles) and, consequently, lead to chocolates with different flavor sensations (Clapperton et al. 1994; Efraim et al. 2013; Moreira et al., 2013; Ramos et al. 2014). This hypothesis was also confirmed in this study.

Afoakwa et al. (2008) stated compounds responsible for flavor formation in cocoa beans and final chocolate have a various generation sources, such as: intrinsic to the bean (genetic origin, growth location, climatic conditions), post-harvest treatment (mainly fermentation and drying processes) and roasting and coaching processes (Maillard and other reactions) during chocolate manufacture (Misnawi, Jinap, Jamilah, & Nazamid, 2004). Furthermore, Afoakwa et al. (2008) indicate that pyrazines components are important to flavor and found mainly in chocolates. Pyrazines detection was very low in this study for all hybrids, precisely two compounds, 2,3,5,6-tetramethylpyrazine and 2,3,5-trimethylpyrazine (Table 1). Recently, Visintin et al. (2017) also observed few pyrazines in chocolates from Brazilian cocoa. Authors stated presence of pyrazines can be influenced by parameters such as weather condition, ripeness of pod, varieties of cocoa, and chocolate processing.

During the fermentation of CEPEC2004, PH15, PS1319 and SJ02 hybrids the total amount of proteins decreased from the beginning to the end. Overall, a drop of 51.25% (CEPEC2004), 47.14% (PH15), 80.32% (PS1319), and 36.20% (SJ02) in the protein concentration in each case was observed. Hue et al. (2016) evaluated the impact of fermentation on nitrogenous compounds of cocoa beans and reported the same results regard to protein content. When the seed embryo is killed by acidification of the pulp and increased temperature, as microbial activities consequence, proteolytic processes start (Schwan & Wheals, 2004). An aspartic endoprotease and a carboxypeptidase degrade the most protein content into the beans which is mainly composed of two proteins: albumin and vicilin (7S)-class globulin (Voigt & Biehl, 1993; Voigt et al., 1994; Voigt & Biehl, 1995; Voigt & Lieberei, 2014).

Furthermore, proteolytic processes into the fermented cocoa beans generate smaller peptides and free amino acids which contribute for formation of cocoa-specific flavor precursors (Marseglia et al., 2014; Hue et al., 2016). The clustering by presence and absence of peaks (different values of m/z) obtained by clustering the MALDI-TOF spectra (Fig. 4) showed that initial fermentation time samples are different from final time samples and also among hybrids. Moreover, regarding to the final fermentation time (144 h) CEPEC2004 and PH15 hybrids were similar between themselves, while PS1319 was different from the other hybrids (Fig. 4). This data is in accordance with results obtained in the sensorial analyzes, chocolates produced from PS1319 hybrid were less accepted presenting particular sensory characteristics which influenced the judges' decision, such as sweet and nut tastes.

5. Conclusions

Different volatile compounds were identified in the fermented beans of CEPEC2004, PH15, PS1319 and SJ02 hybrids and in the chocolate produced with these beans. The different cocoa hybrid CEPEC2004, PH15, PS1319 and SJ02 showed different chemical compositions regarding to the volatile compounds and protein profile observed during fermentation, which consequently influence the chocolates sensory perception.

Chocolate from CEPEC2004 was the most accepted by judges and correlated with sweet and bitter taste, mainly because of the presence of desired flavor compounds, such as 2,3-butanediol and 2-Methyl-1-butanol in chocolate samples from this hybrid. In contrast, a higher concentration of undesirable compounds, such as organic acids, was observed in chocolates samples from PS1319 hybrid. Chocolates samples from PS1319 hybrid generated lower acceptance by judges. In addition, MALDI-TOF MS analysis showed that during fermentation the protein profile was different among the hybrids, which indicates this kind of compound also contributes to the cocoa-specific flavor.

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References

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

Batista, N. N., Ramos, C. L., Ribeiro, D. D., Pinheiro, A. C. M., & Schwan, R. F. (2015). Dynamic behavior of *Saccharomyces cerevisiae*, *Pichia kluyveri* and *Hanseniaspora uvarum* during spontaneous and inoculated cocoa fermentations and their effect on sensory characteristics of chocolate. *LWT Food Science and Technology, 63*, 221–227.

Clapperton, J. F., Lockwood, R., Yow, S. T. K., & Lim, D. H. K. (1994). Effects of planting materials on flavour. *Cocoa Growers' Bulletin, 48*, 47–63.

Crafack, M., Keul, H., Eskildsen, C. E., Petersen, M. A., Saerens, S., Blennow, A., Skovman-Larsen, M., Swiegers, J. H., Petersen, G. B., Heimdal, H., & Nielsen, D.S. (2014). Impact of starter cultures and fermentation techniques on the volatile aroma and sensory profile of chocolate. *Food Research International, 63*, 306–316.

Efraim, P., Pires, J. L., Garcia, A. O., Grimaldi, R., Luccas, V., & Pezoa-Garcia, N. H. (2013). Characteristics of cocoa butter and chocolates

obtained from cocoa varieties grown in Bahia, Brazil. *European Food Research and Technology, 237,* 419–428.

Frauendorfer, F., Schieberle, P. (2008). Changes in key aroma compounds of Criollo cocoa beans during roasting. *Journal of Agricultural and Food Chemistry, 56*, 10244–10251.

Freire, E. S., Schwan, R. F., Mororó, R. C. (1999). The cocoa pulp agroindustry and the use of its residues in Bahia: Progress achieved in the last ten years. *Proceeding of the 12th International Cocoa Research Conference* (pp. 1013–1020). Kuala Lumpur: Cocoa Producers Alliance.

Ho, V., Zhao, J., & Fleet, G. (2014). Yeasts are essential for cocoa bean fermentation. *International Journal of Food Microbiology, 174*, 72–87.

Hue, C., Gunata, Z., Breysse, A., Davrieux, F., Boulanger, R., & Sauvage, F. X. (2016). Impacto f fermentation on nitrogenous compounds of cocoa beans (Theobroma cacao L.) from various origins. *Food Chemistry, 192*, 958-964.

Lima-Neto, R., Santos, C., Lima, N., Sampaio, P., Pais, C., & Neves, R. P. (2014) Application of MALDI-TOF MS for requalification of a *Candida* clinical isolates culture collection. *Brazilian Journal of Microbiology, 45*, 515-22.

Lopes, U. V., Monteiro, W. R., Pires, J. L., Clement, D., Yamada, M. M., & Gramacho, K. P. (2011). Cacao breeding in Bahia, Brazil: strategies and results. *Crop Breeding and Applied Biotechnology, 11*, 73-81.

Lopes, U. V., & Pires, J. L. (2014). Botany and production of cocoa. In R.F. Schwan & G.H. Fleet (Eds), *Cocoa and Coffee Fermentation* (pp. 43–71). Boca Raton, FL: CRC Press.

López-Fernández, H., Santos, H. M., Capelo, J. L., Fdez-Riverola, F., Glez-Peña, D., & Reboiro-Jato, M. (2015). Mass-Up: an all-in-one open software application for MALDI-TOF mass spectrometry knowledge discovery. *BMC Bioinformatics, 16*, 1-12.

Luna, F., Crouzillat, D., Cirou, L., & Bucheli, P. (2002). Chemical Composition and Flavor of Ecuadorian Cocoa Liquor. *Journal of Agricultural and Food Chemistry, 50*, 3527–3532.

Marseglia, A., Sforza, S., Faccini, A., Bencivenni, M., Palla, G., & Caligiani. (2014). Extraction, identification and semi-quantification of oligopeptides in cocoa beans. *Food Research International, 63*, 382-389.

Misnawi, Jinap, S., Jamilah, B., & Nazamid, S. (2004) Sensory properties of cocoa liquor as affected by polyphenol concentration and duration of roasting. *Food Quality and Preference, 15,* 403-409.

Moreira, I. M. V., Miguel, M. G. C. P., Duarte, W. F., Dias, D. R., & Schwan, R.F. (2013). Microbial succession and the dynamics of metabolites and sugars during the fermentation of three different cocoa (*Theobroma cacao* L.) hybrids. *Food Research International, 54*, 9–17.

Moreira, I. M. V., Miguel, M. G. C. P., Ramos, C. L., Duarte, W. F., Efraim, P., & Schwan, R. F. (2016). Influence of cocoa hybrids on volatile compounds of fermented beans, microbial diversity during

fermentation and sensory characteristics and acceptance of chocolates. *Journal of Food Quality, 39*, 839-849.

Moreira, I. M. V., Vilela, L. F., Miguel, M. G. C. P., Santos, C., Lima, & Schwan, R. F. (2017). Impact of a Microbial Cocktail Used as a Starter Culture on Cocoa Fermentation and Chocolate Flavor. *Molecules, 22*, 766.

Oliveira, M., Santos, C., Sampaio, P., Romeo, O., Almeida-Paes, R., Pais, C., Lima, N., & Zancopé-Oliveira, R. M. (2015). Development and optimization of a new MALDI-TOF protocol for identification of the *Sporothrix* species complex. *Research Microbiology, 166*, 102-110.

Passarini, M. R., Santos, C., Lima, N., Berlinck, R. G., & Sette, L. D. (2013). Filamentous fungi from the Atlantic marine sponge *Dragmacidon reticulatum*. *Archives of Microbiology, 195*, 99-111.

Ramos, C. L., Dias, D. R., Miguel, M. G. C. P., & Schwan, R. F. (2014). Impact of different cocoa hybrids (*Theobroma cacao* L.) and *S. cerevisiae* UFLA CA11 inoculation on microbial communities and volatile compounds of cocoa fermentation. *Food Research International, 64*, 908–918.

Rodriguez-Campos, J., Escalona-Buendía, H. B., ORozco-Avila, I., Lugo-Cervantes, E., & Jaramillo-Flores, M. E. (2011). Dynamics of volatile and non-volatile compounds in cocoa (*Theobroma cocoa* L.) during fermentation and drying process using principal components analysis. *Food Research International, 44*, 250–258.

Santos, C.; Ventura, J.A.; Costa, H.; Fernandes, P.M.B.; & Lima, N. (2015). MALDI-TOF MS to identify the pineapple pathogen

Fusarium guttiforme and its antagonist *Trichoderma asperellum* on decayed pineapple. *Tropical Plant Pathology, 40*, 227-232.

Santos, C.; Ventura, J. A.; & Lima, N. (2016). New insights for diagnosis of pineapple fusariosis by MALDI-TOF MS technique. *Current Microbiology, 73*, 206-213.

Serra-Bonvehí, J. (2005). Investigation of aromatic compounds in roasted cocoa powder. *European Food Research and Technology, 221*, 19–29.

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

Stone, H.S., & Sidel, J.L., (1993). *Sensory Evaluation Practices* (2nd ed.). California: Academic Press.

Visintin, S., Ramos, L., Batista, N., Dolci, P., Schwan, F., & Cocolin, L. (2017). Impact of Saccharomyces cerevisiae and Torulaspora delbrueckii starter cultures on cocoa beans fermentation. *International Journal of Food Microbiology, 257*, 31-40.

Voigt, J., & Biehl, B. (1993). The major seed proteins of *Theobroma cacao* L. *Food Chemistry, 47*, 145-151.

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

Voigt, J., & Biehl, B. (1995). Precursors of the cocoa specific aroma components are derived from vicilin-class (7S) globulin of the cocoa seed by proteolytic processing. *Botanica Acta, 108*, 283-289.

Voigt, J., Lieberei, R. (2014). Biochemistry of cocoa fermentation. In R.F. Schwan & G.H. Fleet (Eds), *Cocoa and Coffee Fermentation* (pp. 193–227), Boca Raton, FL: CRC Press.

ARTIGO 2 - INFLUENCE OF *SACCHAROMYCES CEREVISIAE* AND *PICHIA KLUYVERI* AS STARTER CULTURES ON CHOCOLATE FLAVOR FROM DIFFERENT COCOA HYBRIDS

Artigo nas normas da revista *Food Research International*

Abstract

Cocoa fermentation is a very important step in the chocolate chain supply. Therefore, the aim of this study was evaluated the impact of *Saccharomyces cerevisiae* and *Pichia Kluyveri* as starter culture on cocoa fermentation of two hybrids (CEPEC2002 and FA13) cultivated in Brazil. For this, physico-chemical changes (volatile compounds and protein profile) during the fermentation process were evaluated, and the chocolates by sensory analyses and volatile compound identification. A total of 80 volatile compounds were identified by HS–SPME GC-MS analysis, chocolate FA13 inoculated with *S. cerevisiae* contained higher number the acids identified, and was considered more sour than chocolate produced from FA13 spontaneous fermentation. CEPEC2002 inoculated with *S. cerevisiae* in co-culture with *P. Kluyveri* generated a chocolate less sour and sweeter chocolate than spontaneous fermentation. Both chocolates from inoculated assays were more accepted by judges. MALDI-TOF analysis proved that protein profiles changed during fermentation and also were influenced by the inoculation process.

Keywords: cocoa fermentation; GC-MS; MALDI-TOF; protein profile; co-culture

1. Introduction

The main raw material for chocolate production is the fermented cocoa beans. Many years ago, Rohan (1964) proved that cocoa flavors are development only from fermented cocoa beans. Unfermented beans are

bitter and astringent, moreover, do not generate the cocoa-specific flavor during chocolate manufacture, mainly in the roasting and conching steps (Schwan and Wheals, 2004; Schwan and Fleet, 2014). Cocoa fermentation, in all producers countries, is conducted spontaneously by action of natural contaminants (microorganisms) of the process (Schwan et al., 2014). Yeasts, lactic acid bacteria and acetic acid bacteria are the main microbial groups found during fermentation. Yeasts species dominate early stages and, subsequently, are surpassed by lactic acid bacteria and acetic acid bacteria (Schwan and Wheals, 2004). The yeasts most commonly isolated from cocoa fermentation around the world belong to the genus *Saccharomyces*, *Pichia*, *Hanseniaspora* (anamorph *Kloeckera*), and *Candida* (Ardhana and Fleet, 2003; Jespersen et al., 2005; Nielsen et al., 2007; Pereira et al., 2012; Moreira et al., 2013, 2016). Yeasts play an important role in the process, absence of these microorganisms generate an acidic chocolate lacking the specifics characteristic aromas (Ho et al., 2014).

Controlled fermentative processes, mainly using starter cultures, can design standardize products with high quality. Inoculation in the cocoa beans fermentation it is not recent, but until now a microorganisms cocktail suitable for application has not been defined and marketed (Schwan et al., 2014). Many recent studies reported different yeasts species influence fermentation process, consequently chocolate flavor. *Saccharomyces cerevisiae* and *Pichia kluyveri* have been applied as starter cultures on cocoa fermentation, alone (Ramos et al., 2014) in the process or in co-culture with other yeasts (e.g. *Hanseniaspora uvarum* and *Torulaspora delbrueckii*) (Batista et al., 2015; Visintin et al., 2017) or

bacteria species (e.g. *Lactobacillus plantarum* and *Acetobacter pasteurianus*) (Moreira et al., 2017).

Well-fermented cocoa beans produce chocolate with quality. In chapter 5 of the book Cocoa and Coffe Fermentations, Voigt and Lieberei (2014), reviewed the biochemistry of cocoa fermentation and showed that proteolytic reactions inside the beans, during fermentation, are responsible by release small peptides and free amino acids considered flavor precursors of the final product (chocolate). Furthermore, different volatile compounds, such as alcohols, aldehydes, ketones, esters, and carboxylic acids, are produced during fermentation, which also influence chocolates sensory attributes (Rodriguez-Campos et al., 2011; Afoakwa et al., 2008).

As describe above, this study aimed to inoculate two yeast species (*Saccharomyces cerevisiae* and *Pichia kluyveri*) during cocoa fermentations of two different hybrids (CEPEC2002 and FA13). To assess the inoculation impact, physico-chemical changes (volatile compounds and protein profile) during the fermentation process were evaluated, as well as the volatile compounds and sensory analyses of chocolate.

2. Materials and Methods

2.1. Fermentation experiments, inoculation and sampling

The fermentation experiments were conducted at the Vale do Juliana cocoa farm in Igrapiúna, Bahia, Brazil. The ripe cocoa pods from

CEPEC2002 and FA13 hybrids were harvested during the main crop of 2014 (September–December). The cocoa pods were manually opened with a machete, and the beans were immediately transferred to the fermentation house. Fermentation process was performed in 0.06 m3 wooden boxes with approximately 100 Kg of cocoa beans for each box. Fermentations of the hybrids were performed with inoculation of CEPEC2002 with *Saccharomyces cerevisiae* UFLA CCMA 0200 (LNF-CA11, LNF Latino America, Bento Gonçalves, Rio Grande do Sul, Brazil) in co-culture with *Pichia Kluyveri* UFLA CCMA 0237 and FA13 with S. cerevisiae UFLA CCMA 0200 as inoculum. Four fermentations were conducted: CEPEC2002 SP, CEPEC2002 (spontaneous fermentation - control), FA13 S and FA13 (spontaneous fermentation - control).

The two yeasts strains used in the study are preserved at the Culture Collection of Agricultural Microbiology of the Federal University of Lavras (CCMA, Lavras, Minas Gerais, Brazil, WDCM 1083). *S. cerevisiae* UFLA CCMA 0200, which is commercialized by LNF (CA11), was weighed (as recommended by the manufacturer's instructions) and mixed in the solution to reach a population of approximately 107 cells/g of cocoa. *P. Kluyveri* was grown in YPD broth [10 g/L Yeast extract (Merck); 20 g/L Peptone (Himedia); 20 g/ L dextrose (Merck)] at 30 C and 150 rpm, and replicated every 24 h. The cells were recovered by centrifugation (7000 rpm, 10 min) and re-suspended in 1 L of sterile peptone water [1 g/L Peptone (Himedia)]. This solution was spread over the cocoa beans, reaching a concentration of approximately 10^5 cells/g of cocoa. These yeasts were reported in

previous studies on cocoa fermentation around the world, mainly in Brazil (Ardhana and Fleet, 2003; Camu et al., 2008; Jespersen et al., 2005; Pereira et al., 2012; Moreira et al., 2013; Visintin et al., 2016).

The samples were taken every 24 h during 144 h of fermentation, placed in sterile plastic pots and stored at -20 ºC. The fermentations were performed in triplicate (Moreira et al., 2017).

2.2. Physicochemical analysis

2.2.1. Volatile Compounds analysis by Headspace-Solid Phase Microextraction Gas Chromatography-Mass Spectrometry

Volatile compounds from cocoa samples were extracted using the Headspace-Solid Phase Microextraction (HS–SPME) technique, as described by Rodriguez-Campos et al. (2011). Cocoa beans samples (2.0 g) from the beginning and end of fermentation (0 h and 144 h) and chocolate samples (2.0 g) were macerated using liquid nitrogen for headspace analysis.

The samples were transferred to a sealed vial and a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 mm SPME fiber (Supelco Co., Bellefonte, PA, USA.) was used to extract volatile compounds. The fiber was balanced for 15 min at 60 °C and then exposed to the samples for 30 min at the same temperature.

The compounds were analyzed using a Shimadzu GC model QP2010 equipped with a mass spectrometry and a capillary column of silica OV Carbowax 20M (0,25 □m X 0.25 □m X 30 m). The

temperature program began with 5 min at 40 ºC, followed by a gradient of 40 ºC to 200 ºC at 10 ºC/min; the temperature was then maintained at 200 ºC for 20 min. The injector and detector temperatures were maintained at 230 ºC. The carrier gas (He) was used at a flow rate of 1.4 mL/min. Injections were performed by fiber exposition for 2 min. Volatile compounds were identified by comparing the mass spectra of the compounds in the samples with the database of the National Institute of Standards and Technology (NIST library, Gaithersburg, MD, USA) and the retention time with literature data using the n-Alkane index. All samples were examined in duplicate.

2.2.2 Extractions and analysis of protein profile by Matrix Assisted Laser Desorption Ionization Time of flight (MALDI-TOF)

In order that other compounds do not interfere in the proteins extraction, samples from the beginning and end (0 h and 144 h) of each fermentation were subjected to extraction of lipids and polyphenols.

2.2.2.1. Lipids extraction

Cocoa beans (6.0 g) were macerated using liquid nitrogen, in sequence were placed in papers cartridges for extraction of lipid part. A Soxhlet apparatus was used as proposed by Voigt and Biehl (1993). After 6h, the solvent [n-hexane (bp 68 ºC)] was evaporated, and recovered later in rotary evaporator.

2.2.2.2. Polyphenols extraction

Acetone was used to prepare a dried defatted cocoa beans powder and prevent polyphenols influence in analysis of protein profile (Hue et al., 2016). 3 g of each sample were extracted successively by 70% acetone, 80% acetone and 100% acetone using each time a volume of 60 mL (sufficient to cover the macerated). Acetone solutions were supplemented by 5 mM ascorbic acid. Mixtures were vortexed for 20 min and supernatant was discarded by centrifugation (12.000 rpm for 20 min at 4 ºC).

2.2.2.3. Proteins extraction and MALDI-TOF MS analysis

After the lipids and polyphenols extractions, the samples (0.5 g) were transformed into a fine powder with a mortar and pestle using liquid nitrogen. The fine powders were transferred to tubes containing 2 mL of organic solution (water/acetonitrile/trifluoro-acetic acid, 50:47.5:2.5) and were immediately and vigorously vortexed for 10 min, beads into the tubes were used to help in protein extraction. Tubes were centrifuged (9.000 rpm for 2 min at 4 ºC) and the proteins into supernatant were quantified by Bradford's method. Then, 1 □L of resulting suspension was transferred to the 96-well MALDI flex target plate (Bruker Daltonics, Bremen, Germany). When the liquid phase was almost evaporated 1 □L matrix solution [saturated solution of a-cyano-4-hydroxy-cinnamic acid (CHCA) in 50% acetonitrile/2.5% trifluoro-acetic acid] was added and the solution was gently mixed (Oliveira et al., 2015).

MALDI-TOF MS external calibration was conducted using as standard an *Escherichia coli* K12 colony, wich was obtained from the Public Portuguese Culture Collection University of Minho Library (MUM, www.micoteca.deb.uminho.pt). Cells of *E. coli* K12 were grown on Nutrient agar medium (Merck) at 34 ºC for 20 h. Briefly, approximately 1 μg of cellular material from a single colony was transferred to MALDI flex plate and CHCA matrix solution was added followed by gently mixing. Each protein extract was applied in triplicate to evaluate reproducibility. Samples were then analysed in a MALDI-TOF microflex LT spectrometer (Bruker Daltonics, Bremen, Germany), using the MALDI Biotyper 3.0 automatic system.

Raw data (specters in mzXML format) were preprocessed and analyzed using the software Mass-up following the protocol presented by López-Fernández et al. (2015). Mass-Up is an open-source mass spectrometry software for proteomics designed to support the preprocessing and analysis of MALDI-TOF mass spectrometry data. Treatment of data involved the following parameters: none for intensity transformation, smoothing by Savitzky-Golay method, baseline correction by Snip method, standardization by TIC (Total Ion Current) method and detection of peaks by Maldiquant method using signal-to-noise ratio (SNR) of 3. After preprocess and peak matching operations, analysis of "Peak List Quality Control" and "Biomarker Discovery" were performed to generate a list with m/z values of each sample and thus evaluate presence and absence of each peak in the spectrograms.

2.3. Sensorial analysis

The dried beans were sent for chocolate production at Sartori and Pedroso Alimentos Ltda. (São Roque, São Paulo, Brazil). The molded chocolate (70% cocoa) was rapped and stored at 4 ºC for four weeks before sensory analysis.

Samples made from controls and inoculated fermentations were evaluated using a consumer acceptance test followed by a check-all-that-apply (CATA) question. The tests were conducted on 71 adults over 18 years of age, 41% male and 59% female and were consumers of dark chocolate. For the acceptance test, the consumers evaluated how much they liked each sample using a 9-point hedonic scale (1 = dislike extremely and 9 = like extremely) (Stone and Sidel, 1993). For the CATA question, the consumers were asked to evaluate seven sensory attributes (sour, fruity, bitter, astringent, coffee, nutty and sweeteness) and select those they considered appropriate to describe the chocolate. The tests were performed as previously described by Batista et al. (2015).

2.4. Statistical analysis

Data obtained from acceptance test and proteins quantification were subjected to ANOVA. The means were compared using Tukey's test at a 5% level of significance. The analyses were carried out using the SISVAR 5.1 software (Federal University of Lavras, Department of Statistic, Lavras, MG, Brazil).

Agglomerative hierarchical clustering (AHC) was carried out using XLSTAT version 7.5.2. AHC graph was assembled with results of Biomarker Discovery analysis (presence and absence of peaks). Pearson Correlation coefficient was used for showing similarities among samples.

3. Results

3.1. Physical-chemical changes

During fermentation process, the temperature was evaluated, and results are presented in Fig. 1. The inoculated fermentation temperatures increased faster, at 72h of fermentation showed values greater than non-inoculated fermentations.

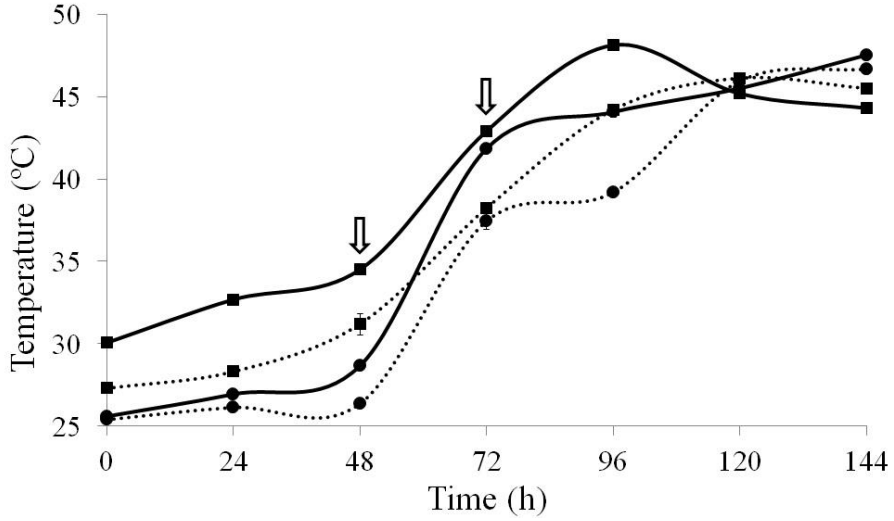


Fig. 1. Evolution of temperature during fermentation of different conditions. CEPEC2002 (●), FA13 (■). Inoculated (CEPEC2002 SP and FA13 S - continuous line) and non-inoculated (CEPEC2002 and FA13 - dotted line) fermentations. Mixing (⇩) of the mass was performed at 48 and 72h.

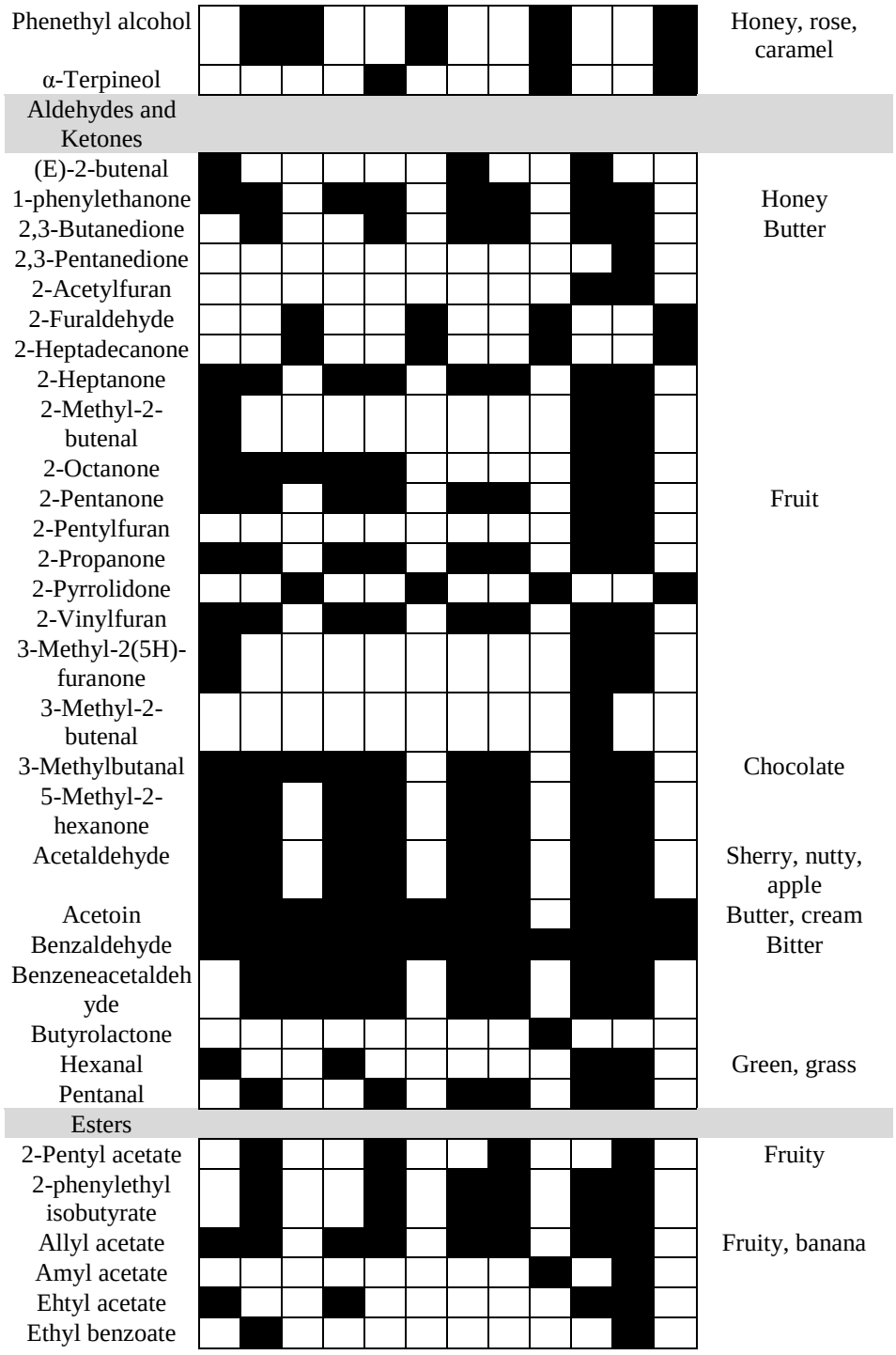
CEPEC200 SP fermentation temperature ranged from 25.6 °C at 0h to a maximum of 47.5 °C at 144 h. The temperature of CEPEC2002 spontaneous fermentation ranged from 25.4 to 46.7 °C at 144 h. FA13 inoculated fermentation (FA13 S) showed maximum temperature with 96h of fermentation (48.12 ºC). FA13 non-inoculated fermentation temperature ranged from 27.3 ºC to 45.5 ºC during the time, with maximum of 46.12 ºC at 120h of fermentation.

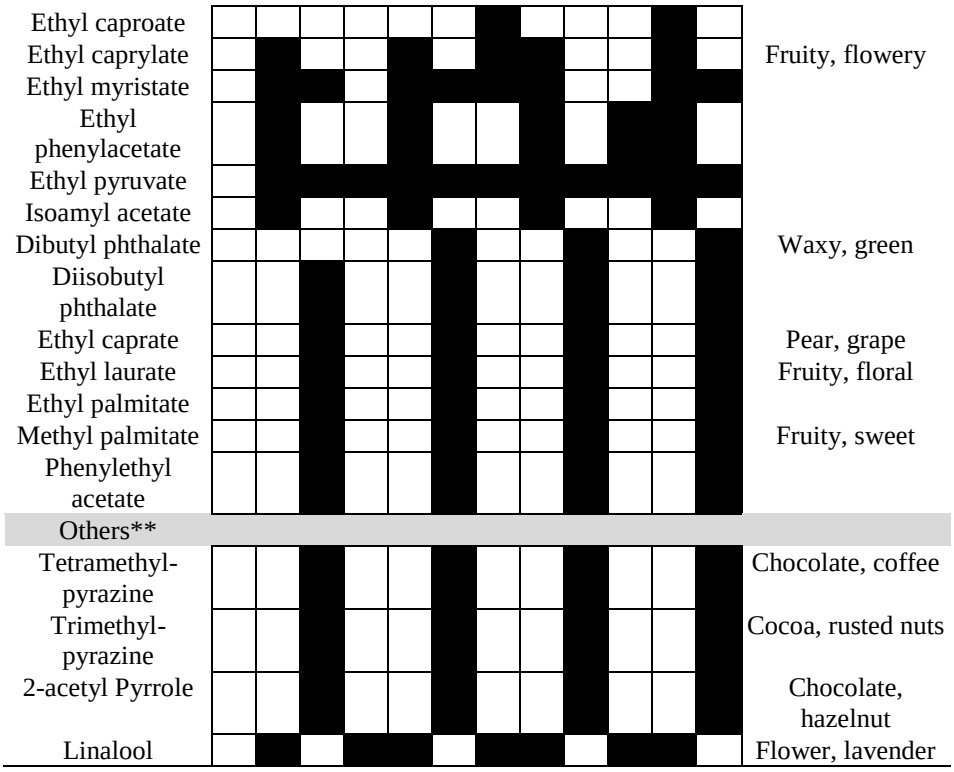
3.1.1. Volatile compounds

A total of 80 volatile compounds were identified by HS–SPME GC-MS analysis. Of these 80 compounds, 9 acids, 22 alcohols, 26 Aldehydes and Ketones, 13 esters and 4 other compounds (inclue: pyrazines, pyrroles and terpenes) were classified. Table 1 presents all the compounds identified (presence and absence) during fermentation and chocolate samples of spontaneous and inoculated fermentations.

Table 1. Volatile compounds identified by Headspace—Solid Phase Microextraction—Gas Chromatography Mass Spectrometry (HS–SPME GC–MS) during fermentation times (0h and 144h) and in chocolate samples, and the reference odor of each compound.

Compounds	Samples*												Odor Description[a]
	A^1	A^2	A^3	B^1	B^2	B^3	C^1	C^2	C^3	D^1	D^2	D^3	
Acids													
Acetic acid	■	□	■	□	□	■	■	□	■	□	■	■	Sour, astringent
Caproic acid	□	■	■	□	■	■	■	■	■	■	■	■	Sweat, pungent
Caprylic acid	□	■	■	□	■	■	□	■	■	□	■	■	Sweaty, fatty
Isobutyric acid	□	■	■	□	■	■	■	■	■	■	■	□	Rancid, butter, cheese
Isovaleric acid	■	■	■	■	■	■	■	■	■	□	■	■	Sweat, rancid
Propanoic acid	□	■	□	□	■	■	■	■	■	■	■	■	
Valeric acid	□	■	□	□	□	□	□	■	■	■	■	□	Sweat, acid, rancid
Caprinic acid	□	□	□	□	□	■	□	□	■	□	■	■	
Phenylacetic acid	□	□	■	□	□	■	□	□	■	□	□	■	
Alcohols													
1-Butanol	□	■	□	□	□	□	■	□	□	□	□	□	
1-Hexadecanol	□	□	□	□	□	■	□	□	■	□	□	□	Fruity, green
1-Pentanol	■	■	□	■	■	□	■	■	□	■	■	□	
1-Phenylethanol	■	■	□	■	■	□	■	■	□	■	■	□	
2,3-Butanediol	□	■	■	□	■	■	□	■	■	■	■	■	Cocoa butter
2-Ethyl-1-hexanol	■	■	□	■	■	□	■	■	□	■	■	□	
2-Heptanol	■	■	■	■	■	□	■	■	■	■	■	□	Sweet, citrusy
2-Hexanol	■	■	□	■	■	□	■	□	■	■	■	□	Fruity, green
2-Methyl-1-butanol	□	□	□	■	■	□	■	■	□	■	■	□	Malty, chocolate
2-Methyl-3-buten-2-ol	■	■	□	■	■	□	■	■	□	■	■	□	
2-Nonanol	□	□	□	□	□	□	□	□	□	■	■	□	
2-Pentanol	■	■	□	■	■	□	■	■	□	■	■	□	
2-Phenylethanol	■	■	□	■	■	□	■	■	□	■	■	□	
3-Methyl-1-butanol	■	■	■	■	■	■	■	■	■	■	■	■	
4-Methyl-1-pentanol	□	□	□	□	□	□	□	□	□	■	□	□	
Benzyl alcohol	■	■	□	■	■	□	■	■	□	■	■	□	Sweet, flower
Ethyl alcohol	■	■	■	■	■	□	■	■	■	■	■	□	
Furfuryl alcohol	□	□	■	□	□	□	□	□	■	■	■	□	
Guaiacol	□	■	■	□	■	□	□	■	■	■	■	□	Smoke, sweet
Isobutyl alcohol	■	■	□	■	■	□	■	■	□	□	■	□	


Phenethyl alcohol
Honey, rose, caramel
α-Terpineol
Aldehydes and Ketones
(E)-2-butenal
1-phenylethanone
Honey
2,3-Butanedione
Butter
2,3-Pentanedione
2-Acetylfuran
2-Furaldehyde
2-Heptadecanone
2-Heptanone
2-Methyl-2-butenal
2-Octanone
2-Pentanone
Fruit
2-Pentylfuran
2-Propanone
2-Pyrrolidone
2-Vinylfuran
3-Methyl-2(5H)-furanone
3-Methyl-2-butenal
3-Methylbutanal
Chocolate
5-Methyl-2-hexanone
Acetaldehyde
Sherry, nutty, apple
Acetoin
Butter, cream
Benzaldehyde
Bitter
Benzeneacetaldehyde
Butyrolactone
Hexanal
Green, grass
Pentanal
Esters
2-Pentyl acetate
Fruity
2-phenylethyl isobutyrate
Allyl acetate
Fruity, banana
Amyl acetate
Ehtyl acetate
Ethyl benzoate



*Samples: CEPEC2002 SP 0h (A^1); CEPEC2002 SP 144h (A^2); CEPEC2002 SP Ch (A^3); CEPEC2002 0h (B^1); CEPEC2002 144h (B^2); CEPEC2002 Ch (B^3); FA13 S 0h (C^1); FA13 S 144h (C^2); FA13 S Ch (C^3); FA13 0h (D^1); FA13 144h (D^2); FA13 Ch (D^3). **Inclued: Pyrazines, pyrroles and terpenes. [a]Obtained from literature.

At the beginning of fermentation, a total of 31 compounds were identified in both spontaneous and inoculated fermentations of CEPEC2002 hybrid. FA13 inoculated with S. cerevisiae presented 40 compounds at beginning, while in the FA13 control were found 48 compounds. The amount volatile compounds at the end of fermentation process (144h) increased for all samples, accurately 46 compounds for CEPEC2002 SP, 43 for CEPEC2002 control, 42 for FA 13 S and 58 for FA13 control. Concerning the chocolate samples, 29 compounds were

identified in spontaneous fermentations (CEPEC2002 and FA13), chocolates from CEPEC2002 inoculated with mix of yeasts showed 30 identified compounds and chocolates produced from FA13 inoculated presented a total of 35 volatile compounds.

Acids and esters groups gradually increased from fermentation process to chocolate for all samples, while alcohols and aldehydes and ketones decreased as shown in Fig. 2.

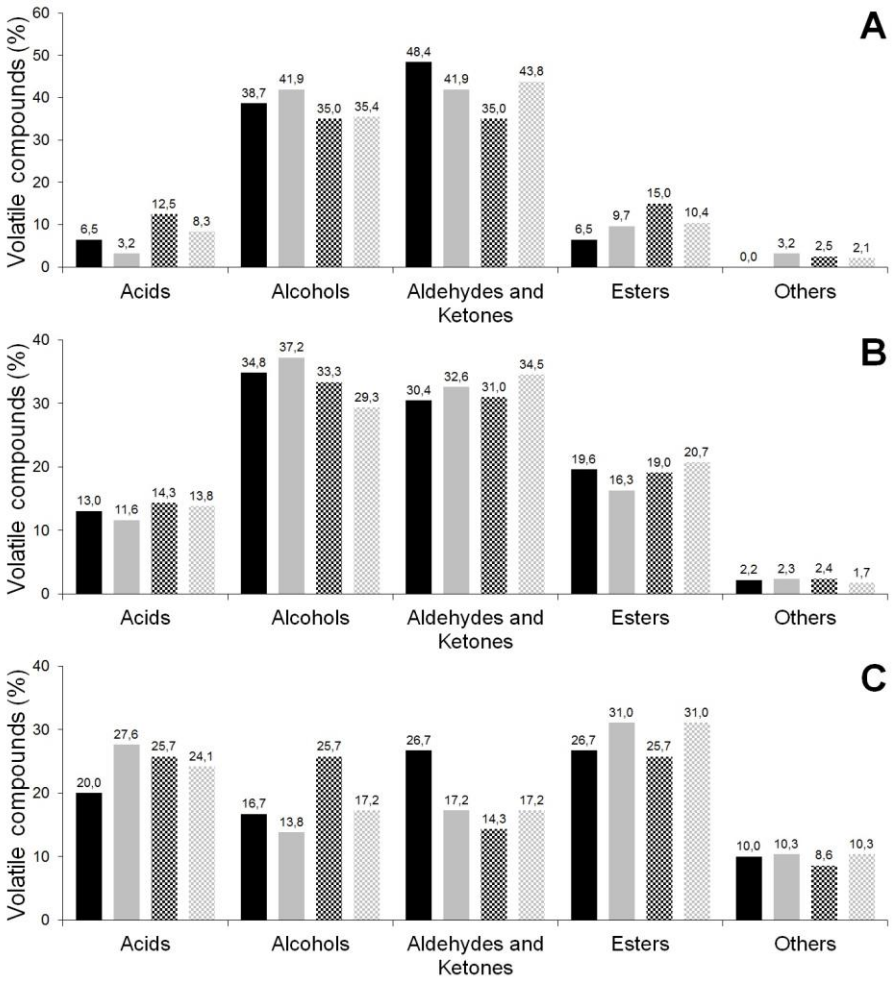


Fig. 2. Percentage of volatile compounds identified by HS-SPME GC-MS during fermentation and in the chocolates of CEPEC2002 SP (■), CEPEC2002 control (▒), FA13 S (▩) and FA13 control (▒).Fermentation times: 0h (A) and 144h (B). Chocolate samples (C).

3.1.2. Protein degradation kinetics and clustering by MALDI-TOF analysis results

Before MALDI-TOF analysis, the protein extracts were quantified by Bradford's method and the results are shown in the Table 2. For all fermentations, proteins amount decreased during the time. The greatest proteins degradation was observed in the CEPEC2002 spontaneous fermentation, a drop in 0.42 mg of protein per mL of extract.

Table 2. Proteins quantification by the Bradford's method of inoculated and non-inoculated fermentation at beginning and at the end of process.

Samples	mg/mL of extract
CEPEC2002 SP 0h	0,71±0,01
CEPEC2002 SP 144h	0,39±0,01
CEPEC2002 0h	0,76±0,05
CEPEC2002 144h	0,34±0,01
FA13 S 0h	0,73±0,00
FA13 S 144h	0,38±0,05
FA13 0h	0,64±0,03
FA13 144h	0,33±0,03

The treatments of the spectra obtained by MALDI-TOF analysis, allowed identify a minimum mass (m/z), a maximum mass (m/z), and total of peaks for each sample. Table 3 shows these results.

Table 3. Minimum and maximum mass (m/z), and total of peaks found at beginning and at the end of inoculated and spontaneous fermentations.

Samples	Min. Mass	Max. Mass	Total of peaks
CEPEC2002 SP 0h	1979.647	11861.734	60
CEPEC2002 SP 144h	2041.405	11859.670	54
CEPEC2002 0h	2026.938	11864.830	62
CEPEC2002 144h	1981.326	11867.925	56
FA13 S 0h	1980.487	12026.340	58
FA13 S 144h	2042.684	9701.327	35
FA13 0h	1970.004	12083.544	65
FA13 144h	1980.066	12077.297	57

The peak matching operation in Mass-up software generated a list with 131 peaks of different m/z values (data not shown). Of these 131 peaks, only three were commom among all samples (m/z 2818.716, 4195.56, and 9538.966). Peaks with mass values m/z 2723.138, 3127.244, and 3735.043 were found only in CEPEC2002 inoculated with *S. cerevisiae* and *P. Kluyveri*. Concerning to FA13 inoculated with *S. cerevisiae*, a greater number of peaks with different m/z values was particular to this sample: 2103.681, 2302.024, 2879.490, 2929.855, 3256.854, 3421.062, and 4797.277.

The Fig. 3 shows a clustering by similarity of the samples using data of presence and absence of the generated peak list in Mass-up software.

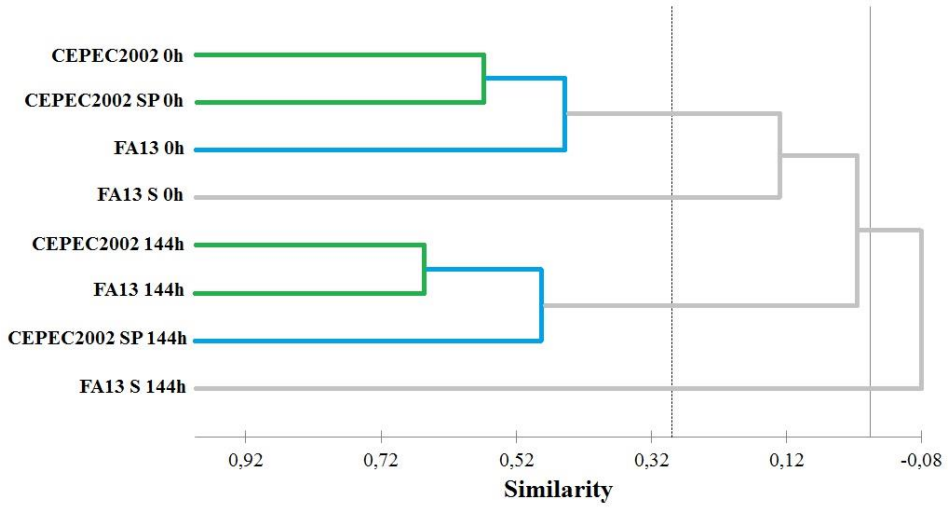


Fig. 3. Agglomerative hierarchical clustering by presence and absence of peaks with different m/z values.

3.2. Sensorial analysis

Both acceptance test and CATA question, allowed to differentiate between the chocolates produced from spontaneous and inoculated fermentation. Chocolates from inoculated fermentations were the most appreciated by judges, but there were no significant differences between the two samples ($p < 0.5$), as shows Table 4.

Table 4. Scores of the acceptance test for the chocolate samples of each fermentation, with and without inoculation.

Chocolates samples	Acceptance test
CEPEC2002 I	7,27[a]
CEPEC2002 NI	6,79[b]
FA13 I	7,32[a]
FA13 NI	6,83[b]

Values followed by the same letter in the same row are not different at the 5% level of significance by Tukey's test.

The parameters used in CATA question analysis to describe the chocolates were bitter, sweetness, coffee and nutty. On this, judges responded which attributes better describe the chocolates samples, and the results are showed in the Fig. 5.

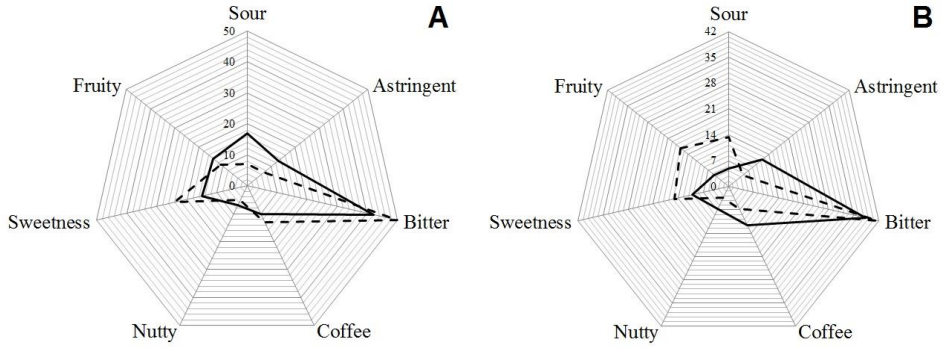


Fig. 4. Flavor profiles of the chocolates produced from cocoa beans spontaneously fermented (continuous line) and cocoa beans fermented with inoculations of yeasts starter culture (dotted line). CEPEC2002 (A) and FA13 (B). The center of the diagram corresponds to the lowest flavor intensity and the perimeter to the highest flavor intensity.

Concerning to fermentation of CEPEC2002 hybrid, the starter culture (*S. cerevisiae* in co-culture with *P. Kluyveri*) influenced the

sensorial profile, reducing the perception of sour taste and increasing perception, by judges, of bitter and sweet tastes. Chocolates from FA13 inoculated with *S. cerevisiae* were considered more sour, fruity, sweetness and less astringent than chocolates from spontaneous fermentation.

4. Discussion

The use of starter cultures in fermentative processes is very common for a number of industrialized products, such as wine, beer, cheese, bread, distilled beverages, sausages and fermented milks (Wood, 1998; Pereira et al., 2012). In the cocoa production chain, many studies selected and applied strains of yeasts and bacteria in the fermentation process (Samah et al., 1992; Schwan, 1998; Leal et al., 2008; Ramos et al., 2014; Moreira et al., 2017) but until now the cocoa fermentation process is still spontaneous and uncontrolled. Under these circumstances, this study evaluated the inoculation effect of two yeasts (*S. cerevisiae* UFLA CCMA 0200 and *P. Kluyveri* UFLA CCMA 0237) on fermentation of two different cocoa hybrids, using as parameters volatile compounds and protein (semi-qualitative) profiles, as well sensorial characteristics of produced chocolates.

Yeast is a very important group of microorganisms during cocoa fermentation. Ho et la. (2014) reported that these microorganisms are essential to fermentation process, consequently for generation of chocolate characteristic flavor. The yeasts are responsible for alcoholic fermentation of cocoa pulp, the fermentation product is ethanol, which is

converted in acetic acid (exothermic reaction) by acetic acid bacteria, this acid together with temperature increase causes the death of seed embryo, proteolytic processes inside the cotyledons begin, and chocolate aroma precursors are released (Schwan and Fleet, 2014).

In addition to influencing embryo death process, yeasts present secondary products of metabolism that contribute to chocolate flavor, such as organic acids, aldehydes, ketones, higher alcohols, and esters (Schwan and Wheals, 2004; Ramos et al., 2014). This study made possible to chemically and sensorially differentiate the samples (fermented cocoa beans and chocolate) from the inoculated and non-inoculated fermentations. Chocolates produced from the inoculated fermentations were considered sweeter than chocolates from the spontaneous fermentations (Fig. 5), this result correlate with greatest amount of alcohols components found in the chocolate samples from CEPEC2002 SP and FA13 S (Fig. 2), moreover, the compounds 2-heptanol and guaiacol were identified only in inoculated fermentations (Table 1). Alcohols are considered desirable compounds during fermentation and in the chocolate, because confer to final product, flavor notes as sweet, fruity, malty, honey and caramel (Table 1) (Serra-Bonvehí, 2005; Afoakwa et al., 2008; Rodriguez-Campos et al., 2011).

Recent studies showed that yeasts inoculation in cocoa fermentation modified the chemical composition of fermented beans, consequently the chocolates sensorial profiles. Batista et al. (2015) applied a yeasts cocktail (*S. cerevisiae*, *P. kluyveri* and *Hanseniaspora uvarum*) in cocoa fermentation, and reported that inoculation accelerated carbohydrate consumption and ethanol production during the process, and

consumers reported stronger coffee and sour attributes of chocolate produced from inoculated assay. Crafack et al. (2014) studied the impact of starter cultures on sensory profile of chocolate, as results, fermentation inoculated with *P. kluyveri* in association with *Kluyveromyces marxianus* produced chocolates characterized as fruity, acid and bitter. *P. kluyveri* is considered highly aromatic yeast, while *S. cerevisiae*, besides producing aromatic compounds, is considered large alcohol producer and highly resistant to environmental conditions (e.g. pH, temperature, ethanol concentration) (Kurtzman et al., 2011).

As reported by Batista et al. (2015), *S. cerevisiae* and *P. kluyveri* probably inhibited the growth of another yeast (*H. uvarum*) used as inoculum, the authors justify this fact by the ability of these species to produce toxins against other yeast genera (Branco et al., 2014). This hypothesis may explain difference found in the chocolates produced from the inoculated fermentations, chocolates from FA13 inoculated only with *S. cerevisiae* suffered more changes in flavor than chocolates produced from CEPEC2002 inoculated with yeast mix culture (Fig. 5). Furthermore, FA13 inoculated chocolate contained higher number the acids identified by GC-MS (Table 1), which may have given the sour characteristic described by judges (Fig. 5), on the other hand, the inoculation of CEPEC2002 hybrid decreased acids amount in chocolate, and thus reflected in the sour taste perception (Fig. 5). Overall, the inoculation process improved the chocolates sensorial qualities, because they were more accepted by the judges (Table 4).

The aromas that characterize chocolate are not formed only by the presence of volatile compounds. As mentioned above, proteolytic

processes inside the beans begin after embryo death. Some studies confirmed the proteases presence, such as endoprotease and carboxypeptidase, which degrade the most protein content inside beans during fermentation, composed mainly by two proteins, albumin and vicilin-class(7S) globulin (Voigt et al., 1993; Voigt et al., 1994; Voigt and Biehl, 1995; Voigt and Lieberei, 2014). Voigt et al. (2016) proved that precursors of cocoa-specific flavors are formed during fermentation and, moreover, showed that free amino acids, hydrophilic peptides derived from the vicilin-class(7S) globular storage protein are necessary for the formation of the cocoa-specific flavors during the roasting process. Therefore, as effect of the proteolytic processes inside the beans, the content of protein during fermentation decreased for all fermentations, both inoculated and non-inoculated (Table 2). Furthermore, initial and final times of fermentation showed different protein profiles, as presents Fig. 4. In agreement to results of sensorial analyzes, the yeasts inoculation also modified the protein profile, the AHC graph made it possible to identify similarities among protein profiles of the inoculated fermentations at end of process (144h).

5. Conclusion

This study proved that the inoculation process can influence the composition of the volatile compounds and the sensory profile of chocolates and analysis by MALDI-TOF corroborated that protein profiles also are changed. *S. cerevisiae*, when used alone as inoculum in cocoa fermentation, showed more effect on changes of sensory attributes,

although the two chocolates produced from inoculated fermentations were most appreciated by judges. Future studies should be conducted for a better understanding of protein degradation during inoculated fermentation, thereby defining which microorganism cultures positively affect the chocolates sensory characteristics.

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References

Afoakwa, E.O., Paterson, A., Fowler, M., Ryan, A. (2008). Flavor formation and character in cocoa and chocolate: A critical review. *Crit. Rev. Food Sci. Nutr, 48*, 840–857

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

Batista, N. N., Ramos, C. L., Ribeiro, D. D., Pinheiro, A. C. M., Schwan, R. F., (2015). Dynamic behavior of *Saccharomyces cerevisiae*,

Pichia kluyveri and *Hanseniaspora uvarum* during spontaneous and inoculated cocoa fermentations and their effect on sensory characteristics of chocolate. *LWT Food Sci. Technol, 63,* 221–227.

Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M. G., Caldeira, J., Albergaria, H. (2014). Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol. Biotechnol, 98,* 843-853.

Camu, N., De Winter, T., Addo, S. K., Takarama, J. S., Bernaert, H., De Vuyst, L. (2008). Fermentation of cocoa beans: Influence of microbial activities and polyphenol concentrations on the flavour of chocolate. *J. Sci. Food Agric, 88,* 2288–2297.

Crafack, M., Keul, H., Eskildsen, C. E., Petersen, M. A., Saerens, S., Blennow, A., Skovman-Larsen, M., Swiegers, J. H., Petersen, G. B., Heimdal, H., Nielsen, D. S. (2014). Impact of starter cultures and fermentation techniques on the volatile aroma and sensory profile of chocolate. *Food Res. Int, 63,* 306–316.

Hue, C., Gunata, Z., Breysse, A., Davrieux, F., Boulanger, R., Sauvage, F. X. (2016). Impact of fermentation on nitrogenous compounds of cocoa beans (Theobroma cacao L.) from various origins. *Food Chem, 192,* 958-964.

Ho, V., Zhao, J., Fleet, G. (2014). Yeasts are essential for cocoa bean fermentation. *Int. J. Food Microbiol, 174,* 72–87.

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

Leal, G. A, Gomes, L. H., Efraim, P., Tavares, F. C., 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.

López-Fernández, H., Santos, H. M., Capelo, J. L., Fdez-Riverola, F., Glez-Peña, D., Reboiro-Jato, M. (2015). Mass-Up: an all-in-one open software application for MALDI-TOF mass spectrometry knowledge discovery. *BMC Bioinformatics, 16*, 1-12.

Moreira, I. M. V., Miguel, M. G. C. P., Duarte, W. F., Dias, D. R., Schwan, R.F. (2013). Microbial succession and the dynamics of metabolites and sugars during the fermentation of three different cocoa (*Theobroma cacao* L.) hybrids. *Food Res. Int, 54*, 9–17.

Moreira, I. M. V., Miguel, M. G. C. P., Ramos, C. L., Duarte, W. F., Efraim, P., Schwan, R.F. (2016). Influence of cocoa hybrids on volatile compounds of fermented beans, microbial diversity during fermentation and sensory characteristics and acceptance of chocolates. *J. Food Qual, 39*, 839-849.

Moreira, I. M. V., Vilela, L. F., Miguel, M. G. C. P., Santos, C., Lima, N., Schwan, R. F. Impact of a Microbial Cocktail Used as a Starter Culture on Cocoa Fermentation and Chocolate Flavor. *Molecules, 22*, 766.

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

Oliveira, M., Santos, C., Sampaio, P., Romeo, O., Almeida-Paes, R., Pais, C., Lima, N., Zancopé-Oliveira, R. M. (2015). Development and optimization of a new MALDI-TOF protocol for identification of the Sporothrix species complex. *Res. Microbiol, 166*, 102-110.

Pereira, G. V. M., Miguel, M. G. C. P., Ramos, C. L., Schwan, R. (2012). Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast and bacteria strains to develop a defined starter culture. *Appl. Environ. Microbiol*, *78*, 5395–5405.

Ramos, C. L., Dias, D. R., Miguel, M. G. C. P., Schwan, R. F. (2014). Impact of different cocoa hybrids (*Theobroma cacao* L.) and *S. cerevisiae* UFLA CA11 inoculation on microbial communities and volatile compounds of cocoa fermentation. *Food Res. Int*, *64*, 908–918.

Rodriguez-Campos, J., Escalona-Buendía, H. B., ORozco-Avila, I., Lugo-Cervantes, E. and Jaramillo-Flores, M. E. (2011). Dynamics of volatile and non-volatile compounds in cocoa (*Theobroma cocoa* L.) during fermentation and drying process using principal components analysis. *Food Res. Int, 44*, 250–258.

Rohan, T. A. (1964). The precursors of chocolate aroma: A comparative study of fermented and unfermented cocoa beans. *J. Sci. Food Agr, 29*, 456-459.

Samah, O. A, 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.

Serra-Bonvehí, J. (2005). Investigation of aromatic compounds in roasted cocoa powder. *Eur. Food Res. Technol, 221*, 19–29.

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

Schwan, R. F., Wheals, A. E. (2004). The microbiology of cocoa fermentation and its role in chocolate quality. *Crit. Rev. Food Sci. Nutr, 44*, 205–221.

Schwan, R.F., Fleet, G.H., 2014. Cocoa and Coffee Fermentations. (Taylor & Francis Group).

Schwan, R. F., Pereira, G. V. M., Fleet, G. H. (2014). Microbioal activities during cocoa fermentation. *In Cocoa and Coffee Fermentation* (R.F. Schwan and G.H. Fleet, eds.) pp. 129–193, CRC Press, Boca Raton, FL.

Stone, H.S., Sidel, J.L., (1993). Sensory Evaluation Practices. Academic Press, California.

Visintin, S., Ramos, L., Batista, N., Dolci, P., Schwan, F., Cocolin, L. (2017). Impact of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* starter cultures on cocoa beans fermentation. *Int. J. Food Microbiol, 257*, 31-40.

Voigt, J., Biehl, B. (1993). The major seed proteins of *Theobroma cacao* L. *Food Chem, 47*, 145-151.

Voigt, J., Biehl, B., Heinrichs, H., Kamaruddin, S., Gaim Marsoner, G., Hugi, A. (1994). In-vitro formation of cocoa-specific aroma precursors: Aroma-related peptides generated from cocoa seed

protein by co-operation of an aspartic endoprotease and a carboxypeptidase. *Food Chem, 49*, 173-180.

Voigt, J., Biehl, B. (1995). Precursors of the cocoa specific aroma components are derived from vicilin-class (7S) globulin of the cocoa seed by proteolytic processing. *Botanica Acta, 108*, 283-289.

Voigt, J., Lieberei, R. (2014). Biochemistry of cocoa fermentation. *In Cocoa and Coffee Fermentation* (R.F. Schwan and G.H. Fleet, eds.) pp. 193–227, CRC Press, Boca Raton, FL.

Voigt, J., Janek, K., Textoris-Taube, K., Niewienda, A., Wöstemeyer, J. (2016). Partial purification and characterization of the peptide precursors of the cocoa-specific aroma components. *Food Chem, 192*, 706-713.

Wood, B. J. B. (1998). Microbiology of fermented foods, 2nd ed Thompson Science, London, United Kingdom.

ANEXO A – ARTIGO CIENTÍFICO RESULTADO DA DISCIPLINA PESQUISA ORIENTADA II PUBLICADO NO PERIÓDICO *MOLECULES*

Impact of a Microbial Cocktail Used as a Starter Culture on Cocoa Fermentation and Chocolate Flavor

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Abstract: Chocolate production suffered a vast impact with the emergence of the "witches' broom" disease in cocoa plants. To recover cocoa production, many disease-resistant hybrid plants have been developed. However, some different cocoa hybrids produce cocoa beans that generate chocolate with variable quality. Fermentation of cocoa beans is a microbiological process that can be applied for the production of chocolate flavor precursors, leading to overcoming the problem of variable chocolate quality. The aim of this work was to use a cocktail of microorganisms as a starter culture on the fermentation of the ripe cocoa pods from PH15 cocoa hybrid, and evaluate its influence on the microbial communities present on the fermentative process on the compounds involved during the fermentation, and to perform the chocolate sensorial characterization. According to the results obtained, different volatile compounds were identified in fermented beans and in the chocolate produced. Bitterness was the dominant taste found in non-inoculated chocolate, while chocolate made with inoculated beans showed bitter, sweet, and cocoa tastes. 2,3-Butanediol and

2,3-dimethylpyrazine were considered as volatile compounds making the difference on the flavor of both chocolates. *Saccharomyces cerevisiae* UFLA CCMA 0200, *Lactobacillus plantarum* CCMA 0238, and *Acetobacter pasteurianus* CCMA 0241 are proposed as starter cultures for cocoa fermentation.

Keywords: chocolate quality; GC-MS; sensory analysis; culture-independent analysis; starter culture

1. Introduction

The cocoa (*Theobroma cacao* L.) supply chain for the production of chocolate is complex. It involves several post-harvest steps, which can determine the quality of the final product. In Brazil, cocoa production suffered a vast impact with the emergence of "witches' broom" disease [1,2]. In order to recover the cocoa production, many disease-resistant hybrid plants, such as PH9, PH15, PH16, PS1030, PS1319, CCN51, CEPEC2002, CEPEC2004, and FA13, have been developed [3,4].

As a matter of consequence, different cocoa hybrids generate cocoa beans that produce chocolate with variable quality [5–7]. In this context, PH15 hybrid has great relevance due to high-productivity, adaptation, and resistance to some diseases, such as "witches' broom" and ceratocystis wilt [8–10].

The fermentation of cocoa beans is a microbiological process with enzymatic activity and the development of chocolate flavor precursors [11–13]. This traditional process is spontaneous and uncontrolled. After opening of the cocoa pods, the beans are transferred to the area of fermentation and placed in heap or fermentation boxes. These methods are the most commonly used among the cocoa producer countries [14,15].

Yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) are the main microbial communities involved during cocoa fermentation. Yeast species are reported as the primary colonizers of cocoa fermentation. *Saccharomyces*, *Hanseniaspora* (anamorph *Kloeckera*), and *Pichia* are the prevalent genera found in cocoa fermentation in different countries. *Saccharomyces cerevisiae* is particularly the most reported species in many fermentations [16–20].

Simultaneously with the yeast growth, LAB colonize the cocoa mass and degrade the pulp's glucose into lactic acid and assimilate the citric acid also present in the pulp. Several studies concerning the microbial fermentation reported two LAB species as the most prevalent in this process: *Lactobacillus plantarum* and *Lactobacillus fermentum* [19–21].

Yeast populations, which are responsible for the ethanol production, decline together with the LAB populations. AAB dominates the process and are responsible to the exothermic reaction of ethanol conversion into acetic acid. *Acetobacter pasteurianus*

is the most frequent species of AAB found in cocoa fermentation, but other species, such as *Acetobacter aceti*, *Acetobacter ghanensis*, *Acetobacter fabarum*, *Gluconobacter oxydans*, and *Gluconobacter xylinus*, have also been reported in the literature [16,17,20,22].

Species of *Bacillus* (e.g., *Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus flexus*) may also grow during fermentation and can affect bean quality and cocoa flavor [16,17,23].

Different compounds, such as alcohols (e.g., 2-methyl-1-propanol, 2-phenylethanol, methanol), aldehydes (e.g., acetaldehyde, benzaldehyde), ketones (e.g., 2-pentanone, phenylmethyl ketone), esters (e.g., ethyl acetate, 2-phenylethyl acetate), and carboxylic acids (e.g., butanoic acid, nonanoic acid), are produced during fermentation, affecting the final flavor character in chocolate [13,17,24–26].

The aim of this work was to use a cocktail of microorganisms as a starter culture on the fermentation of the ripe cocoa pods from PH15 cocoa hybrid, and evaluate its influence on the microbial communities present on the fermentative process, on both the volatile and non-volatile compounds produced during the fermentation, and to perform the chocolate sensorial characterization.

2. Results

2.1. Culture-Independent Analysis with PCR–DGGE

Analyses of the microbial communities on both inoculated and non-inoculated samples of the PH15 cocoa hybrid were performed by Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR–DGGE) for prokaryote (Figure 1A) and eukaryote (Figure 1B) microorganisms. Identification of DGGE bands are shown in Table 1.

Table 1. Identification of the bands based on Basic Local Alignment Search Tool (BLAST) in comparison with those in GenBank as obtained by PCR–DGGE using universal primers for yeasts and bacteria.

Identification	Bands [a]	Similarity (%)	Accession Number	Sample Found
Prokaryotes				
Fructobacillus pseudoficulneus	1, 5, 6	98	AB498052.1	PH15NI, PH15I
Leuconostoc sp.	2	97	DQ523491.1	PH15NI
Lactobacillus plantarum	3, 4, 8, 9	99	KT327866.1	PH15NI, PH15I
Lactobacillus helveticus	7	99	KP764179.1	PH15I
Zymomonas mobilis	10	100	CP003715.1	PH15NI
Uncultured bacterium	11, 12, 13, 14	99	LN875309.1	PH15NI, PH15I
Acetobacter pasteurianus	15, 16, 17, 18, 19, 20	100	KM983001.1	PH15NI, PH15I
Acetobacter sp.	21	98	KC796695.1	PH15NI
Gluconobacter oxydans	22, 23, 24	99	CP003926	PH15NI, PH15I
Bacillus sp.	25, 26	99	JF309224	PH15NI
Eukaryotes				
Hanseniaspora uvarum	1, 2	99	KC544511	PH15NI
Saccharomyces cerevisiae	3, 5, 6, 7, 8, 9, 10	99	KT229544.1	PH15NI, PH15I
Saccharomyces sp.	4	98	KU350335.1	PH15NI
Theobroma cacao	11	97	JQ228377.1	PH15NI
Rhodotorula mucilaginosa	12, 13	100	HM588765	PH15I
Lentinula edodes	14	98	KM015456.1	PH15I
Pichia kluyveri	15, 16, 17, 18, 20	99	FM864201	PH15NI, PH15I
Trichosporon asahii	19	97	JQ425402	PH15I

[a]Bands are numbered as indicated on the DGGE gel. PH15NI: PH15 non-inoculated; PH15I: PH15 inoculated.

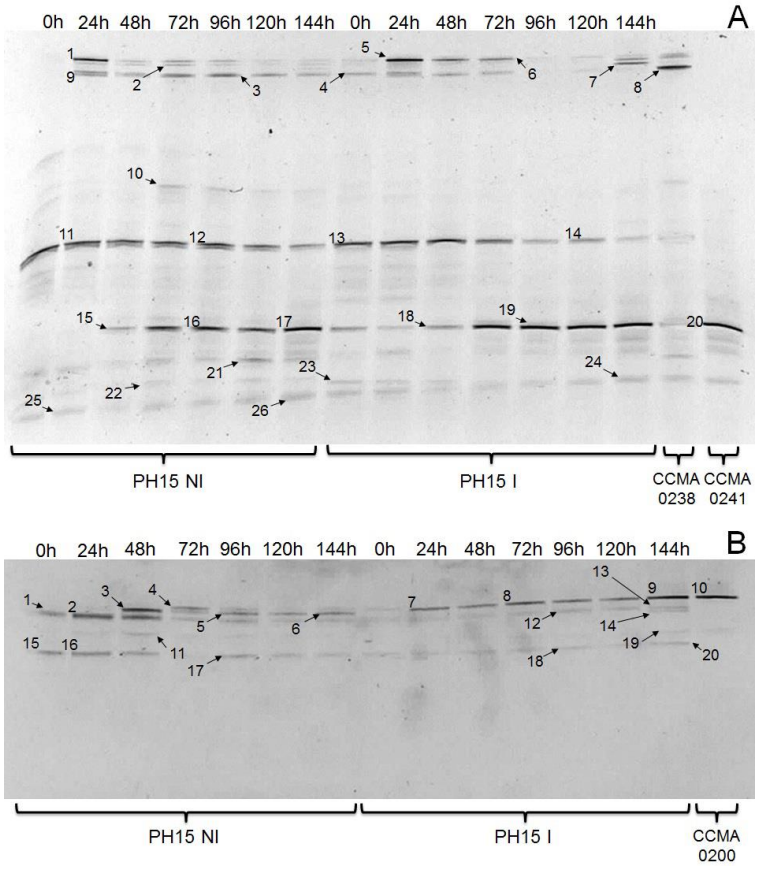

Figure 1. Changes in prokaryote (**A**) and eukaryote (**B**) communities during fermentation of the PH15 cocoa hybrid without (PH15 NI) and with (PH15 I) inoculation, and fingerprints of the starter cultures (CCMA 0238, CCMA 0241, and CCMA 0200). The identities of the bands are presented in Table 1. Bands marked with numbers were excised, re-amplified, and sequenced.

The bacterial and yeast communities changed according to different fermentation processes (inoculated or non-inoculated). The bacterial species *Gluconobacter oxydans* (bands 22–24), *Lactobacillus plantarum* (bands 3, 4, 8, and 9), uncultured bacterium (bands 11–15), *Acetobacter pasteurianus* (bands 15–20), and *Fructobacillus pseudoficulneus* (bands 1, 5, and 6) were detected for both inoculated and non-inoculated fermentations.

The species *Leuconostoc* sp. (band 2), *Zymomonas mobilis* (Band 10), *Acetobacter* sp. (Bands 21), *Bacillus* sp. (Bands 25 and 26) were detected in the non-inoculated fermentation. The *Lactobacillus helveticus* (Band 7) was only detected in inoculated

fermentation. The yeast species *Saccharomyces cerevisiae* (Bands 3 and 5–10) and *Pichia kluyveri* (Bands 15–18 and 20) were detected for both fermentations. *Saccharomyces* sp. (Band 4), *Hanseniaspora uvarum* (Bands 1 and 2), and *Theobroma cacao* (Band 11) were only detected in the non-inoculated fermentation: this was possible because universal eukaryote primers were used. In the inoculated fermentation *Rhodotorula mucilaginosa* (Bands 12 and 13), *Trichosporon asahii* (Band 19), and *Lentinula edodes* (Band 14) were detected.

2.2. Chemical Changes During Fermentation

Temperature and pH values were measured during 120 h of fermentation. In the fermentation of PH15 NI the temperature varied from 30.7 °C at 0 h (with the maximum of 49.1 °C at 96 h) to 47.9 °C at 120 h. On the other hand, in the fermentation of PH15 I, the temperature varied from 26.62 °C at 0 h (with the maximum of 50.06 °C at 72 h) to 49.88 °C at 120 h. The pH value outside the bean (pulp) varied during fermentation. The pH value of PH15 NI ranged from 3.27 to 4.45, and the fermentation PH15 I pH value ranged from 3.27 to 4.81.

2.2.1. Sugar Consumption and Metabolite Production

During the six days of fermentation the concentrations of glucose, fructose, and citric acid were evaluated in the pulp, and the results are shown in Figure 2. Citric acid was fully metabolized at 24 h of fermentation in both assays (Figure 2A,B). In both fermentations, glucose and fructose were completely consumed at 72 h. After 24 h of inoculated fermentation the PH15 sample showed greater sugar consumption compared to the non-inoculated one, but in the following hours, both inoculated and non-inoculated fermentations presented the same profile of sugar consumption (Figure 2A,B).

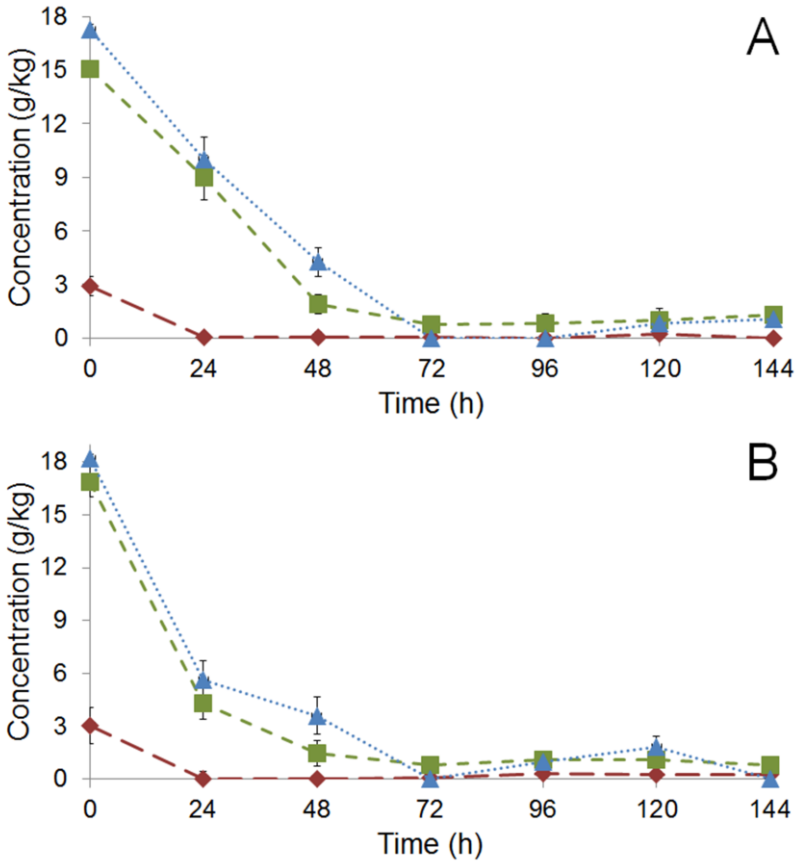

Figure 2. Course of glucose ( ), fructose ( ), and citric acid ( ) during fermentation of PH15 NI (**A**) and PH15 I (**B**).

Ethanol, lactic acid, and acetic acid were evaluated in the pulp and inside the beans, and are shown in Figure 3. The inoculated fermentation of PH15 showed the maximum value of ethanol in the pulp (8.44 g/kg at 48 h), and this compound inside the beans also reached higher values (6.65 g/kg at 48 h) when compared to the control fermentation (PH15 NI) (Figure 3A). However, at the end of the fermentation, the ethanol concentration was higher (4.34 g/kg) inside the beans in the non-inoculated fermentation.

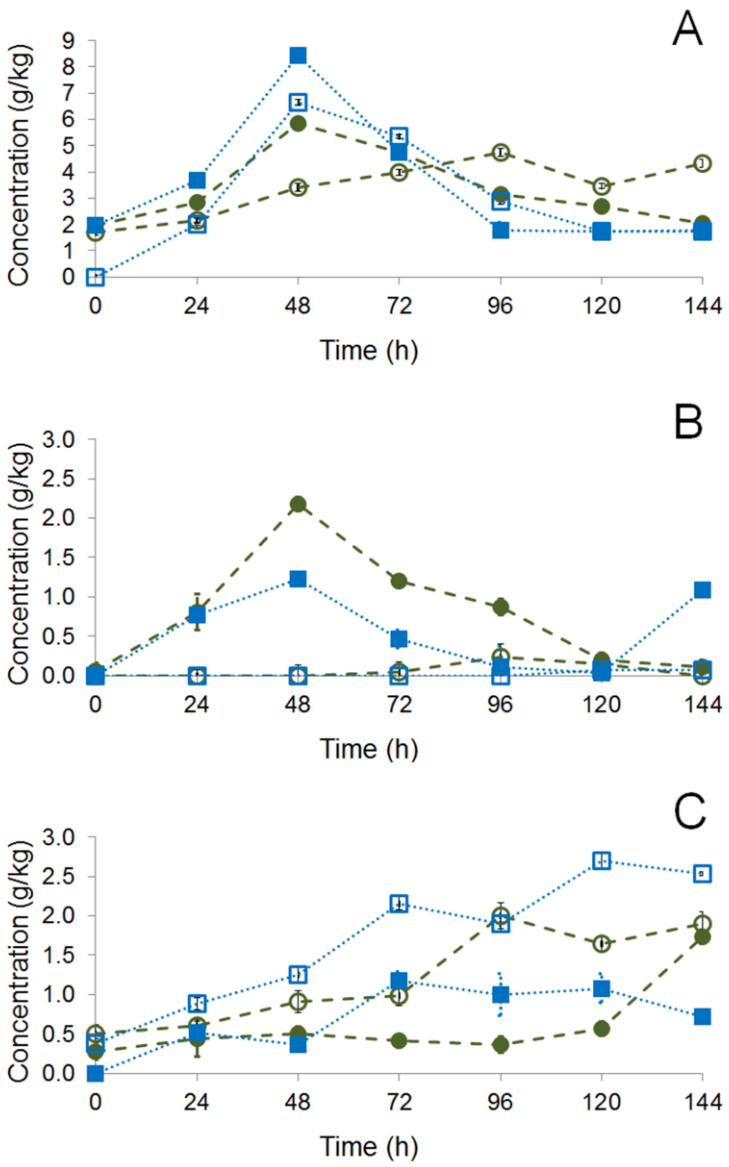

Figure 3. Detection by HPLC of ethanol (**A**), lactic acid (**B**), and acetic acid (**C**) during 144 h of fermentation of PH15 NI (—●—) and PH15 I (···■···); full symbols correspond to metabolites detected in the pulp while open symbols to those detected inside the beans.

The microbial inoculation accelerated the sugar consumption in the first 24 h of cocoa fermentation, whereas the ethanol production was accelerated in the first 48 h of fermentation (Figures 2B and 3A). The pulp of PH15 I showed the highest acetic acid concentration between 48 h and 120 h of cocoa fermentation, and the lowest concentration at the end of the fermentation (144 h, Figure 3C). In contrast, PH15 NI showed the highest acetic acid concentration in the residual pulp at the end of the fermentation (144 h, Figure 3C). Overall, the concentration of acetic acid was higher in the fermentation of PH15 I (Figure 3C).

After 144 h, the pulp of PH15 I showed greater concentration of lactic acid than the pulp of PH15 NI (Figure 3B). In both PH15 I and PH15 NI samples there was no lactic acid penetration in the cotyledon. Acetic acid was higher inside the beans in PH15 I at later stages, at 120 h (2.70 g/kg), and at 144 h (2.53 g/kg).

2.2.2. Volatile Compounds

A total of 37 volatile compounds were detected by Gas Chromatography Mass Spectrometry (GC–MS) at the beginning (0 h), and a total of 38 volatile compounds at the end (144 h), of the non-inoculated fermentation. While in the inoculated fermentation, a total of 37 volatile compounds were detected at the beginning (0 h) and a total of 34 volatile compounds at the end (144 h), as presented in the Table 2.

Table 2. Volatile compounds identified by Headspace - Solid Phase Microextraction - Gas Chromatography Mass Spectrometry (HS–SPME GC–MS) during fermentation times (0 h and 144 h) and in chocolate samples, and the reference odor of each compound**.**

Compounds	Odor Description [a]	Sample Found
Acids		
4-Hydroxybutanoic acid		PH15I Ch
4-Hydroxybutyric acid		PH15NI Ch
Acetic acid	Sour, astringent	PH15NI 144 h
Benzeneacetic acid		PH15NI Ch, PH15I Ch
Butanoic acid	Rancid, butter, cheese	PH15NI Ch, PH15I Ch
Hexanoic acid	Sweat, pungent	All
Isovaleric acid	Sweat, rancid	All
Octanoic acid	Sweaty, fatty	PH15NI (0 h and 144 h), PH15I 144 h, PH15NI Ch
Pentanoic acid		PH15NI Ch, PH15I Ch
Valeric acid	Sweat, acid, rancid	PH15NI Ch, PH15I Ch
Alcohols		
2-Ethyl-1-hexanol		PH15NI Ch
1-Hexanol	Fruity, green	PH15NI (0 h and 144 h), PH15I 0 h
1-Methoxy-2-butanol		PH15NI Ch, PH15I Ch
1-Nonanol		PH15NI Ch, PH15I Ch
1-Octanol	Fatty, waxy	All
1-Penten-3-ol		PH15NI 0 h
2,3-Butanediol	Cocoa butter	PH15NI (0 h and 144 h), PH15I 144 h, PH15I Ch
2,4-Pentanediol		PH15NI 0 h, PH15I 0 h, PH15NI Ch, PH15I Ch
2-Furanmethanol		PH15NI Ch

Compounds	Odor Description [a]	Sample Found
2-Heptanol	Sweet, citrusy	PH15NI (0 h and 144 h), PH15I (0 h and 144 h)
2-Hexanol	Fruity, green	PH15NI 0 h, PH15I 0 h
2-Pentanol	Green, mild green	PH15NI Ch, PH15I Ch
3-Methyl-1-butanol	Malty, chocolate	PH15NI (0 h and 144 h), PH15I (0 h and 144 h)
Benzyl alcohol	Sweet, flower	All
Furfuryl alcohol		PH15I Ch
Guaiacol	Smoke, sweet	PH15NI 144 h, PH15I 144 h
Phenylethyl Alcohol	Honey, rose, caramel	All
Aldehydes and Ketones		
3-methylpentanal		PH15NI 0 h, PH15I 0 h, PH15NI Ch, PH15I Ch
(*E*)-2-Heptenal		PH15NI 0 h, PH15I 0 h
(*E*)-2-Nonenal	Tallowy green	PH15NI 0 h, PH15I 0 h
(*E*)-2-Octanal	Fatty, waxy	PH15NI 0 h, PH15I 0 h
(*E*)-2-Undecenal		PH15NI Ch, PH15I Ch
(*E*,*E*)-2,4-heptadienal		PH15NI 0 h, PH15I 0 h, PH15NI Ch, PH15I Ch
1-(2-hydroxyphenyl)ethanone		PH15NI 0 h, PH15I 0 h
2(5*H*)-Furanone	Caramel-like	PH15NI Ch, PH15I Ch
2-Heptanone		PH15NI (0 h and 144 h), PH15I (0 h and 144 h)
2-Hydroxyphenyl methyl ketone		PH15NI 144 h
2-Nonanone		PH15NI 144 h, PH15I (0 h and 144 h)
2-Phenyl-2-butenal	Sweet, roasted	PH15NI 144 h, PH15I 144 h, PH15NI Ch, PH15I Ch
2-Undecenal		PH15NI (0 h and 144 h), PH15I (0 h and 144 h)
3-Methyl-1,2-cyclopentanedione		PH15NI Ch, PH15I Ch
3-Penten-2-one		PH15NI Ch
4-hydroxy-3-methylbutanal		PH15NI 0 h, PH15I 0 h
4-Methylhexanal		PH15NI Ch, PH15I Ch
5-Methyl-2-furaldehyde		PH15NI Ch, PH15I Ch
5-Methyl-2-phenyl-2-hexenal		PH15NI Ch, PH15I Ch
3-methylpentanal		PH15NI 0 h, PH15I 0 h, PH15NI Ch, PH15I Ch
Acetophenone	Floral	PH15NI (0 h and 144 h), PH15I (0 h and 144 h)
Benzaldehyde	Bitter	All
Benzeneacetaldehyde		PH15NI Ch, PH15I Ch
Nonanal		PH15NI 0 h, PH15I 0 h, PH15NI Ch, PH15I Ch
Octanal		PH15NI 0 h, PH15I 0 h, PH15NI Ch, PH15I Ch
Pyranone		PH15NI Ch, PH15I Ch
Acetoin	Butter, cream	PH15NI 144 h, PH15I 144 h, PH15NI Ch, PH15I Ch
Esters		
1-methylbutyl benzoate		PH15NI 144 h, PH15I 144 h
1-Methylhexyl acetate		PH15NI 144 h
2-Ethyl-1-hexyl acetate		PH15I Ch
2-Pentanyl benzoate		PH15NI 0 h, PH15I 0 h, PH15NI Ch, PH15I Ch
2-Phenethyl acetate	Fruity	PH15NI 144 h, PH15I 144 h
3-methylbutyl formate		PH15NI 0 h
Amyl acetate	Fruity, banana	PH15NI 144 h, PH15I 144 h
Dibutyl phthalate		PH15NI 144 h, PH15I (0 h and 144 h)
Diisobutyl phthalate		PH15NI Ch, PH15I Ch
Ethyl 2-hydroxypropanoate		PH15NI 144 h, PH15I 144 h
Ethyl benzeneacetate		PH15NI 144 h
Ethyl caprate	Pear, grape	PH15NI 144 h, PH15I 144 h
Ethyl caprylate	Fruity, flowery	PH15I (0 h and 144 h), PH15I Ch
Ethyl laurate	Fruity, floral	PH15NI 144 h, PH15I 144 h, PH15NI Ch, PH15I Ch
Ethyl myristate	Waxy, soapy	PH15NI 144 h, PH15I 144 h
Ethyl palmitate	Waxy, green	PH15NI (0 h and 144 h), PH15I (0 h and 144 h)

Compounds	Odor Description [a]	Sample Found
Ethyl phenylacetate		PH15NI 0 h
Hexyl acetate		PH15NI 144 h
Isoamylformate		PH15I 0 h
Isobutyl phthalate		PH15NI (0 h and 144 h), PH15I (0 h and 144 h)
Isopropyl palmitate		PH15I 0 h
Methyl Palmitate		PH15NI 144 h, PH15I (0 h and 144 h)
Phenylethyl acetate	fruity, sweet	PH15NI Ch, PH15I Ch
Pyrazines		
2,3,5,6-Tetramethylpyrazine	Chocolate, coffee	PH15NI Ch, PH15I Ch
2,3,5-Trimethyl-6-isopentylpyrazine		PH15NI Ch, PH15I Ch
2,3,5-Trimethylpyrazine	Cocoa, rusted nuts	PH15NI Ch, PH15I Ch
2,3-Dimethylpyrazine	Caramel, cocoa	PH15I Ch
2,5-Dimethyl-3-isoamylpyrazine		PH15NI Ch, PH15I Ch
2,5-Dimethylpyrazine	Cocoa, rusted nuts	PH15NI Ch, PH15I Ch
2,6-Dimethylpyrazine	Nutty, coffee, green	PH15I Ch
2-Acetyl-3,5-dimethylpyrazine		PH15NI Ch, PH15I Ch
2-Ethyl-3,6-dimethylpyrazine	Roasted, smoky	PH15NI Ch, PH15I Ch
2-Ethyl-6-methylpyrazine		PH15NI Ch, PH15I Ch
2-Ethylpyrazine	Peanut butter, nutty	PH15NI Ch
2-Methyl-3,5-diethylpyrazine		PH15NI Ch
2-Methyl-6-vinylpyrazine		PH15NI Ch
2-Methylpyrazine	Chocolate, cocoa, nuts	PH15NI Ch, PH15I Ch
Pyrroles		
1,3-Dimethyl-5-pyrazolinone		PH15NI Ch, PH15I Ch
2-Acetylpyrrole	Chocolate, hazelnut	PH15NI Ch, PH15I Ch
2-Pyrrolidinone		PH15NI Ch, PH15I Ch
Pyrrole-2-carboxaldehyde		PH15NI Ch, PH15I Ch
Terpenoids		
(*E*)-Linalool oxide	Floral, green	PH15NI (0 h and 144 h), PH15I (0 h and 144 h)
(*Z*)-Linalool oxide	Floral	PH15NI 0 h, PH15I 0 h
Linalool	Flower, lavender	PH15NI (0 h and 144 h), PH15I (0 h and 144 h)
Others [b]		
1-methoxy-2-methylpropane		PH15NI (0 h and 144 h), PH15I 144 h
2-Butyltetrahydrofuran		PH15NI Ch, PH15I Ch
2-Pentylfuran		All
7-methyl pentadecane		PH15I (0 h and 144 h)
Hexadecane		PH15I (0 h and 144 h)

[a] Obtained from literature; [b] Includes: furans and alkanes; Abbreviations: Ch: chocolate.

Both non-inoculated and inoculated hybrid PH15 (0 h and 144 h) showed the following identified compounds: aldehydes and ketones, acids, alcohols, esters, terpenoids, and furans. Aldehydes and ketones occurred at the beginning (0 h) and esters occurred at the end (144 h). In both fermentations, the most important groups of volatile compounds were detected (Figure 4).

Chocolate samples of PH15 I and PH15 NI presented 58 and 54 volatiles compounds, respectively (Table 2 and Figure 4). The compounds identified were aldehydes and ketones, acids, alcohols, esters, pyrazines, pyrroles, and furans, and the most important groups detected, in both chocolate samples, were aldehydes and ketones, alcohols, and pyrazines (Figure 4).

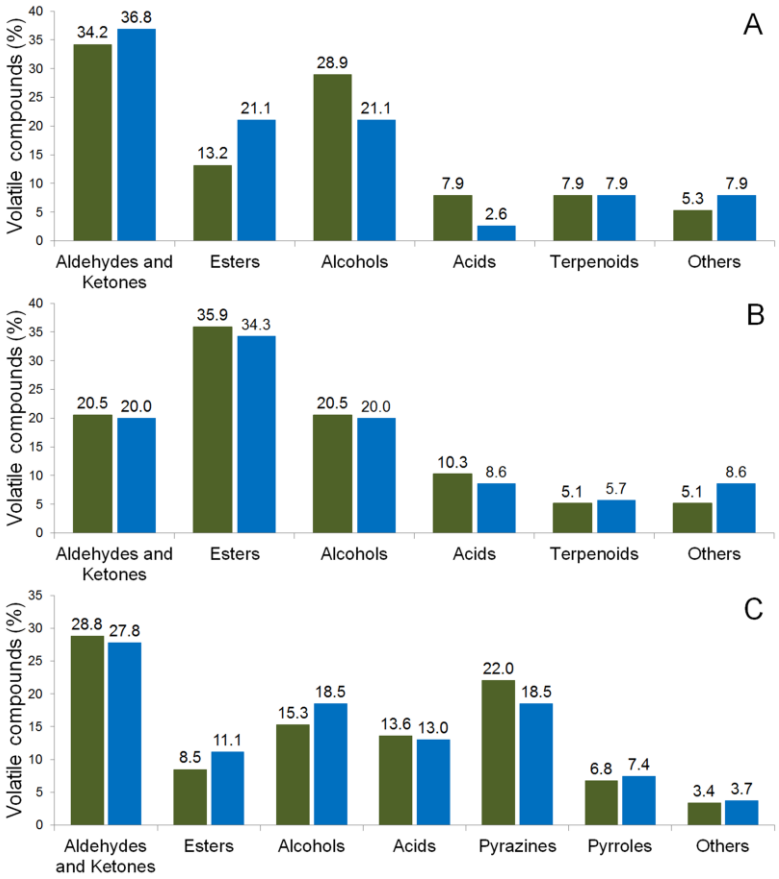

Figure 4. Profile of volatile compounds identified by HS-SPME GC-MS during non–inoculated fermentation (PH15 NI), inoculated fermentation (PH15 I), and in the chocolate samples. Fermentation times: 0 h (**A**) and 144 h (**B**). Chocolate samples (**C**). Total amount of compounds: PH15 SI 0 h (37), PH15 SI 144 h (38), PH15 SI Ch (58), PH15 I 0 h (37), PH15 I 144 h (34), and PH15 I Ch (54).

2.3. Sensorial Analyses of Chocolate

The chocolate analyses by the Temporal Dominance of Sensations (TDS) technique are shown in Figure 5. The judges noted difference between the two samples of chocolate during the tasting time. The bitterness was the dominant taste in the final time (25–35 s) of PH15 NI Ch (no-inoculated chocolate). However, the fruity and cocoa flavors were significant at 17 and 22 s, respectively (Figure 5A).

The sample PH15 I Ch showed a mixture of sensations, alternating between bitterness, cocoa taste and sweetness (Figure 5B). The bitterness is the more dominant taste in the initial (5 to 10 s) and final time (25 to 35 s), while the cocoa taste was dominant in the intermediate (15 to 20 s) and final time (25 to 35 s). The sweetness taste showed significant levels in the final time (25 to 35 s) (Figure 5B).

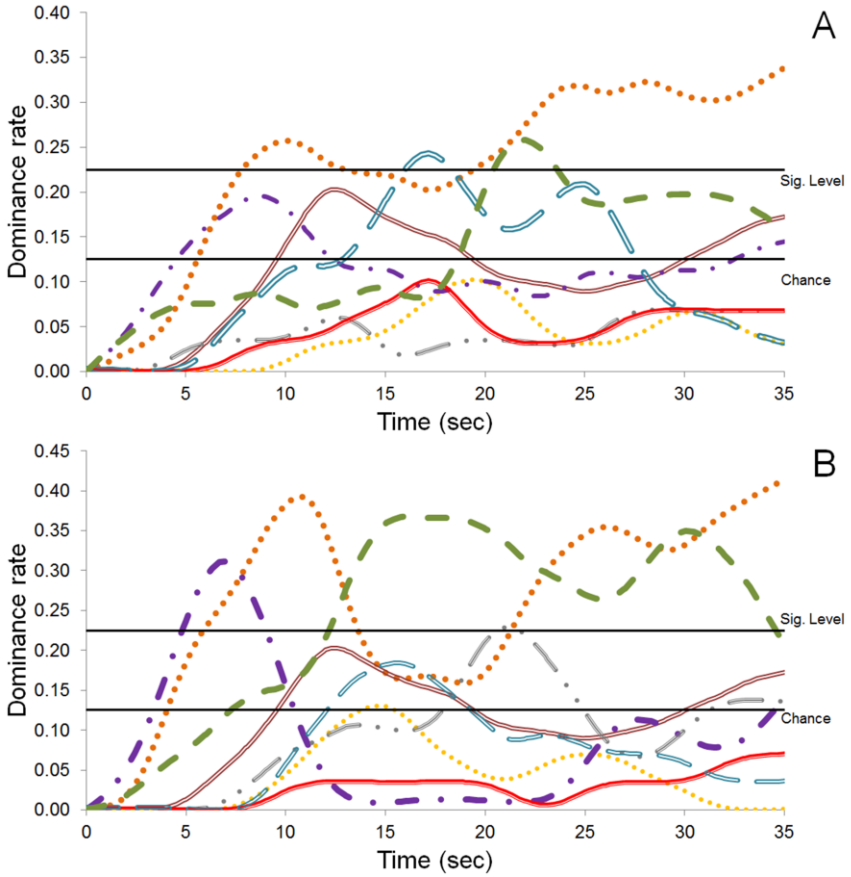

Figure 5. Temporal Dominance Sensory of chocolate produced from cocoa fermented beans PH15 NI (**A**) and PH15 I (**B**). Sensorial attributes: Acid ( ), Bitterness ( ), Nutty ( ), Sweetness ( ), Astringent ( ), Coffee ( ), Fruity ( ), and Cocoa ( ).

3. Discussion

In order to evaluate their influence on the fermentation of cocoa beans and on the final sensorial characteristics of produced chocolate, *S. cerevisiae* UFLA CCMA 0200, *Lactobacillus plantarum* CCMA 0238, and *Acetobacter pasteurianus* CCMA 0241 were used as starter cultures for the cocoa PH15 fermentation. The organic compounds and

microbial communities involved during the fermentation of non-inoculated and inoculated cocoa were analyzed. Furthermore, the sensorial characterization of chocolate (PH15 I Ch and PH15 NI Ch) produced from the fermented beans was evaluated.

The PCR–DGGE analyses showed that the bacterial and yeast communities were different according to the fermentation process. This may be explained by the use of starter cultures that may have generated changes in the natural microbiota, as shown in Figure 1A,B. Species of LAB and AAB were identified in both fermentations.

The *L. plantarum* and *Fructobacillus pseudoficulneus* species were the bacteria present in both fermentations. However, in PH15 I these species were only identified until in the middle of the fermentation period (72 h), but after this time, they seem not to be present. It is reported that the increase of ethanol concentration during cocoa fermentation inhibited *L. fermentum* growth [15, 25]. This could explain the low population rate of *L. plantarum* in PH15 NI.

Gluconobacter oxydans and *A. pasteurianus* were AAB species identified in both fermentation processes. In addition, *A. pasteurianus* (Figure 1A—bands 15, 16, 17, 18, 19, and 20) was present at all fermentation times of PH15 I, and this did not happen in the fermentation without inoculum. These species have been described in cocoa bean fermentation in Brazil, Ghana, and Indonesia [3,16,19,20].

The LAB species *Leuconostoc* sp., *Lactobacillus helveticus* were detected in non-inoculated and inoculated fermentations, respectively. The *Zymomonas mobilis*, an ethanol strain producer, *Acetobacter* sp., an AAB species, and *Bacillus* sp. were only detected in non-inoculated fermentation (Figure 1A). The *Bacillus* sp. present in PH15 I fermentation can be explained as the fermentation was not in aseptic conditions. *Bacillus* sp. and filamentous fungi may participate in the spontaneous cocoa bean fermentation process after four or five days of fermentation [14,16,17,20,25].

Fingerprinting based on PCR–DGGE showed that the most common yeast *Saccharomyces cerevisiae* was present during the fermentation in both fermentations. This fact indicated that this yeast may be a promising starter culture used for the cocoa fermentation process. Some works using *S. cerevisiae* as a starter culture have been reported [4,7], and concluded that yeast inoculation accelerated the fermentation process.

The microbial activity and metabolites produced during the cocoa beans' fermentation leads to an increase of temperature and pH value [19]. Therefore, this may explain the temperature and pH increase in both fermentations, but in PH15 I there was a greater increase.

According to the chemical results, carbohydrates were consumed faster in the inoculated assay (Figure 2B). This is likely due to the higher microorganism population in the inoculated assay than in the control. Further, higher ethanol concentrations (almost two times the concentration detected in the control) were observed in this assay (Figure 3A). However, this was not the case for acetic acid, similar to results previously described elsewhere [4]. However, in inoculated fermentation, acetic acid was detected in the cotyledon at the end of fermentation (Figure 3C). Sucrose was not detected in the

fermentation probably because it was hydrolyzed into glucose and fructose when the pods were broken open, as previously described [4].

In addition, to produce primary metabolites, such as ethanol, and lactic and acetic acids during cocoa fermentation, starter cultures also produce a vast array of volatile secondary metabolites, such as higher alcohols, acids, esters, aldehydes, ketones [24,27], and others that could influence cocoa flavor [25].

The most important volatile compound groups detected were esters and alcohols, in both fermentations (Table 2 and Figure 4). These compounds are already described as important in cocoa products [24]. The esters are correlated to fruity notes [28] and the alcohols with flowery and candy notes [29,30], e.g., 2,3-butanediol, 2-heptanol, guaiacol, benzyl alcohol, and phenylethyl alcohol found in this study, being that the latter two compounds were found in all chocolate process stages of both fermentations (PH15 I and PH15 NI) (Table 2) [4].

Acids are generally related to unpleasant odors present in cocoa products [24,30,31]. A total of five compounds were identified, some being related to rancid, sour, or fatty odors. However, some acids detected here may present pleasant odors, e.g., 4-hydroxybutanoic acid and hexanoic acid, with sweetish odors, as shown in Table 2 [4].

In order to investigate the influence of a starter culture on the final product, two chocolates were produced and their sensory analyses were evaluated. The judges noted differences between the two chocolate samples (PH15 I Ch and PH15 NI Ch) during the tasting time. Significant differences were observed as described in Figure 5. Bitter was the dominant taste in PH15 NI Ch (Figure 5A) and, in PH15 I Ch, bitter, sweet, and cocoa tastes were dominant (Figure 5B). These results can be corroborated by the analysis of the volatile compounds in the chocolate samples. The 2,3-butanediol, which gives flavor to cocoa butter (sweet chocolate), and 2,3-dimethylpyrazine, which gives caramel and cocoa flavors, were detected only in PH15 I Ch (Table 2). Therefore, that dominant flavor detected in PH15 I Ch could be related to these compounds, indicating the inoculation influence in the final product.

4. Materials and Methods

4.1. Fermentation Experiments, Inoculation, and Sampling

The fermentation experiments were conducted at the Vale do Juliana cocoa farm in Igrapiúna, Bahia, Brazil. The ripe cocoa pods from PH15 were harvested during the main crop of 2015 (November).

The cocoa pods were manually opened with a machete, and the beans were immediately transferred to the fermentation house. The fermentation started approximately 4 h after the breaking of the pods and was performed in 0.06 m^3 wooden boxes [17]. The fermentations were conducted with 100 kg of PH15 cocoa beans. The fermentations were performed using a cocktail of microorganisms (PH15 I) as starter culture containing *S. cerevisiae* UFLA CCMA 0200 (LNF-CA11, LNF Latino America,

Bento Gonçalves, Rio Grande do Sul, Brazil), *Lactobacillus plantarum* CCMA 0238 and *Acetobacter pasteurianus* CCMA 0241 at the beginning of the process and without inoculation (PH15 NI-control). These microorganisms were reported in previous studies on cocoa fermentation around the world, mainly in Brazil [3,16,18,20,21]. The pH value and temperature were evaluated during the fermentations.

All of the microbial strains used in the study are preserved at the Culture Collection of Agricultural Microbiology of the Federal University of Lavras (CCMA, Lavras, Minas Gerais, Brazil, WDCM 1083). The *S. cerevisiae* UFLA CCMA 0200, which is commercialized by LNF (CA11), was weighed (as recommended by the manufacturer's instructions) and mixed in the solution to reach a population of approximately 10^7 cells/g of cocoa.

The *Lactobacillus plantarum* and *Acetobacter pasteurianus* species were grown in MRS broth (De Man, Rogosa and Sharpe, Merck, Darmstadt, Germany) and YPD broth (10 g/L yeast extract (Merck); 20 g/L peptone (Himedia); 20 g/L dextrose (Merck), respectively, at 30 °C and 150 rpm, and replicated every 24 h. The cells were recovered by centrifugation (7000 rpm, 10 min) and re-suspended in 1 L of sterile peptone water (1 g/L peptone (Himedia, Mumbai, India)). This solution was spread over the cocoa beans, reaching a concentration of approximately 10^5 cells/g of cocoa.

The samples were taken every 24 h during 144 h of fermentation and placed in sterile plastic pots. The samples were stored at −20 °C. The fermentations were performed in triplicate [32].

4.2. Culture-Independent Microbiological Analysis

4.2.1. DNA Extraction and Polymerase Chain Reaction

The total DNA extraction and PCR reaction from the cocoa pulp were conducted as previously described [3]. Cocoa pulp DNA total was extracted with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and stored at −20 °C.

The bacterial DNA was amplified with the primers 338fgc (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3′) (the GC clamp is underlined) and 518r (5′-ATT ACCGCG GCTGCT GG-3′). The DNA from the eukaryotic community was amplified with the primers NL1GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGGGCA TAT CAA TAA GCG GAG GAA AAG-3′) (the GC clamp is underlined) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′). All reactions were performed in 25 µL containing 0.625 U Taq DNA polymerase (Promega, Madison, WI, USA), 2.5 µL 10 X buffer, 0.1 mMdNTP, 0.2 mM of each primer, 1.5 mM $MgCl_2$, and 1 µL of extracted DNA. The amplification was performed as previously described [4]. The amplified products (2 µL) were analyzed by electrophoresis on 1% agarose gels before the DGGE analysis.

4.2.2. PCR–DGGE Analysis

To conduct the DGGE analyses, the PCR products were analyzed using a Bio-Rad DCode universal mutation detection system (Bio-Rad, Richmond, CA, USA). The PCR products were purified, sequenced, and available according to the procedures previously described [3]. Denaturant solutions containing 35–70% (100% denaturant contains 7 M urea and 40% (*v*/*v*) formamide) were used for bacteria, and containing 30–60% for yeast. The electrophoresis was run at 60 °C for 6 h at a constant voltage of 120 V.

4.3. Chromatographic Analysis

4.3.1. Sugars, Alcohols, and Organic Acid Extraction and HPLC Analyses

The carbohydrates, alcohols, and organic acids were extracted (from pulp and from the content inside the beans) and analyzed as described in previous work [3]. The analyses were determined by HPLC (Shimadzu, model LC-10Ai, Shimadzu Corp., Kyoto, Japan) equipped with a dual detection system consisting of a Ultraviolet-Visible (UV–Vis) detector (SPD 10Ai) and a refractive index detector (RID-10Ai). The HPLC was operated at 50 °C for acids and detected via UV absorbance (210 nm), while the alcohols and carbohydrates were examined at 30 °C and detected via Refractive Index Detector (RID). The column used for separation was a Shimadzu ion exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm, Shimadzu, Kyoto, Japan) with a mobile phase of Perchloric acid (100 mM) at a flow rate of 0.6 mL/min. All samples were analyzed in triplicate.

The chemical compounds used as standards (purity N 99.8%), glucose, fructose, and citric acid, were purchased from Sigma-Aldrich (Saint Louis, MO, USA); acetic acid and ethanol were purchased from Merck (Darmstadt, Germany); and lactic acid was purchased from Fluka Analyticals (Seelze, Germany).

4.3.2. Characterization of Volatile Compounds by Headspace-Solid Phase Microextraction Gas Chromatography-Mass Spectrometry

The volatile compounds from cocoa samples were extracted using the Headspace-Solid Phase Microextraction (HS–SPME) technique, as described in previous research [24], with modifications. Briefly, cocoa samples (2.0 g) from the beginning and end of fermentation (0 h and 144 h) and chocolate samples (2.0 g) were macerated using liquid nitrogen for headspace analysis. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 mm SPME fiber (Supelco Co., Bellefonte, PA, USA.) was used to extract volatile constituents from the cocoa and chocolate headspace. The fiber was equilibrated for 15 min at 60 °C and then exposed to the samples (cocoa and chocolate) for 30 min at the same temperature.

The compounds were analyzed using a Shimadzu GC model QP2010 equipped with a mass spectrometry and a capillary column of silica DB-FFAP (25 m × 0.25 mm i.d. × 0.25 mm). The temperature program began with 5 min at 50 °C, followed by a gradient of 50 °C to 190 °C at 3 °C/min; the temperature was then maintained at 190 °C for 10 min. The injector and detector temperatures were maintained at 230 °C.

The carrier gas (He) was used at a flow rate of 1.2 mL/min. Injections were performed by fiber exposition for 5 min. Volatile compounds were identified by comparing the mass spectra of the compounds in the samples with the database of the National Institute of Standards and Technology (NIST library, Gaithersburg, MD, USA) and the retention time with literature data using the n-Alkane index. All samples were examined in duplicate.

4.4. Sensory Analysis

After fermentation, the beans were dried in the sun inside drying greenhouses. Thereafter, the dried beans were sent for chocolate production at Sartori and Pedroso Alimentos Ltda. (São Roque, São Paulo, Brazil). Dark chocolate (100 g chocolate bar) was produced (62% liquor, 30% icing sugar, 8% cocoa butter). The molded chocolate was rapped and stored at 4 °C for four weeks before sensory analysis.

For sensory analysis, the chocolate was kept at room temperature (±20 °C) for two hours before the tests. The attributes involved in the TDS analysis were established by the Kelly grid method ("Kelly's repertory grid method") [33]. The TDS analysis was performed with 31 selected and trained judges. The judges evaluated differences between the two chocolate samples (PH15 I Ch (from inoculated fermentation) and PH15 NI Ch (from non-inoculated fermentation)) during the tasting time (the analysis time was 35 s, with an addition of delay time 2 s) and the attributes selected were acid, bitterness, nutty, sweetness, astringent, coffee, fruity, and cocoa.

The judges were asked to choose the dominant flavor over the analysis time. The dominant flavor is that perceived with greater clarity and intensity among the other ones [34]. The samples (approximately 2.5 g of chocolate) were presented in plastic cups, coded with a three-digit bar. Crackers and water were provided for palate cleansing. The analysis was performed in triplicate.

In order to calculate the TDS curves for all analyses, the software SensoMaker, version 1.8 was used [35]. Two lines were drawn on graphics: the "chance level" and the "significance level". The "chance level" is the dominance rate that an attribute can obtain by chance. The "significance level" is the minimum value of this ratio to be considered significant.

4.5. Statistical Analyses

Analyses of the variance and the Scott–Knott test were performed with SISVAR 5.1 software (Federal University of Lavras, Department of Statistic, Lavras, MG, Brazil). Differences in values were considered significant when the p value was less than 0.05 ($p < 0.05$).

5. Conclusions

The inoculation of microorganisms as a starter culture accelerated the fermentation process. The bacterial and yeast communities were different according to each process (PH15 I and PH15 NI), but the bacteria (*Gluconobacter oxydans*, *Lactobacillus plantarum*, *Acetobacter pasteurianus*, *Fructobacillus pseudoficulneus*) and yeast (*Saccharomyces cerevisiae* and *Pichia kluyveri*) species were found in both processes. Glucose and fructose were consumed faster in the inoculated assay in the first 24 h of fermentation. Different volatile compounds were identified in fermented beans and chocolate produced in the present study. According to the sensory analysis of PH15 I Ch and PH15 NI Ch significant differences on the dominant tastes were observed. The inoculation leads to a chocolate with higher bitter, sweet, and cocoa notes than the chocolate produced by spontaneous fermentation. Bitter was the dominant taste in PH15 NI Ch, whereas bitter, sweet, and cocoa tastes were dominant tastes in PH15 I Ch. These results were corroborated by the analysis of volatile compounds in both chocolate samples. The 2,3-butanediol, which gives flavor to cocoa butter (sweet chocolate), and 2,3-dimethylpyrazine, which gives caramel and cocoa flavors, were detected only in PH15 I Ch. Therefore, that dominant flavor detected in PH15 I Ch was related with these compounds, indicating the inoculation influence in the final product. In this context, the inoculation influenced the fermentation process and the final product. In order to generate a standardized fermentative process and improve the chocolate quality, *Saccharomyces cerevisiae* UFLA CCMA 0200, *L. Plantarum* CCMA 0238, and *A. pasteurianus* CCMA 0241 are, herein, proposed to be used as a cocktail of microorganisms for application in cocoa fermentation.

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Author Contributions: Rosane Freitas Schwan, Nelson Lima, and Cledir Santos conceived and designed the experiments and made the final revision of the paper; Igor Magalhães da Veiga Moreira and Leonardo de Figueiredo Vilela performed the experiments and wrote the paper; Maria Gabriela da Cruz Pedroso Miguel, Igor Magalhães da Veiga Moreira, and Leonardo de Figueiredo Vilela analyzed the data.

Conflicts of Interest: Authors declare no conflicts of interest.

References

1. Pereira, J.L.; Ram, A.; Figueiredo, J.M.; Almeida, L.C.C. The first occurrence of "witches' broom" disease in the principal cocoa growing region of Brazil. *Trop. Agric.* **1990**, *67*, 188–189.
2. Lopes, U.V.; Monteiro, W.R.; Pires, J.L.; Clement, D.; Yamada, M.M.; Gramacho, K.P. Cacao breeding in Bahia, Brazil: Strategies and results. *Crop Breed. Appl. Biotechnol.* **2011**, *1*, 73–81.
3. Moreira, I.M.V.; Miguel, M.G.C.P.; Duarte, W.F.; Dias, D.R.; Schwan, R.F. Microbial succession and the dynamics of metabolites and sugars during the fermentation of three different cocoa (*Theobroma cacao* L.) hybrids. *Food Res. Int.* **2013**, *54*, 9–17.
4. Ramos, C.L.; Dias, D.R.; Miguel, M.G. C.P.; Schwan, R.F. Impact of different cocoa hybrids (*Theobroma cacao* L.) and *S. cerevisiae* UFLA CA11 inoculation on microbial communities and volatile compounds of cocoa fermentation. *Food Res. Int.* **2014**, *64*, 908–918.
5. Clapperton, J.F.; Lockwood, R.; Yow, S.T.K.; Lim, D.H.K. Effects of planting materials on flavour. *Cocoa Grow. Bull*. **1994**, *48*, 47–63.
6. Efraim, P.; Pires, J.L.; Garcia, A.O.; Grimaldi, R.; Luccas, V.; Pezoa-Garcia, N.H. Characteristics of cocoa butter and chocolates obtained from cocoa varieties grown in Bahia, Brazil. *Eur. Food Res. Technol.* **2013**, *237*, 419–428.
7. Menezes, A.G.T.; Batista, N.N.; Ramos, C.L.; Silva, A.R.A.; Efraim, P.; Pinheiro, A.C.M.; Schwan, R.F. Investigation of chocolate produced from four different Brazilian varieties of cocoa (*Theobroma cacao* L.) inoculated with *Saccharomyces cerevisiae*. *Food Res. Int.* **2016**, *81*, 83–90.
8. Oliveira, B.F.; Silva, S.D.V.M.; Damaceno, V.O.; Filho, L.P.S. Identificação de fontes de resistência a *Ceratocystis cacaofunesta* em mudas de cacaueiro. *Agrotópica* **2009**, *21*, 83–88.
9. Pires, J.L.; Rosa, E.S.; Macêdo, M.M. Avaliação de clones de cacaueiro na Bahia, Brasil. *Agrotópica* **2012**, *24*, 79–84.
10. Silva, S.D.V.M.; Pinto, L.R.M.; Oliveira, B.F.; Damaceno, V.O.; Pires, J.L.; Dias, C.T.S. Resistência de progênies de cacaueiro à murcha-de-Ceratocystis. *Trop. Plant Pathol.* **2012**, *37*, 191–195.
11. Biehl, B.; Meyer, B.; Crone, G.; Pollmann, L.; Said, M.B. Chemical and physical changes in the pulp during ripening and post-harvest storage of cocoa pods. *J. Sci. Food Agric*. **1989**, *48*, 189–208.
12. Schwan, R.F. Cocoa fermentations conducted with a defined microbial cocktail inoculum. *Appl. Environ. Microbiol.* **1998**, *64*, 1477–1483.
13. Afoakwa, E.O.; Paterson, A.; Fowler, M.; Ryan, A. Flavor formation and character in cocoa and chocolate: A critical review. *Crit. Rev. Food Sci*. **2008**, *48*, 840–857.
14. Pereira, G.V.M.; Miguel, M.G.C.P.; Ramos, C.L.; Schwan, R.F. Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast

and bacteria strains to develop a defined starter culture. *Appl. Environ. Microb*. **2012**, *78*, 5395–5405.

15. Schwan, R.F.; Pereira, G.V.M.; Fleet, G.H. Microbial activities during cocoa fermentation. In *Cocoa and Coffee Fermentations*; Schwan, R.F., Fleet, G.H., Eds.; CRC Press: Boca Raton, FL, USA, 2014; pp. 129–190.
16. Ardhana, M.; Fleet, G. The microbial ecology of cocoa bean fermentations in Indonesia. *Int. J. Food Microbiol*. **2003**, *86*, 87–99.
17. Schwan, R.F.; Wheals, A.E. The microbiology of cocoa fermentation and its role in chocolate quality. *Crit. Rev. Food Sci.* **2004**, *44*, 205–221.
18. Jespersen, L.; Nielsen, D.S.; Hønholt, S.; Jakobsen, M. Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. *FEMS Yeast Res.* **2005**, *5*, 441–453.
19. Nielsen, D.S.; Teniola, O.D.; Ban-Koffi, L.; Owusu, M.; Andersson, T.S.; Holzapfel, W.H. The microbiology of Ghanaian cocoa fermentations analysed using culture dependent and culture independent methods. *Int. J. Food Microbiol.* **2007**, *114*, 168–186.
20. Pereira, G.V.M.; Magalhães, K.T.; Almeida, E.G.; Coelho, I.S.C.; Schwan, R.F. Spontaneous cocoa bean fermentation carried out in a novel-design stainless steel tank: Influence on the dynamics of microbial populations and physical-chemical properties. *Int. J. Food Microbiol.* **2013**, *161*, 121–133.
21. Camu, N.; De Winter, T.; Addo, S.K.; Takarama, J.S.; Bernaert, H.; De Vuyst, L. Fermentation of cocoa beans: Influence of microbial activities and polyphenol concentrations on the flavour of chocolate. *J. Sci. Food Agric.* **2008**, *88*, 2288–2297.
22. Papalexandratou, Z.; Vranckena, G.; De Bruyneb, K.; Vandammeb, P.; De Vuys, L. Spontaneous organic cocoa bean box fermentations in Brazil are characterized by a restricted species diversity of lactic acid bacteria and acetic acid bacteria. *Food Microbiol.* **2011**, *28*, 1326–1338.
23. Schwan, R.F.; Rose, A.H.; Board, R.G. Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. *J. Appl. Bacteriol.* **1995**, *79*, 96S–107S.
24. Rodriguez-Campos, J.; Escalona-buendía, H.B.; Orozco-Avila, I.; Lugo-Cervantes, E.; Jaramillo-Flores, M.E. Dynamics of volatile and non-volatile compounds in cocoa (*Theobroma cocoa* L.) during fermentation and drying process using principal components analysis. *Food Res. Int.* **2011**, *44*, 250–258.
25. Ho, V.T.T.; Zhao, J.; Fleet, G. Yeasts are essential for cocoa bean fermentation. *Int. J. Food Microbiol.* **2014**, *174*, 72–87.
26. Albertini, B.; Schoubben, A.; Guarnaccia, D.; Pinelli, F.; Vecchia, M.D.; Ricci, M.; Renzo, G.C.D.; Blasi, P. Effect of Fermentation and Drying on Cocoa Polyphenols. *J. Agric. Food Chem*. **2015**, *63*, 9948–9953.
27. Rodriguez-Campos, J.; Escalona-Buendía, H.; Contreras-Ramos, S.; Orozco-Avila, I.; Jaramillo-Flores, E.; Lugo-Cervantes, E. Effect of fermentation time and drying temperature on volatile compounds in cocoa. *Food Chem.* **2012**, *132*, 277–288.
28. Serra-Bonvehí, J. Investigation of aromatic compounds in roasted cocoa powder. *Eur. Food Res. Technol.* **2005**, *221*, 19–29.

29. Aculey, P.; Snitkjaer, P.; Owusu, M.; Bassompiere, M.; Takrama, J.; Nørgaard, L. Ghanaian cocoa bean fermentation characterized by spectroscopic and chromatographic methods and chemometrics. *J. Food Sci.* **2010**, 75, S300–S307.
30. Frauendorfer, F.; Schieberle, P. Changes in key aroma compounds of Criollo cocoa beans during roasting. *J. Agric. Food Chem.* **2008**, *56*, 10244–10251.
31. Luna, F.; Crouzillat, D.; Cirou, L.; Bucheli, P. Chemical Composition and Flavor of Ecuadorian Cocoa Liquor. *J. Agric. Food Chem*. **2002**, *50*, 3527–3532.
32. Batista, N.N.; Ramos, C.L.; Ribeiro, D.D.; Pinheiro, A.C.M.; Schwan, R.F. Dynamic behavior of *Saccharomyces cerevisiae*, *Pichia kluyv*eri and *Hanseniaspora uvarum* during spontaneous and inoculated cocoa fermentations and their effect on sensory characteristics of chocolate. *LWT Food Sci. Technol*. **2015**, *63*, 221–227.
33. Moskowitz, H.R. *Product Testing and Sensory Evaluation of Foods: Marketing and R &D Approaches*; Food & Nutrition Press: Westport, CT, USA, 1983.
34. Pineau, N.; Schich, P.; Cordelle, S.; Mathonnièrea, C.; Issanchouc, S.; Imbertd, A.; Köster, E. Temporal dominance of Sensations: Construction of the TDS curves and comparison with time intensity. *Food Qual. Pref.* **2009**, *20*, 450–455.
35. Nunes, C.A.; Pinheiro, A.C.M. *SensoMaker Software Version 1.8*; Universidade Federal de Lavras: Lavras, Brazil, 2002.