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GILSON CELSO ALBUQUERQUE CHAGAS JUNIOR

FERMENTAÇÃO DE CACAU:
PROCESSO CONDUZIDO COM LEVEDURAS SELECIONADAS, DIVERSIDADE MICROBIOLÓGICA E PERFIL AROMÁTICO

BELÉM – PA

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Tese apresentada ao Programa de Pós-graduação em Ciência e Tecnologia de Alimentos da Universidade Federal do Pará como requisito para a obtenção do título de Doutor em Ciência e Tecnologia de Alimentos.

Orientadora: Profª. Drª. Alessandra Santos Lopes

Coorientador: Prof. Dr. Nelson Rosa Ferreira

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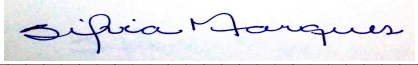

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“Depois de plantada a semente do bambu chinês, não se vê nada por aproximadamente 5 anos – exceto um diminuto broto. Todo o crescimento é subterrâneo; uma complexa estrutura de raiz, que se estende vertical e horizontalmente pela terra, está sendo construída. Então, ao final do 5º ano, o bambu chinês cresce até atingir a altura de 25 metros. Muitas coisas na vida pessoal e profissional são iguais ao bambu chinês. Você trabalha, investe tempo, esforço, faz tudo o que pode para nutrir seu crescimento e, às vezes, não vê nada por semanas, meses ou anos. Mas, se tiver paciência para continuar trabalhando, persistindo e nutrindo, o seu 5º ano chegará; com ele virão mudanças que você jamais esperava. Lembre-se que é preciso muita ousadia para chegar às alturas e, ao mesmo tempo, muita profundidade para agarrar-se ao chão.”

(Autor desconhecido)

“Quando tudo parecer estar contra ti, lembra. Os aviões não decolam com o vento a favor, mas sim, contra.”

Henry Ford

Foto: Gilson Chagas Junior®

RESUMO

As sementes fermentadas e secas de cacau são a matéria-prima para a produção de um dos alimentos mais consumidos a nível mundial: o chocolate. Para que ocorra a formação dos compostos químicos essenciais para os sabores e aromas característicos do chocolate de boa qualidade, a fermentação das sementes é a etapa crucial, onde serão formados tais compostos, resultado da atividade microbiana desenvolvida ao longo do processo. Uma sucessão microbiana, muito bem definida, envolvendo leveduras, bactérias láticas e acéticas torna o meio fermentativo propício para as inúmeras transformações físicas e químicas no interior da semente, que serão finalizadas nas etapas posteriores de secagem e torração. O Estado do Pará é o maior produtor brasileiro de cacau e desponta como referência na produção de amêndoas fermentadas e secas de excelente qualidade no mercado nacional e internacional. Visando em conhecer o papel que duas espécies de leveduras previamente isoladas e identificadas em fermentação de cacau no município de Tomé-Açu exercem durante o processo, este trabalho teve como objetivo principal avaliar a adição de culturas starter de *Saccharomyces cerevisiae* e *Pichia kudriavzevii* nas propriedades microbiológicas, físicas e químicas ao longo do tempo do processo. Reativou-se as culturas de ambas as espécies e após a produção dos inóculos, adicionou-se às fermentações in loco, onde foram avaliadas por períodos de 7 dias, exceto para a fermentação que recebeu um inóculo misto (proporções iguais das duas espécies), que teve duração de 6 dias. Ao longo do tempo das fermentações, notou-se um rápido consumo de açúcares redutores totais, incremento na temperatura, redução dos teores de compostos fenólicos, acidez e aumento do pH nos tratamentos que receberam inóculos com *P. kudriavzevii*, além de proporcionarem a obtenção de amêndoas fermentadas e secas com maiores quantidades de catequina e epicatequina (atividade antioxidante), metilxantinas (atividade metabólica), baixos teores de acidez e de aminas bioativas putrefativas como cadaverina e putrescina. A formação de feniletilamina foi observada também nesses tratamentos com *P. kudriavzevii*, evidenciando uma sinergia microbiana com a microbiota naturalmente encontrada nas fermentações. Com relação aos compostos voláteis identificados, a quantidade de cetonas, aldeídos e ésteres foram maiores nas amêndoas fermentadas com a adição de inóculos de *P. kudriavzevii* o que evidencia a importância dessa espécie de levedura em produção de compostos voláteis com atribuições de aromas frutados, florais e doces. Todos os achados nesta pesquisa estão fortemente ligados com a microbiota existente durante etapa de fermentação de cacau. Foi possível identificar espécies de bactérias láticas que nunca antes isoladas em fermentações de cacau na região amazônica, o que pode ser um dos diferenciais para a produção de amêndoas de cacau de boa qualidade. Pôde-se concluir que a adição de inóculos microbianos proporcionou a obtenção de amêndoas fermentadas e secas de boa qualidade, reforçando a necessidade de mais investimentos a pesquisas para auxiliar os produtores rurais da região amazônica a despontarem na concorrência do mercado de chocolate.

Palavras-chave: Chocolate. Compostos voláteis. Microrganismos. Aminas bioativas. Culturas starter.

ABSTRACT

Fermented and dried cocoa beans are the raw material for the production of one of the most consumed foods worldwide: chocolate. For the formation of the essential chemical compounds for the characteristic flavors and aromas of good quality chocolate, seed fermentation is the crucial stage, where such compounds will be formed, as a result of the microbial activity developed throughout the process. A very well-defined microbial succession involving yeasts, lactic and acetic bacteria makes the fermentation medium conducive to the numerous physical and chemical reactions inside the seed, which will be finalized in the subsequent drying and roasting steps. The State of Pará is the largest Brazilian cocoa producer and stands out as a reference in the production of fermented and dried cocoa beans of excellent quality in the national and international market. To understand the role that two yeast species previously isolated and identified in cocoa fermentation in the city of Tomé-Açu play during the process, this study aimed to evaluate the addition of *Saccharomyces cerevisiae* and *Pichia kudriavzevii* starter cultures in the microbiological properties physical and chemical over the process time. The cultures of both species were reactivated and after the inoculum production, it was added to the fermentations in loco, where they were evaluated for periods of 7 days, except for the fermentation that received a mixed inoculum (equal proportions of the two species), that lasted 6 days. Over the time of the fermentations, there was a rapid consumption of total reducing sugars, an increase in temperature, a reduction in the contents of phenolic compounds, acidity and an increase in pH in treatments that received inoculations with *P. kudriavzevii*, in addition to providing fermented and dried cocoa beans with higher amounts of catechin and epicatechin (antioxidant activity), methylxanthines (metabolic activity), low levels of acidity and putrefactive bioactive amines such as cadaverine and putrescine. The formation of phenylethylamine was also observed in these treatments with *P. kudriavzevii*, showing a microbial synergy with the microbiota naturally found in fermentations. Regarding the volatile compounds identified, the amount of ketones, aldehydes and esters was higher in fermented almonds with the addition of inocula of *P. kudriavzevii*, which shows the importance of this yeast species in the production of volatile compounds with fruity, floral and sweets flavors. All the findings in this research are strongly linked to the existing microbiota during the fermentation of cocoa. It was possible to identify species of lactic acid bacteria that were never isolated before in cocoa fermentations in the Amazon region, which can be one of the differentials for the production of good quality cocoa beans. It was concluded that the addition of microbial inocula provided good quality fermented and dried cocoa beans, reinforcing the need for more investments in research to help rural producers in the Amazon region to emerge in the competition of the chocolate market.

Keywords: Chocolate. Volatile compounds. Microorganisms. Bioactive amines. Starter cultures.

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LISTA DE ABREVIATURAS E SIGLAS

AAB	Acetic Acid Bacteria
ANOVA	Analysis of Variance
CEPLAC	Comissão Executiva do Plano da Lavoura Cacaueira
CF	Control fermentation
CT	Control treatment
eV	Elétron-volt
FID	Flame Ionization Detector
GC-MS	Gas Chromatography – Mass Spectrometry
GYC	Meio Levedura-Glicose com Carbonato de Cálcio
HCA	Hierarquical Cluster Analysis
HPLC	High Performance Liquid Chromatography
ICCO	International Cocoa Organization
LAB	Lactic Acid Bacteria
LOD	Limit of Detection
LOQ	Limit of Quantification
meq NaOH 0.1N/100 g	Miliequivalente de Hidróxido de Sódio 0.1N por 100 g de amostra
mg ECE/g	Miligramas equivalentes de epicatequina por grama de amostra
MRS	De Man, Rogosa & Sharpe
PCA	Principal Component Analysis
Pk	Fermentação com inóculo de *Pichia kudriavzevii*
PPO	Polifenoloxidase
PT	*P. kudriavzevii* treatment
RI	Retention Index
Sc	Fermentação com inóculo de *Saccharomyces cerevisiae*
ScPk	Fermentação com inóculo de *S. cerevisiae* e *P. kudriavzevii*
SPT	*S. cerevisiae* and *P. kudriavzevii* treatment
SSF	Solid-state fermentation
ST	*S. cerevisiae* treatment
TPC	Total Phenolic Compounds

LISTA DE ABREVIATURAS E SIGLAS

TRS	Total Reducing Sugars
TTA	Total Titratable Acidity
YPD	Yeast Extract Peptone Dextrose

SUMÁRIO

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INTRODUÇÃO GERAL

As pesquisas envolvendo a etapa de fermentação do cacau amazônico, ganharam notoriedade nos últimos anos. Vários fatores são responsáveis por esse acontecimento: maior divulgação do produto no mercado internacional, maiores investimentos na área da pesquisa para descobrir novas técnicas de produção e o desponte do Estado do Pará, na retomada da liderança da produção nacional (CEPLAC, 2019).

Um dos fatores cruciais para se alcançar um chocolate de qualidade é o processo fermentativo, que ocorre nas sementes do cacau, que, quando conduzido corretamente, dará as condições ideais para o desenvolvimento das propriedades sensoriais necessárias para a produção de um chocolate de boa qualidade.

A fermentação é responsável pelas complexas reações bioquímicas que provocam a morte do embrião, a hidrólise de açúcares e proteínas, a liberação de enzimas e substratos, a difusão de compostos fenólicos (que entram em contato com as enzimas) (SCHWAN; WHEALS, 2004). O tempo requerido para a fermentação das sementes é variável, dependendo do material genético da cultura cacaueira. Como exemplo, para a ocorrência das principais reações que levam à formação dos principais precursores de sabor do chocolate, as sementes do cacau do grupo *Forastero* (variedade predominante no Brasil) devem ser fermentadas por um período de cinco a oito dias (OZTURK; YOUNG, 2017).

O acompanhamento de alguns parâmetros é utilizado como referência para uma fermentação bem sucedida, dentre eles: o tempo de processamento, a temperatura do ambiente e da massa fermentativa, a frequência da prática de revolvimento, o pH e a acidez da polpa, o sistema de fermentação e a microbiota existente (CAMU et al., 2007).

As leveduras são os primeiros microrganismos atuantes no processo de fermentação do cacau, as quais convertem os açúcares presentes na polpa em etanol e produzem enzimas que degradam a pectina presente, favorecendo as condições de

crescimento de outros microrganismos (SCHWAN; WHEALS, 2004). As leveduras são microrganismos bastante versáteis, em termos de atividade metabólica, especialmente fisiológica (BADER et al., 2010). Por isso, isolar e identifica-las vem a ser de grande importância para avaliar a sua diversidade e distribuição durante a fermentação do cacau.

A diversidade de espécies de leveduras presente na fermentação do cacau, tem sido frequentemente associada às diferentes localidades de fermentação, bem como as condições do processo (nutrientes disponíveis, pH, temperatura, oxigenação, entre outros). De fato, vários estudos revelam uma ampla diversidade de leveduras na fermentação do cacau, em diferentes países (CHAGAS JUNIOR; FERREIRA; LOPES, 2020; FIGUEROA-HERNÁNDEZ et al., 2019).

Além de serem responsáveis pela produção do etanol e liberação de enzimas que auxiliam na hidrólise de polissacarídeos (como a pectina), as leveduras são produtoras de ésteres e álcoois superiores, que têm sigo associados como contribuintes para a complexa mistura de compostos voláteis, que conferem o aroma e o sabor característicos do chocolate (CRAFACK et al., 2013; RAMOS et al., 2014; SCHWAN; WHEALS, 2004).

A importância deste estudo se dá em conhecer o desempenho que duas espécies de leveduras (anteriormente isoladas e identificadas durante a fermentação de cacau em Tomé-Açu) exerce durante o processo, por serem o primeiro grupo microbiano que atua durante a fermentação. Conhecer os produtos formados durante esta importante etapa do beneficiamento do cacau e suas influências sobre a qualidade das amêndoas fermentadas e secas são objetivos deste estudo. Assim será possível contribuir para a cadeia produtiva do cacau, com alternativas para o melhoramento do processo.

Este trabalho está dividido em 4 capítulos, onde são abordados, no:

- **Capítulo I:** o artigo de revisão *"The microbiota diversity identified during the cocoa fermentation and the benefits of the starter cultures use: an overview"* (https://doi.org/10.1111/ijfs.14740) com as informações necessárias e atuais sobre o tema desta tese para sua melhor compreensão;

- **Capitulo II:** o artigo de pesquisa *"Chemical implications and time reduction of on-farm cocoa fermentation by Saccharomyces cerevisiae and Pichia kudriavzevii"* (https://doi.org/10.1016/j.foodchem.2020.127834) abordando as características físicas e químicas em amêndoas de cacau fermentadas com a adição de inóculos de leveduras isoladas e identificadas previamente durante a fermentação de cacau;
- **Capítulo III:** a *short communication "Profile of Volatile Compounds of On-Farm Fermented and Dried Cocoa Beans Inoculated with Saccharomyces cerevisiae KY794742 and Pichia kudriavzevii KY794725"* (https://doi.org/10.3390/molecules26020344), abordando o perfil de compostos voláteis formados nas amêndoas de cacau fermentadas e secas, após a adição de inóculos de leveduras;
- **Capítulo IV:** o manuscrito da *short communication* (submetida) *"The first report of Lactobacillus farraginis and Lactobacillus parafarraginis identified on-farm cocoa beans fermentation in Brazilian Amazon"* sobre as espécies de bactérias láticas isoladas e identificadas na fermentação espontânea do cacau.

OBJETIVOS E HIPÓTESE

- GERAL
    a) Utilizar inóculos padronizados de leveduras na condução do processo fermentativo do cacau, para melhoramento da qualidade do chocolate.
- ESPECÍFICOS
    a) Confeccionar uma revisão de literatura atualizada sobre o tema desta tese, com o objetivo de mostrar os avanços das pesquisas com inóculos microbianos na fermentação do cacau;
    b) Verificar se os inóculos de leveduras utilizados irão proporcionar mudanças físico-químicas desejadas à amêndoa do cacau, para a produção do chocolate;
    c) Estabelecer o perfil aromático nas amêndoas fermentadas e secas de cacau, conduzidas com inóculos de leveduras;
    d) Isolar e identificar as espécies de bactérias láticas durante a fermentação espontânea e natural do cacau.

- HIPÓTESE

Este estudo baseia-se na hipótese, de que a adição de inóculos de leveduras podem conferir amêndoas de cacau com características peculiares à atuação de determinadas espécies, aliados às técnicas de fermentação conduzidas pelo produtor. Baseia-se também, na sinergia microbiana com a microbiota naturalmente encontrada na fermentação, o que pode ser uma das explicações para os efeitos benéficos da adição de inóculos microbianos.

Capítulo I

The microbiota diversity identified during the cocoa fermentation and the benefits of the starter cultures use: an overview

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Manuscrito do artigo publicado no periódico **International Journal of Food Science & Technology** (Capes Qualis A2, 2019).

DOI: https://doi.org/10.1111/ijfs.14740

“The microbiota diversity identified during the cocoa fermentation and the benefits of the starter cultures use: an overview”

ABSTRACT

Cocoa fermentation is a process that is constantly in evidence in the scientific world, as it is the pillar for the production of one of the most consumed foods in the world: the chocolate. Along with well-applied fermentation techniques, careful handling, variety of fruit, the microbiota (or cocobiota) is responsible for imparting characteristic and desirable aromas to chocolate. In addition to providing the start of essential physical and chemical reactions inside the seed to reduce the astringency and natural bitterness of the seeds. The production of starter crops is an alternative to obtain fermented beans with specific desirable characteristics. In this review, we discuss the latest studies on cocobiota identification and the benefits of using starter cultures and we conclude that advances have been made in recent years with regard to improving the fermentation process after using starter cultures, like reducing time, producing desirable volatile compounds and inhibiting putrefactive compounds.

Keywords: chocolate, yeasts, lactic acid bacteria, acetic acid bacteria, volatile compounds, putrefactive amines.

1 INTRODUCTION

One of the crucial factors to obtain a chocolate of acceptable quality in the market is the fermentative process that occurs in cocoa beans, which when conducted correctly, will give the ideal conditions for the development of the sensory properties necessary for the production of the product. Fermentation is the stage where the complex biochemical reactions occur that cause the death of the cocoa seed embryo, hydrolysis of sugars and proteins, release of enzymes and substrates and diffusion of phenolic compounds that come into contact with enzymes (SCHWAN; WHEALS, 2004).

Fermentation is an essential step towards obtaining good quality cocoa beans. For the International Cocoa Organization (ICCO), good quality almonds are considered to be commercialized, those which: i) are fully fermented and dried; ii) it is free of smoke notes and/or other abnormal and/or uncharacteristic of beans well-fermented; iii) is free from any evidence of tampering; iv) has a uniform size; v) it is free of broken, fragmented grains and/or with pieces of bark; vi) is free of foreign matter (FERREIRA, 2017).

Within biotechnological processes, cocoa fermentation falls into a category of fermentation process called solid-state fermentation (SSF). In this way, we can consider that the wooden boxes (which are normally used in seed fermentations carried out in Brazil) are examples of static bioreactors, and as such, this implies a certain difficulty in aeration, homogeneous distribution of nutrients and difficulty in carrying out the scale-up.

The microbial diversity present in cocoa fermentation has often been associated with different fermentation locations, as well as the conditions of the fermentation process (such as, available nutrients, pH, temperature, oxygen, among others). In fact, several studies reveal a wide diversity of yeasts in cocoa fermentation in different countries (FIGUEROA-HERNÁNDEZ; MOTA-GUTIERREZ; FERROCINO, 2019).

Despite this, cocoa is one of the few fruits that has a complex and defined microbial succession during the fermentation process. Three most reported groups of microorganisms gain prominence during cocoa fermentation, among which they exert influence during the process: yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB), and more recently the participation of filamentous fungi is increasingly being studied. It is known that some species of filamentous fungi are natural producers of hydrolases that can corroborate in the fermentation stage (ARAÚJO et al., 2019).

Each group develops according to the good conditions of the environment in which they find themselves. The initial conditions of anaerobiosis allow the growth of yeasts that produce ethanol from the sugars present in the pulp. In sequence, LAB convert ethanol and citric acid present in the pulp into lactic acid and other substances and AAB produce acetic acid from the oxidation of ethanol, generating an increase in noticeable temperature and aromas of vinegar during fermentation. Pectinolytic yeasts secrete pectinase, which removes the pectin present in the pulp, and release of more

sugars in the medium, being fermented and thus enabling aeration, increasing the proliferation of AAB (DE VUYST; LEROY, 2020).

In this review, we aimed to collect data on the main microbiota present during cocoa fermentation in the last five years and to discuss the importance of using starter cultures with selected microorganisms.

2 THE COCOA FERMENTATION

The fermentation of cocoa beans is a biotechnological process that occurs most of the time, naturally and spontaneously. Within the beans processing chain for the production of chocolate, fermentation is the most important process, since it is there that the formation of various aromatic compounds occurs and reduces the astringency and natural bitterness of the seed through physical and chemical reactions initiated by the action groups of very specific microorganisms (SCHWAN; WHEALS, 2004; VISINTIN et al., 2017).

The fermentation stage begins after harvesting the fruit (Figure 1A), which comes into contact with natural sources of yeast, LAB and AAB. After opening the fruits to remove their seeds with seeds with pulp, the microorganisms present in the machetes that served to break the skin occur (Figure 1B), in the worker's hands (Figure 1C), in the contact of the fruit in the soil, and mainly, of the microbial groups. present in the containers that will serve for the process over the necessary time (AGYIRIFO et al., 2019; SCHWAN; WHEALS, 2004).

Figure 1 – Manual harvesting of the cocoa fruit (1A), cutting of the fruit from the peel to remove the seeds with pulp (1B), manual separation of the seeds with pulp from fruit placenta (1C).

Photos: The authors.

Fermentation techniques vary between some countries that are heavily fermented cocoa beans producers. This process can be carried with the seeds in heaps (Ghana and Ivory Coast), baskets (Ivory Coast), fermentation boxes (Brazil and Malaysia), trays (Ghana) and fermentation platforms (both last in Ecuador) (OZTURK; YOUNG, 2017).

In Brazil, the Executive Committee of the Cocoa Crop Plan (CEPLAC) advises that the fermentation be carried out in wooden boxes that are called fermentation troughs, for a period of not less than 5 days and not more than 7 days, in view the non-formation of the compounds necessary for the formation of flavour and aroma precursors and the non-death of the seed, and the formation of beans with brown colour and strong ammonia-like aroma, respectively (MARTINS et al., 2012).

The boxes suggested by CEPLAC are equipped with removable dividers in order to facilitate the practice of seed turning and small holes measuring 6 to 10 mm inside the boxes arranged at a distance of approximately 15 cm from each other, in order to draining of the liquid formed during fermentation (MARTINS et al., 2012).

Banana leaves are used because in addition to being a natural inoculum of microorganisms, they provide a control of the drying of the seeds in the superficial part of the trough and an excellent control of the fermentation temperature (SCHWAN; WHEALS, 2004), this last characteristic can also be combined with the use of burlap sacks.

During fermentation, it is important to practice the so-called rolling of the dough, which consists of moving the dough below to the top, thus favoring the aeration of the medium and evaporation of the acids produced by the microorganisms. In addition, this practice favors the microbial succession at the appropriate fermentation times, providing the development of the desired characteristics, which does not happen in a mass that does not undergo overturning (HAMDOUCHE et al., 2019).

As already mentioned, groups of microorganisms perform specific functions and reactions during fermentation in two distinct phases: the anaerobic phase and the aerobic phase.

2.1 THE ANAEROBIC PHASE

This phase lasts approximately 48 to 72 hours after breaking and opening the fruits. The pulp that surrounds the cocoa seed begins to liquefy, the result of the action of yeasts that consume naturally found sugars (glucose and fructose) and some organic acids, such as citric and malic. These initial reactions result in the production of ethanol and a gradual rise in temperature. Thanks to the action of the invertase enzyme produced by some yeasts, the sucrose present in the pulp is converted into glucose and fructose providing more substrate to the medium (DE VUYST; WECKX, 2016).

The LABs also ferment the sugars present in the pulp. Examples of dominant LAB in the fermentation process are *Lactobacillus plantarum* and *Lactobacillus fermentum* and some of the genera *Leuconostoc* and *Lactococcus*, characterized as aerotolerant bacteria, tolerant to acid and ethanol, in addition to being able to ferment the citric acid present in the pulp. The citric acid and the sugars present in the medium are converted into lactic acid, acetic acid and mannitol, triggering a decrease in pH, microbiological stability, producing taste precursor molecules and colour development within the grains (DE VUYST; WECKX, 2016; OZTURK; YOUNG, 2017).

The pectin present in the mucilaginous pulp that surrounds the cocoa seed is degraded by the action of pectinolytic enzymes that are produced by some yeast species such as *Pichia kudriavzevii* (Figure 2) (BASTOS et al., 2018) and some bacteria of the genus *Bacillus* (FIGUEROA-HERNÁNDEZ; MOTA-GUTIERREZ; FERROCINO, 2019). As a result of this degradation, the pulp is drained and the aeration of the medium begins to increase, thus facilitating the performance of microorganisms in the next phase.

2.2 THE AEROBIC PHASE

Acetic bacteria, especially species of the *Acetobacter* genus, develop during this phase. Acetic acid is considered one of the main metabolites produced by an

exothermic reaction (oxidizing ethanol to acetic acid), promoting embryo inactivation, increasing the permeability of the cell wall of the grain and the release of precursor molecules of aroma and chocolate flavour (BATISTA et al., 2015; CAMU et al., 2007; NIELSEN et al., 2007; SOUMAHORO et al., 2020).

During this phase, the internal temperature of the fermentation mass rises due to exothermic reactions that start with the oxidation of ethanol by acetic bacteria, reaching values above 40 ºC. This increase in temperature also triggers the degradation of phenolic compounds naturally found in cocoa seeds (catechin, epicatechin, for example) with the activation of the polyphenoloxidase enzyme (NAZARUDDIN et al., 2006).

Methylxanthines (caffeine, theobromine and theophylline) also undergo degradation during this phase, and are exudated by the seed husk and released with the upturning movements. Monitoring the decrease in phenolic compounds and methylxanthines is of great importance for the industry, since these substances add bitterness and astringency to the beans (CAMU et al., 2008; GOMES JÚNIOR et al., 2020), on the other hand, at adequate levels of consumption, they have stimulants, vasodilators, diuretics and muscle relaxants (LEITE et al., 2013). The theobromine and caffeine ratio has been used as a parameter of quality and differentiation of fine cocoa, which ensures the implementation of a real price in the market (HASHIMOTO et al., 2018).

3 MICROBIAL DIVERSITY DURING COCOA FERMENTATION

The importance that yeasts, LAB and AAB play throughout the cocoa fermentation process is known and elucidated by the specialized literature. Some species of these microbial groups are reported in fermentations conducted in different locations and this is important because it gives flavour identity to the final product, for example. We seek to bring together some of the species of the main microbial groups reported in the last five years in the scientific literature (2015-2020) in Table 1, for their biotechnological importance for the process.

Table 1 – Yeasts, lactic acid bacteria and acetic acid bacteria identified during the cocoa fermentation distributed in continents over the past five years (2015-2020).

(to be continued)

	Africa	**America**	**Asia**	**Oceania**
		References		
Yeasts				
Candida glabrata		20		
Candida krusei		19		
Candida magnolia		13		
Candida nitrativorans	18			
Candida rugosa		19		
Candida temnochilae		20		
Candida tropicalis	11			
Debaryomyces etchellsii		19		
Galactomyces geotrichum	11			
Hanseniaspora guilliermondii		17		10
Hanseniaspora opuntiae	9, 14	7, 17,19		
Hanseniaspora uvarum		5		
Kluyveromyces lactis	1			
Kluyveromyces marxianus				10
Pichia galeiforms	11			
Pichia kudriavzevii	11	4, 7, 16, 17		10
Pichia manshurica	9	2, 4, 7, 19		
Pichia mexicana			8	
Pichia pijperi	14			
Pichia sp.		19		
Saccharomyces cerevisiae	1, 11, 14	2, 3, 4, 12,16,19, 20		10
Saccharomycopsis amapae		20		
Saccharomycopsis crataegensis		20		
Starmerella bacillaris		20		
Toruslapora delbrueckii		22		
Wickerhamomyces anomalus	11			
Zygosaccharomyces bailii		2, 20		
Lactic Acid Bacteria (LAB)				
Enterococcus casseliflavus	6			
Enterococcus gallinarum		4		
Fructobacillus pseudoficulneus		20		
Lactobacillus amylovorus		17		
Lactobacillus brevis	1			
Lactobacillus cacaoum		17		
Lactobacillus coryniformis		13		

Table 1 – Yeasts, lactic acid bacteria and acetic acid bacteria identified during the cocoa fermentation distributed in continents over the past five years (2015-2020).

(to be continued)

	Africa	**America**	**Asia**	**Oceania**
		References		
Lactic Acid Bacteria (LAB)				
Lactobacillus curvatus		13		
Lactobacillus fermentum	1, 6, 9, 14	4, 12, 13		10
Lactobacillus mali		13		
Lactobacillus murinus		20		
Lactobacillus paraplantarum	6			
Lactobacillus pentosus				10
Lactobacillus plantarum	1, 6, 9, 19	4, 12, 13, 17		10
Lactobacillus pontis		20		
Lactobacillus reuteri		20		
Lactobacillus sakei		13		
Lactobacillus sp.		20		
Lactococcus lactis		4		
Lactococcus sp.		20		
Leuconstoc citreum	1			
Leuconostoc mesenteroides	1			
Leuconostoc sp.		20		
Pediococcus acidilactici		4, 17		
Pediococcus pentosaceus		20		
Weissella confusa		20		
Weissella paramesenteroides	6			
Acetic Acid Bacteria (AAB)				
Acetobacter ghanensis	21			
Acetobacter lovaniensis	9			
Acetobacter malorum	21			
Acetobacter nitrogenifigens	9			
Acetobacter okinawensis sp.	21			
Acetobacter pasteurianus	6, 9, 14, 21	12, 13, 17, 20		10
Acetobacter pomorum		17		
Acetobacter senegalensis	6	17		
Acetobacter sp.	9	20		
Acetobacter syzygii	6			
Acetobacter tropicalis	21	12		
Acidisphaera sp.		20		
Acinetobacter sp.		13		
Gluconobacter frateurii				10
Gluconobacter oxydans	21	13		

Table 1 – Yeasts, lactic acid bacteria and acetic acid bacteria identified during the cocoa fermentation distributed in continents over the past five years (2015-2020).

(conclusion)

	Africa	America	Asia	Oceania
		References		
Acetic Acid Bacteria (AAB)				
Gluconobacter sp.		20		
Komagataeibacter hansenii		20		
Roseomonas sp.		20		

Notes. 1: (AGYIRIFO et al., 2019), **2:** (ALMEIDA et al., 2018), **3:** (ARANA-SÁNCHEZ et al., 2015), **4:** (BASTOS et al., 2018), **5:** (BATISTA et al., 2015), **6:** (BORTOLINI et al., 2016), **7:** (FERNÁNDEZ MAURA et al., 2016), **8:** (GIBE; PANGAN, 2015), **9:** (HAMDOUCHE et al., 2015), **10:** (HO; ZHAO; FLEET, 2015), **11:** (KONÉ et al., 2016), **12:** (LEE et al., 2019), **13:** (MIGUEL et al., 2017), **14:** (MOTA-GUTIERREZ et al., 2018), **15:** (OUATTARA et al., 2017) **16:** (PEREIRA et al., 2017), **17:** (ROMANENS et al., 2018), **18:** (SAMAGACI et al., 2016), **19:** (SCHWENNINGER et al., 2016), **20:** (SERRA et al., 2019), **21:** (SOUMAHORO et al., 2020), **22:** (VISINTIN et al., 2017).

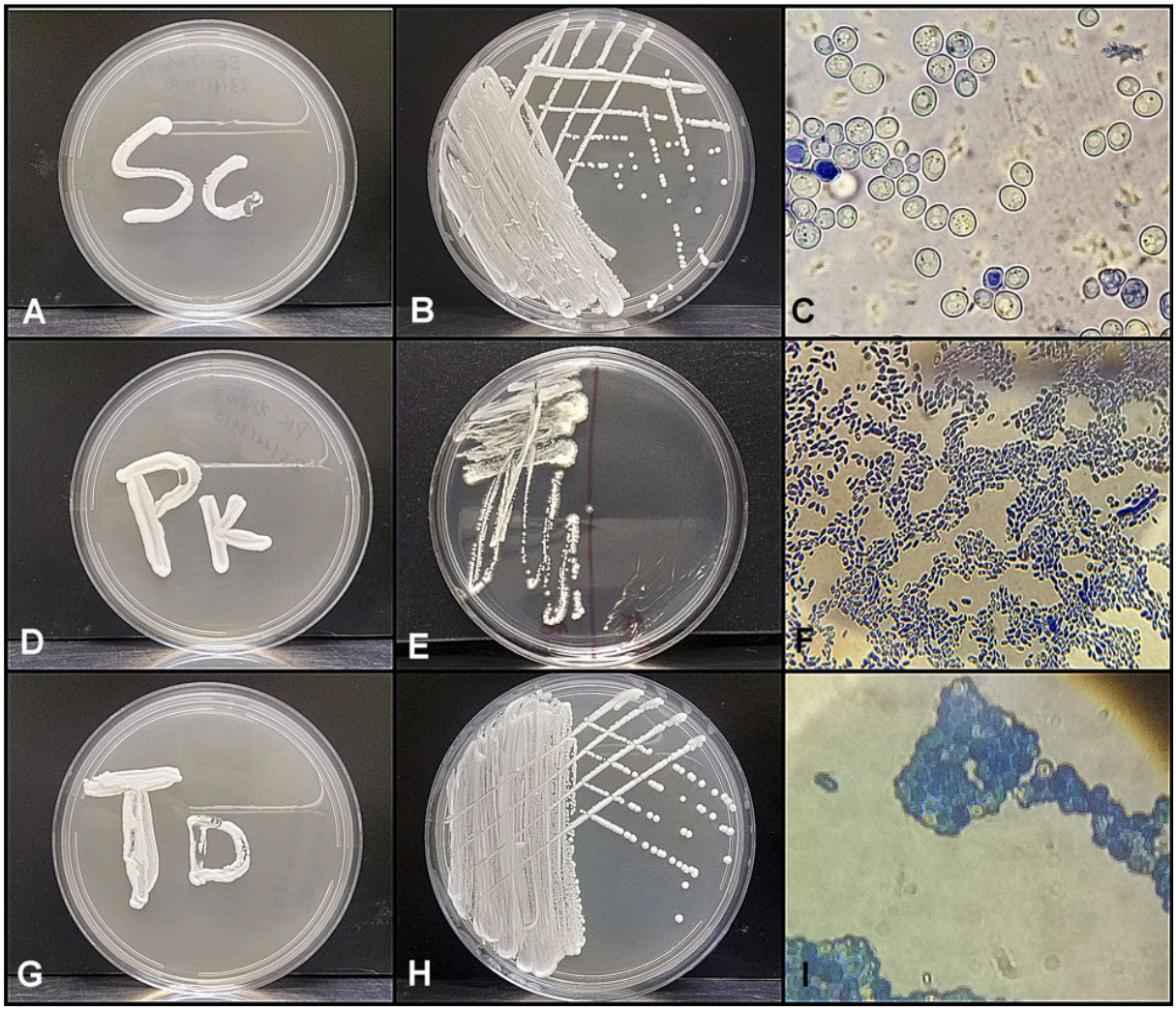
3.1 THE YEASTS

As shown in Table 1, in the last five years, four yeast species have been more present in studies on the identification of these microorganisms during natural cocoa fermentation: *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Pichia manshurica* and *Hanseniaspora opuntiae*.

Possibly, *S. cerevisiae* (Figure 2) is the most common species in cocoa fermentations in several locations: Ghana (AGYIRIFO et al., 2019; FIGUEROA-HERNÁNDEZ; MOTA-GUTIERREZ; FERROCINO, 2019; KONÉ et al., 2016), Mexico (ARANA-SÁNCHEZ et al., 2015), Brazil (BASTOS et al., 2018; SERRA et al., 2019), Honduras (ROMANENS et al., 2018) and in laboratory-scale studies (LEE et al., 2019). The importance of this species during the process is elucidated by the high fermentation capacity of the sugars present in the pulp in the initial moments, as well as result, with the formation of a large amount of ethanol and aromatic compounds that will give desirable notes to the fermented beans as raw material for the manufacture of chocolate, such as esters, aldehydes and other types of alcohols (BATISTA et al., 2015; KONÉ et al., 2016), in addition to producing protective toxins (BATISTA et al., 2015), thus avoiding, the proliferation of microorganisms undesirable to the process.

Species of *P. kudriavzevii* are also important producers of desirable aromas to chocolate and of high fermentative capacity (KONÉ et al., 2016; PEREIRA et al., 2017; SERRA et al., 2019), providing a considerable increase in the fermentation temperature , which does not affect its metabolic activity as it is a thermotolerant species (LI et al., 2018). On the other hand*, P. manshurica* together with *S. cerevisiae* are resistant and adapt to different environmental conditions (ALMEIDA et al., 2018).

Figure 2 – Some yeasts species identified during on-farm cocoa fermentation in the Brazilian Amazon.



Notes. Sc: *Saccharomyces cerevisiae*; **Pk:** *Pichia kudriavzevii*. **Td:** *Torulaspora delbrueckii*. Species reactivated and isolated on Petri dishes with YM agar (1% glucose, 0.3% peptone bacteriological, 0.3% yeast extract, 0.3% malt extract) (A, B, D, E, G, H) and morphological test on microscope (1000×) (C, F, I) with 1% methylene blue solution. **Photos:** The authors.

Hanseniaspora opuntiae is also responsible for the formation of good amounts of aromatic compounds during the first hours of fermentation in some studies (MOTA-GUTIERREZ et al., 2018) and is reported in locations like Ivory Coast (HAMDOUCHE

et al., 2015), Cuba (FERNÁNDEZ MAURA et al., 2016), Honduras (ROMANENS et al., 2018) and Bolivia (SCHWENNINGER et al., 2016).

The first study to identify yeasts in cocoa from the Brazilian Amazon identified distinct species between two locations in the Amazon region, Tucumã (*Pichia fermentans*, *P. kudriavzevii*, *P. manshurica*, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailli*) and Medicilândia (*P. manshurica* and *S. cerevisiae*) (ALMEIDA et al., 2018). This study also reports that *Z. bailli* was identified only 24 hours after the beginning of the fermentation process in Tucumã, which is favorable, since this species is related to the excessive production of CO_2 in wine fermentations, which can trigger undesirable notes of aroma and flavour.

3.2 LACTIC ACID BACTERIA (LAB)

The genus *Lactobacillus* is the most commonly found during the fermentation of cocoa beans (FIGUEROA-HERNÁNDEZ; MOTA-GUTIERREZ; FERROCINO, 2019) with the species *Lactobacillus fermentum* and *Lactobacillus plantarum* being the most identified in several locations (Table 1): Ghana (AGYIRIFO et al., 2019), Cameroon (BORTOLINI et al., 2016; MOTA-GUTIERREZ et al., 2018), Ivory Coast (HAMDOUCHE et al., 2015), Brazil (BASTOS et al., 2018; MIGUEL et al., 2017) and in the studies by (LEE et al., 2019) developed on a laboratory scale.

The role of LABs still provokes some discussion in the scientific world because some studies suggest that they are not necessary for the good performance of the cocoa fermentation process (HO; FLEET; ZHAO, 2018; HO; ZHAO; FLEET, 2014, 2015). However, other studies report the good interaction with yeasts in the first hours of fermentation, degrading the fermentable sugars of the pulp (glucose, fructose and sucrose), having a preference for fructose and converting them into lactic, acetic acid, ethanol, carbon dioxide and even in mannitol (DE VUYST; LEROY, 2020; DE VUYST; WECKX, 2016).

The LABs are directly related to the production of biogenic amines as a way of protecting bacteria from adverse pH and temperature conditions, resulting from the decarboxylation of amino acids caused by the production of decarboxylase enzymes.

For example, ornithine undergoes decarboxylation by ornithine decarboxylase and forms putrescine, responsible for undesirable flavors and aromas that are characteristics of spoilage foods (OZOGUL et al., 2015). The bacterial variability of different locations may be the determining factor that confers good quality fermented beans, thus reinforcing its importance for fermentation (OUATTARA et al., 2017).

3.3 ACETIC ACID BACTERIA (AAB)

The diversity of these species is practically restricted to two genera: *Acetobacter* and *Gluconobacter* (Table 1). The species *Acetobacter pasteurianus* being the most identified in fermentation processes in Cameroon (BORTOLINI et al., 2016; MOTA-GUTIERREZ et al., 2018), Ivory Coast (HAMDOUCHE et al., 2015; SOUMAHORO et al., 2020), Brazil (MIGUEL et al., 2017; SERRA et al., 2019) and Honduras (ROMANENS et al., 2018).

The lactic acid produced in the previous phase by LAB, can be used as the only carbon source by *A. pasteurianus*, thus reinforcing a good interaction and synergy with the other microbial groups present in the fermentation. Thanks to the acetic acid produced from the oxidation of ethanol (produced by yeasts) and the consequent increase in the mass temperature, the cocoa seed loses its germinative power due to the death of the embryo, which initiates several structural and chemical reactions inside (SOUMAHORO et al., 2020). The oxidation reactions of lactic acid to acetoin occur via the α-acetolactate pathway and in a low proportion in acetic acid, since bacteria of the genus *Acetobacter* have low production and activity of the enzyme decarboxylase pyruvate (DE VUYST; LEROY, 2020; PELICAEN et al., 2019).

3.4 FILAMENTOUS FUNGI AND *Bacillus spp.*

There is a strong correlation between the presence of filamentous fungi and the production of enzymes favorable to the fermentation of cocoa beans. One of the identified species and which presented a good activity in the production of amylases

and pectinases, thus contributing to a good conduction in the microbial succession, as well as formation of precursors of aroma and chocolate flavour through the Maillard reaction in the roasting stage (ARAÚJO et al., 2019).

Studies can be suggested, such as the association of a microbial consortium of fungi (yeasts and filamentous fungi) and bacteria already known in the literature, with their verification of the role played in the production of products made from cocoa beans with fermentation conducted with such inocula. Studies already point out that the association of yeasts and lactic and acetic bacteria are beneficial for the production of chocolates with favorable and desirable sensory notes, however the inclusion of filamentous fungi is still scarce.

Little was known, but there are reports in the literature about the beneficial role that some species of the genus *Bacillus* play during fermentation, from where they have been isolated and studied. *Bacillus subtilis*, *B. pumilus* and *B. fusiformis* are isolated during the process and others may be linked to the production of pectinolytic enzymes such as polygalacturonase during fermentation, thus favoring the degradation of the pectin chain that surrounds the seed pulp (FIGUEROA-HERNÁNDEZ; MOTA-GUTIERREZ; FERROCINO, 2019) and also the formation of pyrazines after the Maillard reaction that started in the drying step and ended in the roasting of the cocoa beans. These compounds are responsible for imparting characteristic chocolate aromas and have their precursors formed with bacteria of the genus (ASSI-CLAIR et al., 2019).

4 THE USE OF MICROBIAL INOCULUM IN THE FERMENTATION OF COCOA

The use of starter cultures with the most varied groups and species of microorganisms is quite common in the food industry. Products such as cheeses, yogurts, probiotic drinks, wines and beers, are produced from the addition of pre-selected microorganisms (TAMANG; WATANABE; HOLZAPFEL, 2016). The main aim of this practice is to provide the formation of desirable characteristics for the final product, such as aromas and flavors.

The first report with mixed starter cultures in cocoa fermentations is reported from the late 90s in Brazil (SCHWAN, 1998). Cultures of *Saccharomyces cerevisiae* var. *chevalieri*, *Lactobacillus lactis*, *L. plantarum*, *Acetobacter aceti* and *Gluconobacter oxydans*, were chosen for this study due to the most varied characteristics, such as microorganisms resistant to high temperature, high concentrations of ethanol and acids and, finally, degradation of the pectin chain that involves the sugars of pulp (DE VUYST; LEROY, 2020). Since then, several other studies on the performance of starter cultures in cocoa fermentations have been developed and showing the effectiveness in obtaining fermented beans of good quality.

On-farm and laboratory-scale studies have been extensively developed over the decades and we can verify its importance with some mentioned in Table 2 (The last two years).

Table 2 – The role of strains and starter cultures in the cocoa fermentation (2018-2020).

(to be continued)

Culture Composition	**Findings**	**Reference**
Sc, Td	Inoculated fermentations showed higher levels of volatile compounds and shorter fermentation times conducted in boxes than in piles; microbial modulation.	Mota-Gutierrez et al., (2018)
Hg, Pk, Km, Sc, Lp, Lf, Ap, Gf	No significant differences between inoculated fermentations with respect to volatile compounds, lactic and acetic acids. Suggesting that LAB and AAB are not essential for cocoa fermentation, unlike yeasts.	Ho; Fleet; Zhao, (2018)
Sc, Lp, Aa	Immersion in 10% of inoculum, which provided (during fermentation) a decrease in phenolic compounds and an increase in the content of volatile compounds.	Saunshia et al., (2018)
Sc	Improvement in the time and quality of fermentation (production of desirable phenolic compounds in chocolate).	Assi-Clair et al., (2019)
Lf M017, Lf F223, Ho H17, Sc H29	Inhibition of *Aspergillus flavus* proliferation and its production of aflatoxin on the surface of cocoa beans during fermentation.	Romanens et al., (2019)
Lf, Lp, Sc, Ce	Production of organic acids and protein compounds to inhibit the activity of filamentous fungi harmful to fermentation.	Ruggirello et al., (2019)

Table 2 – The role of strains and starter cultures in the cocoa fermentation (2018-2020).

(conclusion)

Culture Composition	Findings	Reference
Sc, Lp, Aa	Development of experimental models to predict the production of alcohols, acids and degradation of phenolic compounds. Improved cut test and sensory analysis.	Saunshi et al., (2019)
Ht, Pk, Ho, H, W, Sc	*Hanseniaspora thailandica* and *Pichia kudriavzevii* were shown to be able to modulate the antioxidant activity in relation to natural fermentation, conducted without inocula.	Ooi; Ting; Siow, (2020)
Gs and Bc spores	The spore inocula did not influence the microbial fermentation count. Spores can probably be found after fermentation and are detrimental to food stability.	Pereira et al., (2020)
Rm, Td, Cp, Pg, Pkl, Io, Sc, Pm	*Candida parapsilosis*, *Torulaspora delbrueckii* e *Pichia kluyveri* provided significant changes in the reduction of acidity, concentration of sugars and free amino acids, during fermentation and fine cocoa Scavina.	Santos et al., (2020)

Notes. Aa: *Acetobacter aceti*; **Ap:** *Acetobacter pasteurianus*; **Bc:** *Bacillus cereus*; **Ce:** *Candida ethanolica*; **Cp:** *Candida parapsilosis*; **Gf:** *Gluconobacter frateuri*; **Gs:** *Geobacillus stearothermophilus*; **H:** *Hanseniaspora* genus; **Hg:** *Hanseniaspora guilliermondii*; **Ho:** *Hanseniaspora opuntiae*; **Ho H17:** *Hanseniaspora opuntiae* H17; **Ht:** *Hanseniaspora thailandica*; **Io:** *Issatchenkia orientalis*; **Km:** *Kluyveromyces marxianus*; **Lf:** *Lactobacillus fermentum*; **Lf F223:** *Lactobacillus fermentum* F223; **Lf M017:** *Lactobacillus fermentum* M017; **Lp:** *Lactobacillus plantarum*; **Pg:** *Pichia galeiformis*; **Pk:** *Pichia kudriavzevii*; **Pkl:** *Pichia kluyveri*; **Pm:** *Pichia manshurica*; **Rm:** *Rhodotorula mucilaginosa*; **Sc:** *Saccharomyces cerevisiae*; **Sc H29:** *Saccharomyces cerevisiae* H29; **Td:** *Torulaspora delbrueckii*; **W:** *Wickerhamomyces* genus.

What caught our attention was the evidence that some species of LAB and AAB provide inhibition of the growth of microorganisms that produce toxins harmful to health, if consumed in high quantities. Some studies verified the reduction of the aflatoxins production (ROMANENS et al., 2019) and the proliferation of filamentous fungi that produce them (RUGGIRELLO et al., 2019), after the application of yeast, LAB and AAB (Table 2). These results reflect the opposite of studies by (HO; FLEET; ZHAO, 2018; HO; ZHAO; FLEET, 2014, 2015), which suggest that LAB and AAB are not essential for cocoa fermentation.

Yeasts like *Hanseniaspora thailandica* and *Pichia kudriavzevii* can modulate the antioxidant activity of cocoa beans, providing the opportunity to produce products with

high antioxidant power (OOI; TING; SIOW, 2020). However, studies should be carried out regarding the sensory acceptance of the product, since high levels of phenolic compounds can generate bitterness and astringency (EFRAIM; ALVES; JARDIM, 2011). The use of *Pichia kudriavzevii* culture starter was important to verify the increase in the levels of some polyphenolic compounds on-farm fermentations in the study by Chagas Junior et al., (2021), as well as the protection against the production of putrefactive amines.

Studies can be carried out regarding the adaptations that some LAB undergo in the fermentation medium, such as, for example, *Pediococcus acilactici*. However, studies indicate that this species, can maintain the decrease in the production of these amines and favor amines that are considered interesting for chocolate, such as phenylethylamine, known as the *'passion hormone'* (AFOAKWA, 2008; BARTKIENE et al., 2018; OZOGUL et al., 2015). Such factors can be promising in cocoa fermentations, since the production of bioactive amines, for example, is expected by the high activity of LAB (DO CARMO BRITO et al., 2017).

The stages after fermentation (drying and roasting) also deserve special attention, since in them the reactions of formation of flavors and aromas of chocolates end, providing the desirable characteristics to the chocolate (RODRIGUEZ-CAMPOS et al., 2011, 2012; ZZAMAN et al., 2017).

5 CONCLUSIONS

In this review, we carried out a bibliographic survey regarding the identification of the main active microbiota during the cocoa fermentation process in several locations in the past five years. As is already reported by the specialized literature, the cocoa microbiota varies according to the locations, with some species being more present than others, this generates a discussion about the use of starter cultures in the fermentation process. The benefits are numerous if associated with the application of correct production and treatment techniques throughout the process. More detailed studies are suggested regarding the role of lactic acid bacteria and filamentous fungi,

since the indication of a positive influence for obtaining good quality fermented cocoa beans has already been reported.

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Capítulo II

Chemical implications and time reduction of on-farm cocoa fermentation by *Saccharomyces cerevisiae* and *Pichia kudriavzevii*

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“Chemical implications and time reduction of on-farm cocoa fermentation by *Saccharomyces cerevisiae* and *Pichia kudriavzevii*”

ABSTRACT

The use of starters during fermentation has been gaining momentum as it can warrant high-quality chocolate. The objective of this study was to investigate the influence of *Saccharomyces cerevisiae* (Sc) and *Pichia kudriavzevii* (Pk) during on-farm fermentation on physico-chemical and microbiological characteristics and levels of methylxanthines and bioactive amines of cocoa. Four treatments were used: ScPk (1:1), only Sc, only Pk, and no starter (control). The starters lead to changes throughout fermentation, but provided fermented cocoa with similar pH, titratable acidity, reducing sugars and phenolic compounds. ScPk shortened fermentation time by 24 h. The ScPk fermented and dried cocoa had higher levels of monomeric phenols, methylxanthines, phenylethylamine and lower levels of the putrefactive amines – putrescine and cadaverine ($p<0.05$). The results were confirmed by multivariate analysis. Based on these results, the mixture of both yeasts species is a promising starter for cocoa fermentation decreasing duration time and modulating high-quality components.

Keywords: Starter; Phenylethylamine; PCA; HCA; putrefactive amines.

1 INTRODUCTION

Cocoa bean is the main raw material for chocolate production. It undergoes several important processing stages from fruit opening to industrial processing. However, fermentation is outstanding, due to its role in the formation of precursors for high-quality chocolate aroma and flavor (HO; ZHAO; FLEET, 2015).

Cocoa fermentation is a natural and spontaneous process, involving different microorganisms, including yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Each group develops stepwise according to the environmental conditions in which they are present. Yeasts and LAB hydrolyze fermentable sugars (glucose, fructose and sucrose) transforming them into ethanol and lactic acid, leading to increased temperature (FIGUEROA-HERNÁNDEZ et al., 2019). Yeasts, e.g. *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Kluyveromyces marxianus*, also

produce pectinolytic enzymes. These enzymes break down pectin which is responsible for the viscosity and stickiness of cocoa pulp, causing the collapse of the pulp and allowing the formation of void space between the beans, favoring air percolation (DE VUYST; WECKX, 2016; HO; ZHAO; FLEET, 2014; NIAMKE; DIOPOH, 2008; SCHWAN; WHEALS, 2004). The aeration conditions are needed for the growth of AAB which convert ethanol into acetic acid, increasing the temperature and leading to the death of the seed embryo. The high temperature and low pH are needed for protein breakdown and release of amino acids, which are precursors of typical chocolate aromas and flavors and also of bioactive amines (HERNÁNDEZ-HERNÁNDEZ et al., 2016).

The development of starter cultures for use in cocoa fermentation has been gaining momentum due to the numerous benefits they can exert on process standardization and harmonization of chocolate quality. Studies indicate that some yeast species are valued due to the high capacity to produce ethanol and to hydrolyze pectin and proteins, facilitating the release of sugars and nitrogenous compounds which are relevant precursors for high-quality chocolate, mainly regarding flavor compounds, including alcohols, esters, pyrazines (VISINTIN et al., 2017). Among them, *Saccharomyces cerevisiae* and *Pichia kudriavzevii* were highlighted for their ability to produce a wide variety of esters, alcohols and aldehydes that impart desirable aromas to chocolate, such as 'fruity', 'floral' and 'sweet' aromas (KONÉ et al., 2016). Furthermore, they have high capacity to adapt to diverse environmental conditions and are capable of inhibiting the growth of putrefactive microorganisms (BATISTA et al., 2015). In addition, these species can facilitate drainage of the pulp, increasing aeration, allowing fast microbial succession of LAB to AAB, thereby reducing fermentation time (SANDHYA et al., 2016). However, scarce information is available regarding the influence of added starter culture on the fermentation process and on the formation of compounds which are responsible for high-quality chocolate quality, including bioactive amines, phenolic compounds and methylxanthines.

The objective of this study was to investigate, for the first time, the influence of yeast starter cultures, comprised of *Saccharomyces cerevisiae* and *Pichia kudriavzevii*, on the evolution of on-farm cocoa fermentation and on the formation of compounds which are responsible for high-quality chocolate. Four fermentation treatments were undertaken (only *S. cerevisiae*; only *P. kudriavzevii*; both *S. cerevisiae*

and *P. kudriavzevii*; and control – no yeast added). Fermentation was followed by determination of the changes on temperature, pH, total titratable acidity, total phenolic compounds and microbial counts. The fermented cocoa was dried and analyzed for methylxanthines (theobromine, theophylline and caffeine), biogenic amines, catechin and epicatechin in the fermented and dried cocoa beans.

2 MATERIAL AND METHODS

2.1 CHEMICALS AND REAGENTS

Reagents and solvents were of analytical grade (Synth, Diadema, SP, Brazil; Dinâmica, SP, Brazil). Acetonitrile and methanol were HPLC grade (J.T. Baker, Radnor, PA, USA). Glucose, Folin-Ciocalteu reagent, 3,5-dinitrosalicyclic acid and the standards [spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride, agmatine sulfate, cadaverine dihydrochloride, 5-hydroxytryptamine (serotonin), histamine dihydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride, tryptamine, *o*-phthalaldehyde, (+)-catechin, (-)-epicatechin, theobromine, theophylline and caffeine] were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ultrapure water was from Milli-Q (Millipore Corp., Milford, MA, USA). For chromatographic analysis, HPLC solvents were filtered through 0.45 µm nylon membrane (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and the extracts through 0.22 µm (Analítica, São Paulo, SP, Brazil).

Yeast extract, De Man, Rogosa & Sharpe (MRS) agar, peptone water, bacteriological peptone and bacteriological agar were from Kasvi (São José dos Pinhais, PR, Brazil); calcium carbonate and chloramphenicol were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA); and nystatin was from Prati-Donaduzzi (Toledo, PR, Brazil). These components were used to make the culture media: Yeast Extract Peptone Dextrose Agar – YPD [20 g glucose, 20 g bacteriological peptone, 10 g yeast extract (BATISTA et al., 2016), adapted with 20 g bacteriological agar in 1 L distilled water, pH 5.6]; YPD broth [20 g glucose, 20 g bacteriological peptone, 10 g yeast extract in 1 L distilled water (BATISTA et al., 2016), pH 5.6]; and Glucose Yeast Medium with calcium carbonate – GYC [50 g glucose, 10 g yeast extract, 30 g calcium

carbonate, 20 g bacteriological agar in 1 L distilled water, pH 5.6 (MOREIRA et al., 2013)].

2.2 YEASTS STRAINS AND STARTER CULTURES PREPARATION

Cultures of *Saccharomyces cerevisiae* (GenBank Access KY794742) and *Pichia kudriavzevii* (GenBank Access KY794725) were used. These strains were isolated and identified during fermentation of cocoa beans at the same farm in 2016 (CHAGAS JUNIOR, 2016). They were adequately stored in the Bank of Microorganisms of the Laboratory of Biotechnology Processes, Federal University of Pará (LABIOTEC, UFPA, Brazil).

The preserved strains were activated in Petri dishes containing sterile YPD agar incubated in inverted position at 28 ºC for 72 h (BAFFI et al., 2011). After incubation, colonies were transferred to Erlenmeyers containing 100 mL of sterile YPD broth and subsequently incubated in an orbital shaker at 150 rpm, 30 °C for 12 h. The contents of each Erlenmeyer were aseptically transferred to 900 mL of sterile YPD broth in a bench bioreactor (FerMac 320, Electrolab Biotech, Tewkesbury, UK) at 30 °C and 150 rpm (RAMOS et al., 2014) for cell growth ($\approx 10^8$ cells/mL). Subsequently, the contents were centrifuged in sterile falcon-type conical tubes at 10.000 × *g* for 14 min at 4 °C. The supernatant was discarded, and the biomass pellet stored under refrigeration (4 ± 2 ºC) for up to 48 h prior to use.

2.3 COCOA BEANS FERMENTATION AND DEHYDRATION

Forastero cocoa fruits were produced, fermented and dried at a farm in Tomé-Açu, PA, Brazil (02º28'41.3 "S and 48º16'50.7" W), in September of 2017. On-farm cocoa fermentation was conducted according to commercial practices. Immediately after harvest, the fruits were cut open with stainless steel knives and the beans and pulp, without peel and placenta, were placed in wooden boxes with a capacity of 50 kg of cocoa beans/each.

Four fermentation treatments were carried out simultaneously: (i) a control without inoculum (CF), (ii) fermentation with 10^6 cells of *S. cerevisiae* (Sc), (iii) fermentation with 10^6 cells of *P. kudriavzevii* (Pk) and (iv) fermentation with the two

species all together (ScPk), 1:1, 10^6 cells each. All experiments were performed in triplicate with a total of 12 fermentation boxes. The starter cultures (10^6 cells/g cocoa beans) (VISINTIN et al., 2017) were suspended in 500 mL sterile peptone water and they were sprayed uniformly onto the seeds, layer by layer, as they were placed into the wooden boxes. Banana leaves were placed on the surface of the fermentation mass.

Throughout fermentation (0, 24, 48, 72, 96, 120, 144 and 168 h), the temperature was measured with a digital thermometer (model HT-600, Instrutherm, São Paulo, SP, Brazil) at the middle of the wooden boxes. Samples from each fermentation box were taken from five random points, totaling ≈200 g of samples per fermentation time per box. The samples were aseptically packed in sterile bags and taken immediately to the laboratory for microbiological analysis and, afterwards, they were stored at -18 ºC for chemical analyses. Fermentation was terminated based on aspects, color and aroma typical of fermented cocoa beans.

At the end of fermentation (168 h), except for ScPk, which ended at 144 h, the cocoa beans were transferred to trays, and exposed to sunlight for natural dehydration. The drying period lasted approximately 72 h. The dried samples were packed in sterile plastic bags and transported to the laboratory for analysis.

2.4 METHODS OF ANALYSIS

2.4.1 Microbiological analysis during the fermentation

Ten grams of cocoa beans were aseptically ground and homogenized in 90 mL peptone water (0.1% w/v), providing 10^{-1} dilutions. Subsequently, serial decimal dilutions were obtained for the quantification of yeasts in YPD agar with chloramphenicol (100 mg/L) for inhibition of bacterial growth. The spread plate technique was used and the plates were incubated at 28 ºC for 72 h (BAFFI et al., 2011).

For acetic acid bacteria (AAB), serial decimal dilutions were prepared in GYC agar with 0.2% nystatin for the inhibition of fungal growth. The spread plate technique was used, and the plates were incubated at 30 °C for 96 h (HO; ZHAO; FLEET, 2014). For lactic acid bacteria (LAB), the dilutions were made on MRS agar (pH 6.2) with 0.2%

nystatin for inhibition of fungal growth. The pour plate technique was used followed by incubation at 30 °C for 72 h (DE MELO PEREIRA et al., 2013).

2.4.2 Physico-chemical analysis of cocoa beans during fermentation

The peel and germs were removed from the cocoa seeds, which were ground in an analytical mill (A11, IKA Staufen, Germany). The analysis of moisture content (method 931.04), pH (method 970.21) and total titratable acidity (TTA, method 31.06.06) were carried out according to the Association of Official Analytical Chemists (HORWITZ; LATIMER, 2006) and total reducing sugars (TRS) by the 3,5-dinitrosalicylic acid method (MILLER, 1959). All analyzes were performed in triplicate.

2.4.3 Total phenolic compounds in cocoa beans during fermentation and in the dried cocoa beans

The fermented and dried cocoa seeds were freeze-dried (L101 Liotop, São Paulo, SP, Brazil) and defatted with *n*-hexane (DO CARMO BRITO et al., 2017). Total phenolic compounds (TPC) were determined by the Folin-Ciocalteu method (SINGLETON; ORTHOFER; LAMUELA-RAVENTÓS, 1998) using an UV-visible spectrophotometer (EVO 60, Thermo Fisher Scientific, Waltham, MA, USA) at 760 nm. The results were obtained by interpolation in epicatechin calibration curves (20-100 mg/L, $R^2 \geq 0.99$) and expressed in milligram equivalent epicatechin per gram (mg ECE/g). The analyses were performed in triplicate.

2.4.4 Methylxanthines and monomeric phenols in the fermented and dried cocoa beans by HPLC

Dried and defatted samples (250 mg) were placed in polypropylene tubes containing 2.5 mL aqueous ethanol solution (1:1, v/v) and dissolved using an ultrasonic bath for 10 minutes at 25 °C (HE et al., 2010; SANDHYA et al., 2016). The tubes were centrifuged at 1.500 × *g* for 10 min and the extraction was repeated. The supernatants were combined and filtered through 0.22 µm membrane prior to HPLC analysis.

Aliquots of 20 µL were automatically injected into a HPLC coupled with a diode array detector at 280 nm (HE et al., 2010) (1260 Infinity, Agilent Technologies, La Jolla, CA, USA) and a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm, Agilent Technologies, La Jolla, CA, USA) at 25 °C. The mobile phases were (A) water: acetonitrile (99.8:0.2, v/v) and (B) methanol, at a linear gradient of 0 to 50% for 0-12 min, 50 to 100% for 13-20 min at a flow rate of 1.2 mL/min. Identification was based on retention times of the compounds compared to those of standards and addition of the suspected standards to the sample. Quantification was undertaken by interpolation in analytical curves of each standard (Table 1).

Table 1 – Concentrations range of the analytes used for construction of the calibration curves and respective coefficients of correlation and limits of quantification for HPLC analysis.

Compound	**Standard curve**		**Limit of**
	Concentration range (µg/mL)	**Linear equation (R^2)**	**quantification**
Methylxanthines			
Theobromine	3.125 – 50	y=0.0000001x-3.4713 (>0.999)	0.64 mg/g
Theophylline	3.125 – 100	y=0.0000002x-0.055 (>0.999)	0.02 mg/g
Caffeine	3.125 – 100	y=0.0000002x-0.1899 (>0.999)	0.04 mg/g
Monomeric phenols			
Catechin	3.125 – 50	y=0.000001x+0.5831 (>0.999)	0.31 mg/g
Epicatechin	3.125 – 100	y=0.0000004x+0.9615 (>0.999)	0.11 mg/g
Bioactive amines			
Putrescine	0.71 – 21.16	y=0.074x-0.0245 (≥0.998)	0.07 mg/kg
Cadaverine	0.82 – 24.52	y=0.0535x-0.0002 (≥0.999)	0.08 mg/kg
Phenylethylamine	0.97 – 29.08	y=0.0437x+0.0011 (≥0.999)	0.10 mg/kg

R^2 – Correlation coefficient of linear regression.

2.4.5 Bioactive amines in the fermented and dried cocoa beans by HPLC

The determination of bioactive amines was carried out with 5 g samples, after trichloroacetic acid extraction (DO CARMO BRITO et al., 2017). A Shimadzu HPLC

system (LC-10AD, Shimadzu, Kyoto, Japan) with a fluorometric detector (340 and 445 nm of excitation and emission, respectively) and a Novapak C18 column (3.9 × 300 mm, 4 µm, Waters Co., Milford, MA, USA). A gradient of 0.2 M sodium acetate and 0.3 mM sodium octanesulfonate, pH 4.9 and acetonitrile was used (DO CARMO BRITO et al., 2017, 2019). Amines were identified by comparison of retention times and co-elution with standards. The quantification was performed after post column derivatization with *o*-phthalaldehyde, using analytical curves for each amine (Table 1).

2.5 STATISTICAL ANALYSIS

The results were submitted to analysis of variance (ANOVA) and the means were compared by the Duncan test at 5% significance. Multivariate analysis was used to further analyze data. For Principal Component Analysis (PCA), temperature, microbial counts, pH, total acidity, TPC and TRS values, were used as active variables during fermentation with each of the four different treatments (starter cultures). The Hierarchical Cluster Analysis (HCA) was obtained considering the same PCA active variables and the groups were formed based on Euclidean distances (Ward's method). PCA and HCA were also carried out for the fermented and dried cocoa beans considering as active variables: pH, TPC, monomeric phenols, methylxanthines and bioactive amines. The statistical analyses were undertaken using the Statistica 7.0 software (StatSoft Inc., Tulsa, USA).

3 RESULTS AND DISCUSSION

3.1 INFLUENCE OF STARTER CULTURE ON THE PHYSICO-CHEMICAL CHARACTERISTICS DURING FERMENTATION

During fermentation using the different starter culture, all treatments showed evolution typical of cocoa fermentation (Table 2): the temperature increased reaching a maximum followed by a decrease; there was significant increase on TTA and decreases on pH, TRS and TPC.

The end of fermentation, determined by the processor, was 168 h for most treatments (CF, Sc, and Pk) whereas for treatment ScPk fermentation ended earlier,

at 144 h. This result indicates that by using the combination of the starter cultures *S. cerevisiae* and *P. kudriavzevii*, the cocoa beans were ready for drying 24 h earlier. This represents advancement in cocoa fermentation, but the desired quality must be kept.

Table 2 – Changes on temperature, pH, total titratable acidity (TTA), reducing sugars and total phenolic compounds throughout on-farm cocoa fermentation with different starters CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017.

Parameters/ treatments	Mean values */Fermentation time (h)							
	0	24	48	72	96	120	144	168
Temperature (ºC)								
CF	29.7±0.52^{eA}	33.2±0.48^{dB}	40.0±0.62^{bB}	44.2±1.43^{aA}	41.4±2.76^{bB}	41.9±2.28^{bA}	37.2±2.48^{cB}	32.4±2.39^{dA}
Sc	30.9±0.59^{cA}	34.9±0.51^{bA}	43.7±2.61^{aA}	43.9±1.97^{aA}	44.1±2.10^{aAB}	42.6±1.15^{aA}	43.0±0.82^{aA}	34.3±1.58^{bA}
Pk	30.4±1.42^{cA}	35.2±1.38^{bA}	45.2±1.79^{aA}	44.3±1.93^{aA}	44.6±0.87^{aA}	45.3±0.91^{aA}	36.7±1.88^{bB}	35.5±2.11^{bA}
ScPk	31.2±1.03^{bA}	34.0±0.87^{bA}	44.0±1.59^{aA}	43.1±1.24^{aA}	44.1±1.46^{aAB}	34.4±2.84^{bB}	31.4±2.95^{bC}	n.a.
pH								
CF	6.57±0.36^{aA}	4.70±0.03^{bC}	4.20±0.01^{cdB}	3.89±0.03^{eA}	4.03±0.04^{deA}	4.34±0.02^{cA}	4.73±0.03^{bA}	4.90±0.01^{bB}
Sc	6.46±0.04^{aA}	5.42±0.04^{bA}	4.42±0.06^{eA}	3.84±0.04^{gA}	4.09±0.01^{fA}	4.10±0.03^{fB}	4.71±0.01^{dA}	4.96±0.03^{cB}
Pk	6.27±0.04^{aA}	5.30±0.09^{bB}	3.52±0.02^{fC}	3.30±0.04^{gB}	3.34±0.05^{gC}	3.88±0.02^{eC}	4.05± 0.06^{dC}	5.15±0.02^{cA}
ScPk	6.44±0.08^{aA}	5.37±0.02^{bAB}	3.57±0.04^{eC}	3.35±0.03^{fB}	3.57±0.04^{eB}	4.11±0.04^{dB}	4.28 ± 0.04^{cB}	n.a.
TTA (meq. NaOH 0.1 N/100 g) [1]								
CF	4.85±0.49^{fA}	16.06±1.01^{dA}	29.00±0.59^{bA}	39.68±0.55^{aA}	27.68±1.47^{bB}	23.18±1.07^{cA}	16.60±1.03^{dA}	12.53±0.09^{eB}
Sc	4.76±0.29^{gA}	11.01±0.46^{fB}	18.82±1.97^{dC}	32.77±1.24^{aB}	23.93±0.84^{bC}	21.86±1.36^{cA}	12.63±0.96^{fB}	14.75±0.90^{eA}
Pk	4.00±0.11^{gB}	8.55±0.52^{fC}	28.15±0.32^{cA}	40.41±0.75^{aA}	33.32±0.55^{bA}	20.98±0.36^{dA}	17.20±2.95^{eA}	16.85±0.16^{eA}
ScPk	5.28±0.47^{dA}	6.50±0.50^{dD}	25.68±1.35^{bB}	30.83±1.62^{aB}	24.93±1.44^{bC}	17.42±1.70^{cB}	14.90±1.95^{cA}	n.a.
Reducing sugars (mg/g)								
CF	11.72±0.02^{aB}	9.51±0.75^{bB}	2.62±0.39^{eC}	4.87±0.10^{dD}	6.30±0.06^{cB}	4.93±0.08^{dA}	1.59±0.02^{fC}	1.85±0.04^{fA}
Sc	11.70±0.26^{bB}	14.41±0.36^{aA}	5.15±0.40^{dA}	5.27±0.10^{dC}	6.68±0.19^{cA}	4.21±0.02^{eB}	1.93±0.08^{fB}	1.92±0.04^{fA}
Pk	10.61±0.08^{aC}	9.41±0.42^{bB}	3.93±0.00^{dB}	6.37±0.26^{cB}	2.90±0.03^{eD}	2.94±0.29^{eC}	2.08±0.03^{fA}	1.30±0.05^{gB}
ScPk	13.42±0.60^{aA}	9.78±0.44^{bB}	4.20±0.16^{eB}	6.72±0.06^{cA}	5.77±0.16^{dC}	1.43±0.00^{fD}	1.60±0.00^{fC}	n.a.
Total phenolic compounds (mg ECE/g) [2]								
CF	63.99±0.97^{aA}	65.26±1.22^{aB}	50.10±1.44^{bA}	50.13±0.21^{bA}	35.76±2.90^{cB}	38.00±1.55^{cB}	38.80±0.48^{cB}	36.95±1.88^{cB}
Sc	62.20±0.14^{bB}	70.00±0.83^{aA}	52.72±2.99^{cA}	49.53±1.03^{cdA}	47.12±0.68^{deA}	45.78±0.69^{eA}	41.33±0.06^{fA}	41.55±1.22^{fA}
Pk	62.10±0.14^{aB}	45.78±1.78^{cD}	50.91±3.60^{bA}	51.29±0.84^{bA}	30.29±0.94^{eC}	35.37±0.95^{dB}	35.95±0.33^{dC}	30.25±0.47^{eC}
ScPk	63.05±0.06^{aA}	53.40±0.89^{bC}	39.22±1.46^{dB}	45.86±0.02^{cB}	36.98±0.03^{eB}	26.21±0.04^{fC}	25.10±0.70^{fD}	n.a.

[1]meq. NaOH 0.1 N/100 g: milliequivalent sodium hydroxide solution 0.1N per 100 g sample

[2]mg ECE*/g*: milligram equivalent epicatechin per gram sample

* Means (± standard deviation) with different capital letters in the same columns (treatments) and with different lower case letters in the same line (fermentation time) are statistically different (Duncan test, $p \leq 0.05$).

n.a.: not analyzed as fermentation for ScPk was completed in 144 h.

The increase in temperature during cocoa fermentation is important as it causes the seed to lose its germinative power, which is essential for the formation of aroma precursors and characteristic flavors of chocolate (RAMOS et al., 2014; VISINTIN et al., 2017). The increase in the temperature of the cocoa mass during fermentation is associated with the metabolism of ethanol (produced from yeasts) by AAB. These reactions are highly exothermic and raise the temperature of the fermenting mass (DO CARMO BRITO et al., 2017). Maximum temperatures (≈45 °C) were reached at 72 h fermentation for control (CF), decreasing afterwards. However, the treatments with added starter cultures reached higher temperatures earlier in the fermentation process (at 48 h) and it remained high for 96, 120 and 144 h for ScPk, Pk and Sc, respectively. Similar trends were also observed when using the combination of *S. cerevisiae* and *Torulaspora delbrueckii* during cocoa fermentation (VISINTIN et al., 2017). Afterwards, the temperatures decreased reaching similar values (≈34 ºC) at 168 h fermentation for most treatments (CF, SC and Pk) and at 144 h fermentation for ScPk.

Based on these results, by adding the yeast starter cultures, typical high temperatures were reached earlier, compared to CF. Moreover, the temperature remained higher for a longer period of time, probably due to the more intense yeast activity during initial fermentation times (Figure 1) favoring the numerous biochemical reactions that are inherent to cocoa fermentation (HO; ZHAO; FLEET, 2014). The behavior observed for the ScPk treatment indicates that a faster microbial succession took place.

The pH values did not differ statistically at the beginning of fermentation for all treatments (p>0.05). However, the pH decreased reaching lower levels at 72 h fermentation (3.30–3.89), with lower levels found for Pk (3.30–3.35). The pH increased afterwards, and higher values were found for the Pk treatment (5.15) compared to the others (4.90–4.96). The pH values are within the range found in other cocoa fermentation studies (NAZARUDDIN et al., 2006).

In the opposite way, as expected, TTA increased up to 72 h for all treatments (Table 2), and higher levels were observed for treatments without *S. cerevisiae* (CF and Pk treatments). After 72 h fermentation, TTA decreased and, at the end of fermentation, higher values were found for the treatments with starter culture compared to CF (p≤0.05). The increase in TTA is mainly due to the production of lactic and acetic acids from lactic and acetic acid bacteria, respectively during metabolism

of the microorganisms. The acetic acid produced, along with ethanol, at high temperature, diffuse through the seed tissues and cotyledon, killing the germ and activating proteolytic enzymes (DO CARMO BRITO et al., 2017; EFRAIM et al., 2010; RAMOS et al., 2014; VISINTIN et al., 2017). The increase in pH and concomitant decrease in TTA at the end of fermentation can result from the evaporation of volatile acids, mainly acetic acid, which takes place by stirring the cocoa mass (LIMA et al., 2011).

The maintenance of the cocoa pH around 5.0 during fermentation is relevant as it allows activity of proteases which are important in the formation of chocolate aroma (HO; ZHAO; FLEET, 2014). Protein hydrolysis liberates small peptides and amino acids which are substrates for the Maillard reaction during drying and roasting (FIGUEROA-HERNÁNDEZ et al., 2019). In addition, the acid environment induces the decarboxylation of free amino acids by microbial enzymes producing amines. Under favorable conditions (low pH environment, for example), some microbial species can produce decarboxylase enzymes and produce biogenic amines as a result of the decarboxylation of free amino acids. Some bacterial species of the genus *Lactobacillus*, *Salmonella* and *Staphylococcus* can produce enzymes such lysine, ornithine and phenylalanine decarboxylases, thus warranting survival under this stressful situation, by buffering the pH due to the production of cadaverine, putrescine and phenylethylamine, respectively (DO CARMO BRITO et al., 2017; GLORIA, 2005).

With respect to the reducing sugars, during fermentation there was a significant decrease on TRS levels ($p \leq 0.05$), due to the use of sugar by microorganisms (MOREIRA et al., 2017). However, at 72 and 96 h fermentation, there were increases on reducing sugars which could be related to the hydrolysis of sucrose by lactic and acetic acids, produced by lactic and acetic acids bacteria, respectively (DE VUYST; WECKX, 2016; HO; ZHAO; FLEET, 2014; SCHWAN; WHEALS, 2004). Glucose, fructose and sucrose are the carbohydrates that are most readily available in the cocoa pulp during the first fermentation times (SCHWAN; WHEALS, 2004). However, sucrose probably is not assimilated by some species of yeast and bacteria. When there is production of lactic and acetic acids there is hydrolysis of the sucrose molecule into glucose and fructose, and these monosaccharides become available for fermentation. Invertase secreted by yeasts also breakdown sucrose in glucose and fructose (DE VUYST; WECKX, 2016; HO; ZHAO; FLEET, 2014; SCHWAN; WHEALS,

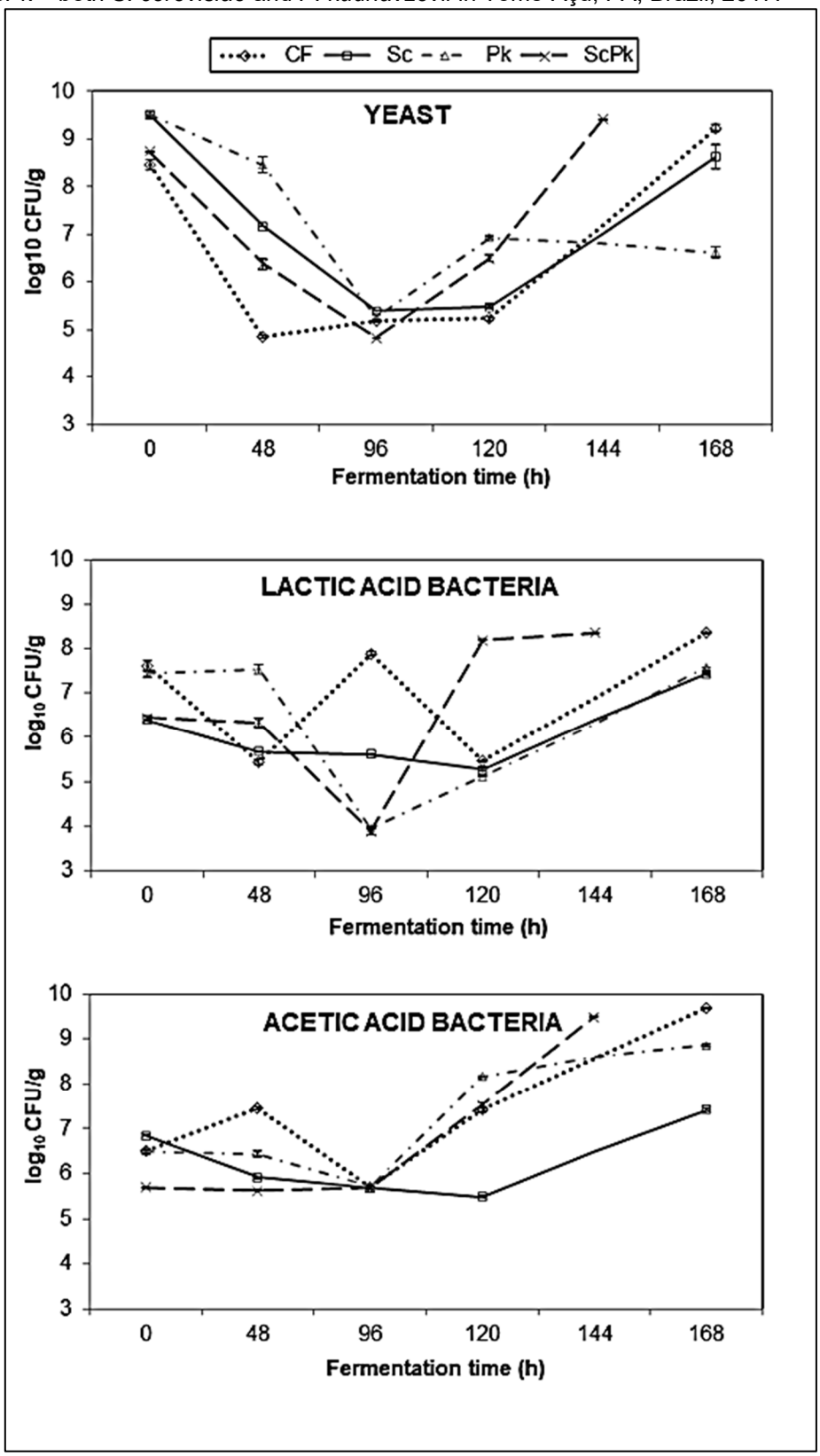
2004). In this study, this behavior can be suggested by increasing the amount of TRS between 72 h and 96 h (Table 1).

There was reduction on the contents of TPC during fermentation, with an overall loss of 33 to 60%, which is within the ranges reported in the literature (DO CARMO BRITO et al., 2017; EFRAIM et al., 2010). The loss of phenolic compounds is associated with polyphenoloxidase (PPO) activity, which is responsible for the enzymatic browning reactions and the typical brown color of cocoa beans. According to the literature (HERNÁNDEZ-HERNÁNDEZ et al., 2016), the ideal temperature for PPO activity is between 42–45 °C. Longer times at this temperature range were observed for treatment Sc (96 h), followed by Pk and CF (72 h), and then ScPk (48 h). Therefore, lower TPC levels would be expected in the Sc treatment, which was not the case. In this context, there might be other factors affecting PPO activity besides temperature, for example, polymerization and exudation of the liquid formed during fermentation (NAZARUDDIN et al., 2006), which can affect phenolic compounds. The understanding of the reactions behind these changes is important as they affect color, bitterness and astringency of chocolate. At the end of fermentation (144 h), higher TPC levels were found for the Sc treatment, followed by CF and Pk, and by fermentation with ScPk ($p \leq 0.05$).

3.2 CHANGES ON MICROBIAL COUNTS DURING FERMENTATION

The microbial population dynamics among the three major groups of cocoa fermentation microorganisms, yeast, LAB and AAB (Figure 1), show the change in the predominance of these microorganisms throughout fermentation. This is relevant to warrant the desirable cocoa and chocolate quality. The counts of yeast during on-farm indigenous fermentation (CF) decreased in the first 48 h, remained constant for up to 120 h, and increased afterwards (Figure 1). The yeast count decrease is probably due to the consumption of readily fermentable carbohydrates by the yeasts in the first fermentation times (SCHWAN; WHEALS, 2004), suggesting that there are no more readily substrates available after 48 hours fermentation. However, the microbial count increases again after 96 h can be associated with invertase activity and conversion of sucrose into glucose and fructose (DE VUYST; WECKX, 2016), once again providing substrate for microbial growth.

Figure 1 – Counts of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) during on-farm cocoa fermentation with different starters: CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017.



However, when yeasts were used as starter cultures (treatments Sc, Pk and ScPk), higher yeasts counts were observed at the beginning of fermentation compared

to control and it took longer (96 h) for the yeasts counts to decrease, reaching lower counts at 96 h. This behavior can be explained by the yeast species present in the control fermentation (CF) and in the Sc (*Saccharomyces cerevisiae*) fermentation. It is known that *S. cerevisiae* is one of the most common yeast species present in cocoa fermentations in Brazil (FIGUEROA-HERNÁNDEZ et al., 2019). The inoculum of *S. cerevisiae* may have found a synergy with *S. cerevisiae* naturally present in the CF treatment at 96 h. Future studies on molecular biology are needed to elucidate the microbial dynamics with these inoculants. There was a significant increase on yeasts counts afterwards, and the rates were higher for the treatments with *P. kudriavzevii* (Pk and ScPk) compared to control (CF) and *S. cerevisiae* (Sc) treatments. The final yeast counts (168 h) were lower for Pk compared to the others. In the treatment with both yeasts (ScPk), the higher counts were reached at 144 h, 24 h earlier compared to the other treatments.

For the lactic acid bacteria – LAB, during control fermentation (CF), the counts varied throughout fermentation (Figure 1), decreasing up to 48 h, increasing up to 96 h, decreasing again up to 120 h, and increasing until the end of fermentation. However, when yeasts were inoculated, no increase on LAB counts was observed at 96 h; moreover, there was a decrease on LAB counts at 96 h when *P. kudriavzevii* was inoculated (both Pk and ScPk treatments). This result suggests that the inoculation of yeast inhibited the initial growth of LAB (up to 96 h), and that the inhibition was more pronounced when *P. kudriavzevii* was in the starter culture. However, the combination of yeasts (ScPk) provided a faster recovery of LAB counts, reaching higher counts earlier, at 120 h fermentation.

The changes on acetic acid bacteria – AAB also varied throughout fermentation. There was an initial increase on AAB counts in the control fermentation (CF) in the first 48 h, followed by a decrease at 96 h and increased afterwards (Figure 1). However, when yeasts starter cultures were inoculated (Sc, Pk and ScPk), the counts remained similar up to 96 h, increasing afterwards with a sharper increase for the ScPk treatment, reaching higher counts (≈10 log10 CFU/g) at 144 h. Similar counts were obtained for CF and Pk at 168 h; whereas in the Sc treatment, final AAB counts only reached 7 log10 CFU/g at the end of fermentation.

Briefly, during natural cocoa fermentation (CF), the succession of microorganisms was as follows: initially, AAB, LAB and yeasts were present with

higher counts for yeasts, followed by LAB and AAB (10^9, 10^7 and 10^6, respectively); at 48 h there was predominance of AAB; followed by LAB at 96 h; and, at the end of fermentation (168 h), AAB, LAB and yeast were present with higher counts for yeasts and AAB (10^9) followed by LAB (10^8) (Figure 1). These results are similar to literature reports (VISINTIN et al., 2016). The use of yeasts as starter cultures modulated the counts of microorganisms throughout fermentation. It attenuated the increases on LAB and AAB counts, reduced the decrease in yeasts, resulting in similar counts at the end of fermentation. In addition, by adding both *S. cerevisiae* and *P. kudriavzevii* simultaneously, the time required for fermentation was shortened.

3.3 INFLUENCE OF STARTER CULTURE ON THE PHYSICO-CHEMICAL CHARACTERISTICS OF THE FERMENTED AND DRIED BEANS

The reduction on the moisture content immediately after fermentation is required to terminate microbial activities; some chemical reactions start or continue during the drying process, including Maillard reaction, which plays an important role in cocoa and chocolate quality (EFRAIM et al., 2010). The characteristics of the on-farm fermented and dried cocoa are indicated in Table 3. Fermented cocoa beans had moisture content values from 5.56% to 5.72%, which were not significantly different ($p>0.05$, Duncan's test) among treatments. However, significant difference was observed for the other parameters analyzed ($p \leq 0.05$, Duncan's test).

The pH values varied from 5.26 to 5.56, with higher values for control (CF) and the treatment containing both yeasts (ScPk) (5.56 and 5.54, respectively), whereas the treatments with individual yeasts provided lower values, with the lowest pH for the Sc treatment. Total titratable acidity varied from 17.55 to 22.86 meq NaOH/100 g. In accordance with pH values, lower acidity was observed for CF and for the treatment containing both yeasts (ScPk), and higher values were observed for the Sc treatment. Based on these results, the treatment with both yeasts (ScPk) was not different ($p \geq 0.05$) from the typical natural on-farm fermentation (CF), and had the lowest acidity, which is desirable for high quality chocolate. The product with the highest acidity was obtained with *S. cerevisiae*, suggesting that the predominance of this yeast can lead to lower pH compared to control fermentation.

Table 3 – Mean values of moisture content, pH, total titratable acidity, methylxanthines and bioactive amines in fermented and dried cocoa beans submitted to on-farm fermentation with different starters: CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017.

Parameters	Mean values*/ Fermentation treatment			
	CF	Sc	Pk	ScPk
Moisture content (%)	5.65±0.08[a]	5.72±0.07[a]	5.56±0.12[a]	5.71±0.06[a]
pH	5.56±0.02[a]	5.26±0.04[c]	5.39±0.01[b]	5.54±0.03[a]
Total titratable acidity (meq. NaOH 0.1 N /100 g) [1]	18.02±0.60[c]	22.86±0.57[a]	19.63±0.06[b]	17.55±0.99[c]
Total phenolic compounds (mg ECE/g) [2]	26.35±0.93[c]	26.59±1.07[c]	32.25±0.35[a]	29.05±0.54[b]
Monomeric compounds (mg/g)				
Catechin	0.05±0.00[d]	0.61±0.03[c]	1.88±0.11[b]	2.19±0.07[a]
Epicatechin	0.32±0.05[c]	2.39±0.01[b]	2.44±0.09[b]	3.14±0.13[a]
Methylxanthines (mg/g)*				
Theobromine	7.40±0.02[a]	5.53±0.74[b]	7.19±0.01[a]	8.05±0.64[a]
Caffeine	0.93±0.18[c]	1.27±0.04[b]	1.22±0.05[b]	2.78±0.09[a]
Total	8.33±0.19[b]	6.8±0.70[c]	8.41±0.04[b]	10.83±0.73[a]
Bioactive amines (mg/kg)**				
Putrescine	1.13±0.08[b]	2.22±0.07[a]	1.05±0.14[b]	0[c]
Cadaverine	1.79±0.22[b]	3.32±0.12[a]	1.49±0.36[b]	0[c]
Phenylethylamine	1.64±0.20[b]	1.56±0.02[b]	2.30±0.00[a]	2.42±0.02[a]
Total	4.56±0.51[b]	7.10±0.21[a]	4.84±0.21[b]	2.42±0.02[c]

[1] meq. NaOH 0.1 N/100 g: milliequivalent sodium hydroxide solution 0.1N per 100 g sample
[2] mg ECE/*g*: milligram equivalent epicatechin per gram sample
*Means (± standard deviation) with different letters in the same line (treatment) are statistically different (Duncan test, $p \leq 0.05$).
*Theophylline was not detected in any treatment. LOQ = 0.02 mg/g.
**Only 3 out of 9 amines were detected. Tyramine, histamine, serotonin, agmatine, spermidine and tryptamine were not detected in any sample. LOQ = 0.04 mg/kg.

The levels of total phenolic compounds varied from 26.35 to 32.25 mg ECE/g, with lower levels for CF and Sc treatments (≈26 mg ECE/g) and higher levels for the Pk treatment (32.25 mg ECE/g). Higher levels of total phenolic are desirable due to the antioxidant properties of these compounds, but they are also associated with the bitterness of the product (LIMA et al., 2011). Therefore, the possibility of modulating total phenolic levels in cocoa during fermentation would be relevant in the optimization of chocolate quality.

Catechin and epicatechin are also phenolic compounds which contribute to the antioxidant activity, bitterness and astringency (EFRAIM et al., 2010). The CF

treatment provided the lowest levels (0.05 and 0.32 mg/g, respectively), whereas the treatment with both yeasts (ScPk) the highest levels (2.19 and 3.14 mg/g, respectively). These compounds also affect product quality, therefore, the possibility of modulating their concentration in the final product is desirable. During cocoa fermentation, the microorganisms can hydrolyze phenolic complexes (which are linked to sugars reducing availability) into free and simple-soluble phenols increasing the levels of these compounds. The modification of the cellular structure of the cocoa cotyledon during the drying process, can release these compounds thus increasing their quantity (HAILE; KANG, 2019; OOI; TING; SIOW, 2020). There is evidence that yeasts species like *Pichia kudriavzevii* provide the release of these compounds as well, as the drying process in recent laboratory scale studies (OOI; TING; SIOW, 2020).

Among the three methylxanthines investigated (Table 3), only theobromine and caffeine were detected, whereas theophylline was not (LOQ=0.02 mg/g). Higher levels of methylxanthines were present in the ScPk treatment (10.83 mg/g) compared to the others. These methylxanthines are exuded from the cocoa bean testa (cocoa bean shell) (NAZARUDDIN et al., 2006) and the use of both yeasts (ScPk) could have facilitated its release. The possibility of modulating theobromine and caffeine levels in cocoa is also interesting as these compounds are associated with several functional and pharmacological properties, including reduction of stress and beneficial neurophysiological effects (SANSONE et al., 2017; TODOROVIC et al., 2015). However, in large amounts, caffeine can trigger agitation, hypertension and insomnia (MARTÍNEZ-LÓPEZ et al., 2014).

Among the nine bioactive amines investigated, only three were detected in the fermented and dried cocoa beans: putrescine, cadaverine and phenylethylamine (Table 3). Higher total amines levels were found in the Sc treatment (7.10 mg/kg) followed by CF and Pk (4.6-4.8 mg/kg), which were higher compared to the treatment with mixed starter culture – ScPk (2.42 mg/kg).

The highest levels of cadaverine and putrescine were observed in the Sc treatment, suggesting that added *S. cerevisiae* could induce production of these amines during fermentation. Putrescine can be formed directly from ornithine by decarboxylase activity and it is the precursor in the formation of the polyamines which are relevant in several vital functions for cell growth and maintenance (GLORIA, 2005; TACHIHARA et al., 2005). The formation of cadaverine was reported by *S. cerevisiae*

exposed to 12% ethanol for 24 h (WALTERS; COWLEY, 1998). Since both putrescine and cadaverine can contribute with putrefactive sensory characteristics to the product (TUFARIELLO et al., 2019), the minimization of the formation and accumulation of these amines is desirable. When using the treatment with both yeast – ScPk, putrescine and cadaverine were not detected, therefore, it would be the treatment of choice to prevent the formation and accumulation of these putrefactive amines.

Phenylethylamine was also detected in every dried fermented cocoa. Higher levels (2.30-2.42 mg/kg) were detected in *P. kudriavzevii* treatments (Pk and ScPk), suggesting that this yeast can be associated with the buildup of this amine. Furthermore, phenylethylamine can be formed during cocoa dehydration, due to release from conjugated amines or to the oxidative decarboxylation of the precursor amino acid (phenylalanine) at high temperatures (ORACZ; NEBESNY, 2014). Phenylethylamine is an important hypothalamic stimulating amine and, thus, can induce feelings of well-being and affect the levels of serotonin and brain endorphins, improving mood (AFOAKWA, 2008). But at high levels (≥30 mg/kg), this amine can be detrimental to human health, inducing migraines (JEON; LEE; MAH, 2018; ORACZ; NEBESNY, 2014). The levels of phenylethylamine obtained with the different treatments are far below the values capable of causing adverse effect to human health.

Based on these results, by using the ScPk treatment, a product with better characteristics was obtained compared to control and the use of the yeasts individually: higher pH, levels of total phenolic compounds, monomeric compounds, methylxanthines and phenylethylamine, and lower titratable acidity and levels of the putrefactive amines (putrescine and cadaverine). Furthermore, this product would have better functional properties associated with methylxanthines, monomeric compounds and phenylethylamine.

3.4 DIFFERENTIATION OF FERMENTED COCOA FROM DIFFERENT TREATMENTS BY MULTIVARIATE ANALYSIS

In order to better understand which factors were affected by the different starter cultures used during on-farm cocoa fermentation, PCA and HCA were used. In the four fermentation treatments, the first two components (PC1 + PC2) accounted for

approximately 83, 88, 75 and 92% of the data variance for CF, Sc, Pk and ScPk, respectively (Figure 2).

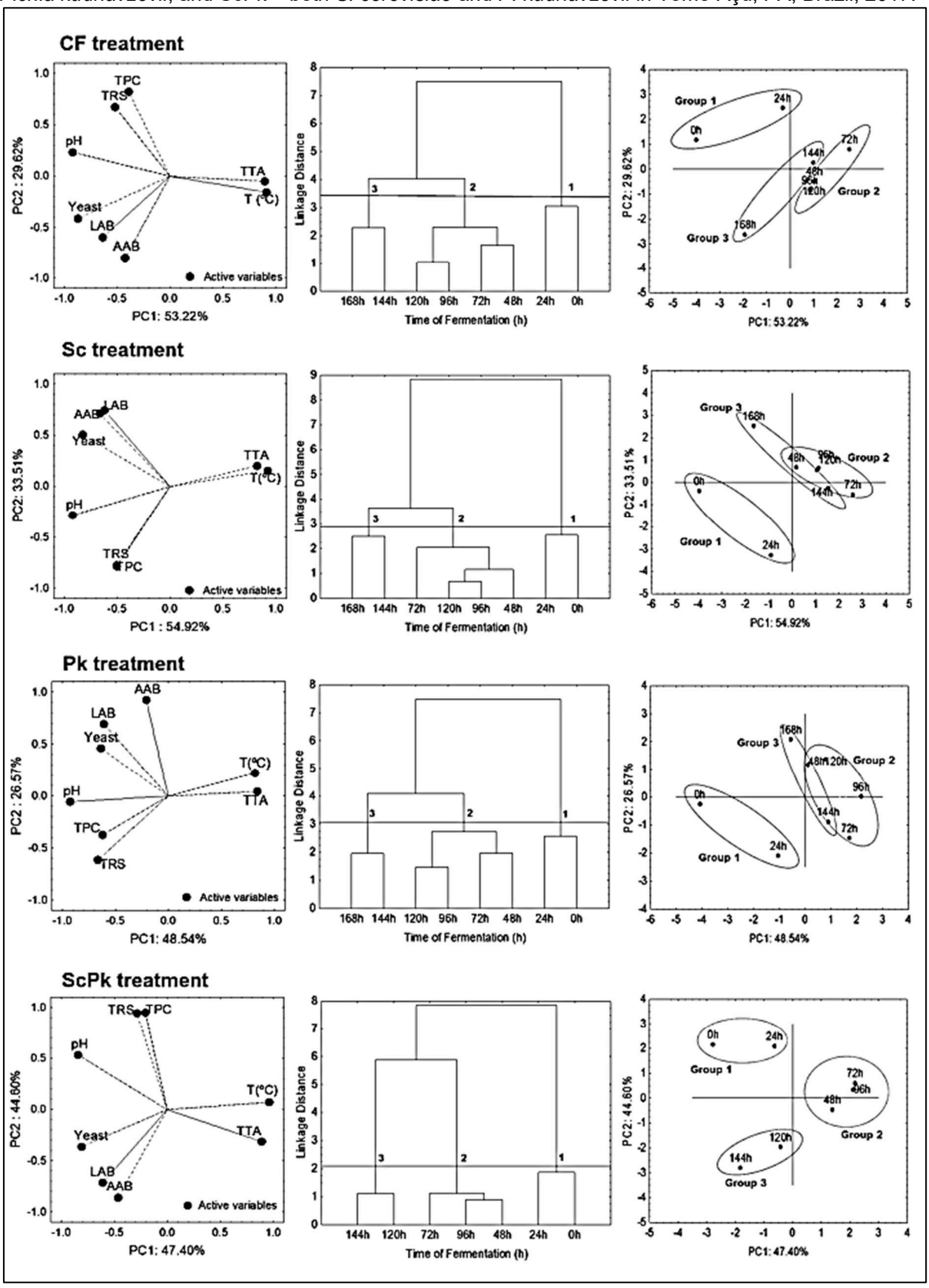
For all the treatments, fermentation was separated into three clusters (HCA), which, based on the parameters included in this study, represented three fermentation momentums: (i) group 1 – 0 and 24 h, which was characterized by highest pH, TRS and TPC, i.e., the beginning of the fermentation process; (ii) group 2 – 48, 72, 96 and 120 h for CF, Sc and Pk treatments; and 48, 72 and 96 h for ScPk treatment, intermediate fermentation times, which were characterized by lower TPC and pH, and higher TTA and temperature; and (iii) group 3 – 144 and 168 h for CF, Sc and Pk treatments; and 120 and 144 h for ScPk treatment, which were characterized by the high counts of yeasts, LAB and AAB. Based on these results, ScPk differed from the other treatments as the fermentation process was sped up and the end of fermentation was achieved faster (144 h compared to 168 h). Moreover, the three groups were better segregated compared to the others and PC1+PC2 accounted for the highest percentage of the data variance (92%).

A second PCA and HCA study (Figure 3) compared dried cocoa submitted to the four different fermentation treatments and involved several components but also included monomeric phenols, methylxanthines and bioactive amines. According to Figure 3, PC1 + PC2 accounted for approximately 91% of the data variance. There was a negative relationship between pH and cadaverine (r=-0.76) and putrescine (r=-0.79), indicating that low pH is a favorable environment for the formation of these amines (GLORIA, 2005). The same behavior was found in the fermentation of cocoa and manipueira for tucupi (DO CARMO BRITO et al., 2017, 2019). CF and Sc treatments were characterized by higher TTA, putrescine and cadaverine, whereas Pk and ScPk were characterized by higher concentrations of TPC, monomeric phenolic compounds (catechin and epicatechin), methylxanthines (theobromine and caffeine) and phenylethylamine. HCA separated well these two groups. Based on these results, the treatment with *S. cerevisiae* (Sc) was similar to control and the presence of *P. kudriavzevii* affected the quality of the dried cocoa, setting them apart from CF. In fact, the treatments containing *P. kudriavzevii* provided products with better functional properties, associated with methylxanthines (neurophysiological and cardiovascular properties), phenolic compounds (antioxidant activity) and phenylethylamine (mood modulation). These characteristics were restricted to fermented cocoa beans with the

addition of *P. kudriavzevii* – Pk and ScPk, therefore, enhancing the role of this yeast in cocoa fermentation.

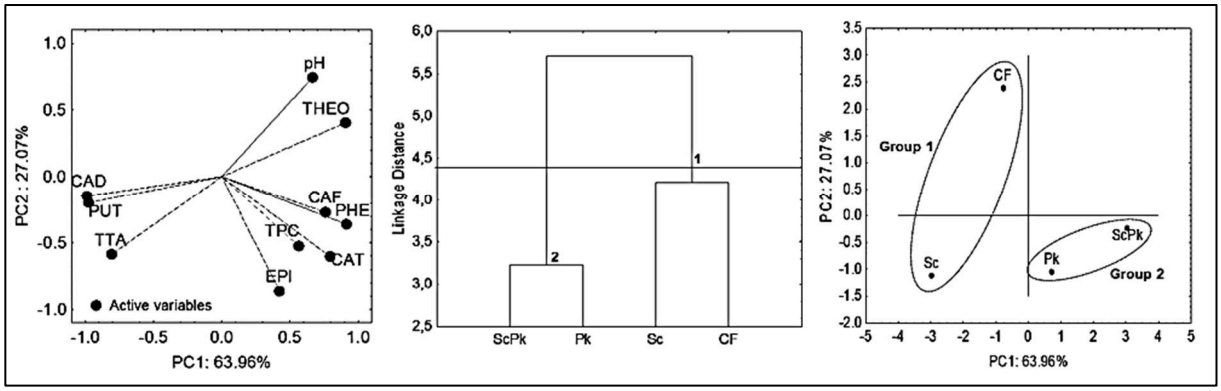
When considering the time taken for fermentation to end, the ScPk treatment achieved final desirable characteristics 24 h shorter compared to the other treatments. Based on these results, the use of the two yeast species, together and in equal proportions, provided conditions that favored the active microbial performance generating acceptable and unprecedented indices to the process, reported for the first time in the literature on yeast starter cultures for cocoa fermentation in the Amazon region.

Figure 2 – Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) during on-farm cocoa fermentation with different starters: CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; and ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017.



Notes. T (ºC) – temperature, TTA – total titratable acidity, TPC – total phenolic compounds, TRS – total reducing sugars, LAB – lactic acid bacteria, AAB – acetic acid bacteria.

Figure 3 – Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) of on-farm dried and fermented cocoa beans with different starters: CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; and ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017.



Notes. TTA – total titratable acidity, TPC – total phenolic compounds, EPI – epicatechin, CAT – catechin, THEO – theobromine, CAF – caffeine, CAD – cadaverine, PUT – putrescine, PHE – phenylethylamine.

4 CONCLUSIONS

Significant differences were observed between the four treatments (CF, Sc, Pk, ScPk) with respect to pH, total titratable acidity, total phenolic compounds, total reducing sugars and temperature during fermentation. In addition, fermentation with ScPk was completed 24 h earlier. Fermented and dried cocoa beans from treatments with *P. kudriavzevii* (Pk and ScPk) showed higher amounts of phenolic compounds and methylxanthines, showing the influence of this species in increasing antioxidant capacity. The synergy of ScPk inhibited putrefactive amines and enhanced phenylethylamine, a mood modulating amine. PCA and HCA analyses confirmed previous results and provided clusters which clearly differentiated the characteristics of the treatments. Therefore, the addition of starter culture to cocoa prior to fermentation can modulate chocolate quality and functional properties. The promising results observed show the need of investment in studies aimed at modulating the quality of fermented and dried cocoa beans to warrant high-quality chocolate with desirable chemical and flavor characteristics associated with the new consumer demands.

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Capítulo III

Profile of Volatile Compounds of On-Farm Fermented and Dried Cocoa Beans Inoculated with *Saccharomyces cerevisiae* KY794742 and *Pichia kudriavzevii* KY794725

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"Profile of Volatile Compounds of On-Farm Fermented and Dried Cocoa Beans Inoculated with *Saccharomyces cerevisiae* KY794742 and *Pichia kudriavzevii* KY794725"

ABSTRACT

This study aimed to identify the volatile compounds in the fermented and dried cocoa beans conducted with three distinct inoculants of yeast species due to their high fermentative capacity: *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, the mixture in equal proportions 1:1 of both species, and a control fermentation (with no inoculum application). Three starter cultures of yeasts, previously isolated and identified in cocoa fermentation in the municipality of Tomé-Açu, Pará state, Brazil. The seeds with pulp were removed manually and placed in wooden boxes for the fermentation process that lasted from 6 to 7 days. On the last day of fermentation, the almonds were packaged properly and placed to dry (36 °C), followed by preparation for the analysis of volatile compounds by GC-MS technique. In addition to the control fermentation, a high capacity for the formation of desirable compounds in chocolate by the inoculants with *P. kudriavzevii* was observed, which was confirmed through multivariate analyses, classifying these almonds with the highest content of aldehydes, esters, ketones and alcohols and low concentration of off-flavours. We conclude that the addition of mixed culture starter can be an excellent alternative for cocoa producers, suggesting obtaining cocoa beans with desirable characteristics for chocolate production, as well as creating a product identity for the producing region.

Keywords: chocolate; GC-MS; PCA; HCA

1 INTRODUCTION

Being among the most appreciated products in different locations in the world, chocolate is the result of a complex processing process where different physical, chemical and microbiological reactions occur, from the collection of the cocoa fruit until the final product (OZTURK; YOUNG, 2017; RAMOS et al., 2014).

In 2019, Pará state was the largest Brazilian cocoa producer, with a total of 135 thousand tons of harvested fruit expected, representing a 25% growth in five years (CEPLAC, [s.d.]). Recently, the city of Tomé-Açu was awarded the Geographical Indication certification, being the only municipality in Pará to have it, thus allowing greater representation in the trade of products related to the fruit (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE, 2019).

The processing of cocoa beans has well-defined stages: harvesting, breaking and opening the fruit, fermentation, drying, roasting, grinding and refining, conching and tempering. Among the aforementioned stages, fermentation is one of the essential stages, since it is there that the formation of the precursors of chocolate aroma and flavour occurs. It is a natural and spontaneous biological process in which there is the participation of a microbial consortium, in which groups of yeasts, lactic and acetic acid bacteria (AFOAKWA et al., 2008; SCHWAN; WHEALS, 2004).

Several studies in the literature report the performance of a wide variety of yeasts, lactic and acetic bacteria during fermentation in different locations around the world (CHAGAS JUNIOR; FERREIRA; LOPES, 2020; FIGUEROA-HERNÁNDEZ et al., 2019); however, in the Amazon, these studies are still scarce and few studies see reporting the cacao fermentative microbiota (ALMEIDA et al., 2018; DE ARAÚJO et al., 2019; SERRA et al., 2019).

The literature reports several studies using starter cultures for the fermentation of cocoa beans and subsequent manufacture of chocolates (BATISTA et al., 2016; HO; ZHAO; FLEET, 2014; MOREIRA et al., 2018; VISINTIN et al., 2017). The chemical compounds formed during fermentation by the different microbial groups active in the process are responsible for the characteristic aroma and flavour of chocolate. Esters, ketones, alcohols and aldehydes are responsible for the design of fruity, floral and sweet aromas formed during the fermentation process by different yeast species, such as *Pichia kudriavzevii*, *Saccharomyces cerevisiae* and *Candida tropicalis*, for example (KONÉ et al., 2016; MOREIRA et al., 2013).

Recent studies have used yeast starter cultures and were able to elucidate the importance of these microorganisms in the formation of volatile compounds in fermented almonds, thus providing almonds with desirable flavour and flavour characteristics to chocolate.

The use of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* mixed inoculum were responsible for the formation of higher amounts of alcohols and esters, responsible for notes of floral, fruity and sweet aromas (VISINTIN et al., 2017). The same behaviour was registered by Moreira et al., (2017) when using mixed inoculum of *S. cerevisiae*, *Lactobacillus plantarum* and *Acetobacter pasteurianus* and in the study by Pereira et al., (2017) with inoculants of variations of *Pichia kudriavzevii* that were also capable of accelerating fermentation on a laboratory scale.

Recent research papers with inoculants of the yeast species *S. cerevisiae* and *P. kudriavzevii* have been reporting numerous benefits for the quality of fermented cocoa beans such as obtaining almonds with fruity, sweet, floral aromas, reduced levels of acidity, increased phenolic compounds and methylxanthines levels (antioxidant and metabolic capacity) and, above all, capacity to reduce the time of the seed fermentation process, which on a large scale can favour greater productivity for the producer of the fruit (CHAGAS JUNIOR et al., 2021; MOREIRA et al., 2017; OOI; TING; SIOW, 2020; PEREIRA et al., 2017; SANDHYA et al., 2016). In this study, we aimed to identify the volatile compounds, for the first time, formed in the on-farm fermentation of Amazonian cocoa beans, from the use of *Saccharomyces cerevisiae* and *Pichia kudriavzevii* starter cultures, through GC-MS analysis.

2 MATERIAL AND METHODS

2.1 MATERIAL

Cocoa beans, fermented with *Saccharomyces cerevisiae* (Genbank KY794742) and *Pichia kudriavzevii* (Genbank KY794725) by Chagas Junior et al., (2021) in Tomé-Açu city, Pará state, Brazil (02°28′41.3″ S and 48°16′50.7″ W) in September of 2017, were used in this research.

2.2 FERMENTATION ASSAY, DRYING PROCESS AND MOISTURE DETERMINATION

Four fermentation treatments were carried out being one without the addition of inoculum (control treatment—CT) and three treatments with different inocula: *Saccharomyces cerevisiae* inoculum (ST); *Pichia kudriavzevii* inoculum (PT) and fermentation with the 1:1 addition of both species (SPT). All treatments were performed in triplicate (n = 3) according to the standard procedures established by the local producer with no external interference, followed by seven days of fermentation for CT, ST and PT treatments and six days for SPT treatment once the producer noticed the completion of the process (CHAGAS JUNIOR et al., 2021). All the fermentations were carried out in wooden boxes with a dimension of 0.12 m^3, each containing 90 kg of cocoa beans in each fermentation treatment.

At the end of the process, the fermented cocoa beans were packed in sterile polyethylene bags under freezing (−18 °C) for subsequent artificial drying in an air circulation oven (DeLeo, Porto Alegre, RS, Brazil) at 35 °C until at a constant moisture of 6% (EFRAIM et al., 2010).

The shells and embryo of the fermented and dried cocoa beans of each treatment were removed and the cotyledons were ground in an analytical mill (model A11B, Ika, Staufen, Germany) for moisture determination. This process was performed by direct analyse on an infrared moisture analyser (model IV2500, Gehaka, São Paulo, SP, Brazil). The analysis was performed in triplicate.

2.3 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) IN FERMENTED AND DRIED COCOA BEANS

Samples of fermented and dried cocoa beans (20 g) of each fermentation treatments were subjected to the simultaneous distillation/extraction process for 2 hours, using pentane (Sigma-Aldrich, St. Louis, MO, USA) as a solvent (DE SOUSA et al., 2019; MAIA; ANDRADE, 2009; MAIA et al., 2008).

The volatile concentrate obtained was analysed by GC-MS (model QP-2010 Plus, Shimadzu, Tokyo, Japan), equipped with a DB-5MS column (30 m × 0.25 mm × film thickness = 0.25 μm). The oven temperature was adjusted from 60–250 °C, using a ramp of 3 °C/min, injector temperature of 250 °C. Helium gas was used as a mobile phase, with a flow rate of 1.2 mL/min. An electron ionization mass spectrometer (model GC-2010A, Shimadzu, Tokyo, Japan) at 70 eV with the ion source temperature and other parts at 220 °C was used. Quantitative analysis of the chemical constituents was performed by peak-area normalization using a flame ionization detector (FID—Shimadzu, QP 2010 system) under the same conditions as GC-MS, except that nitrogen was used as a mobile phase. The components were identified based on the retention index (RI), which was calculated using the retention times of a homologous series of n-alkanes (C8–C40, Sigma-Aldrich, St. Louis, MO, USA).

The pattern of fragmentation observed in the spectra was compared with existing data in the system library and with data from the literature (ADAMS, 2007; MONDELLO, 2015; STEIN et al., 2011).

2.4 STATISTICAL ANALYSIS

The means of the moisture determination were verified according to the Analysis of Variance (ANOVA) and compared with Tukey's test at 5% significance. The Principal Component Analysis (PCA) was performed to group the volatile compounds identified and quantified in the cocoa samples in all treatments used (CT, ST, PT, SPT). The sum of the compounds of each chemical class was considered as the active variables. For Hierarchical Cluster Analysis (HCA), the hierarchical tree was obtained taking into account the same groups of active variables as the PCA, based on the Euclidean distances (Ward's method) for the grouping. The statistical analyses were performed with Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA).

3 RESULTS AND DISCUSSION

3.1 MOISTURE DETERMINATION OF FERMENTED AND DRIED COCOA BEANS

The moisture values in each treatment were 5.70% (control treatment—CT), 5.65% (*S. cerevisiae* inoculum—ST), 5.45% (*P. kudriavzevii* inoculum—PT) and 5.55% (1:1 inoculum—SPT), showing a statistical difference between them (Tukey's test, $p < 0.05$) (Table 1). All the cotyledons samples of fermented and dried cocoa beans were below 7% (important to prevent insect attack) and below 8% (important to prevent mould proliferation) (FERREIRA, 2017). Studies report the importance of low moisture values to promote the Maillard reaction along with protein levels (AGUS; MOHAMAD; HUSSAIN, 2018; UTRILLA-VÁZQUEZ et al., 2020; ZZAMAN; BHAT; YANG, 2014).

Table 1 – Moisture (%) values* in fermented and dried cocoa beans, inoculated with different yeasts inocula: CT—control fermentation, ST—*Saccharomyces cerevisiae* inoculum, PT—*Pichia kudriavzevii* inoculum, SPT—inoculum with both species (1:1).

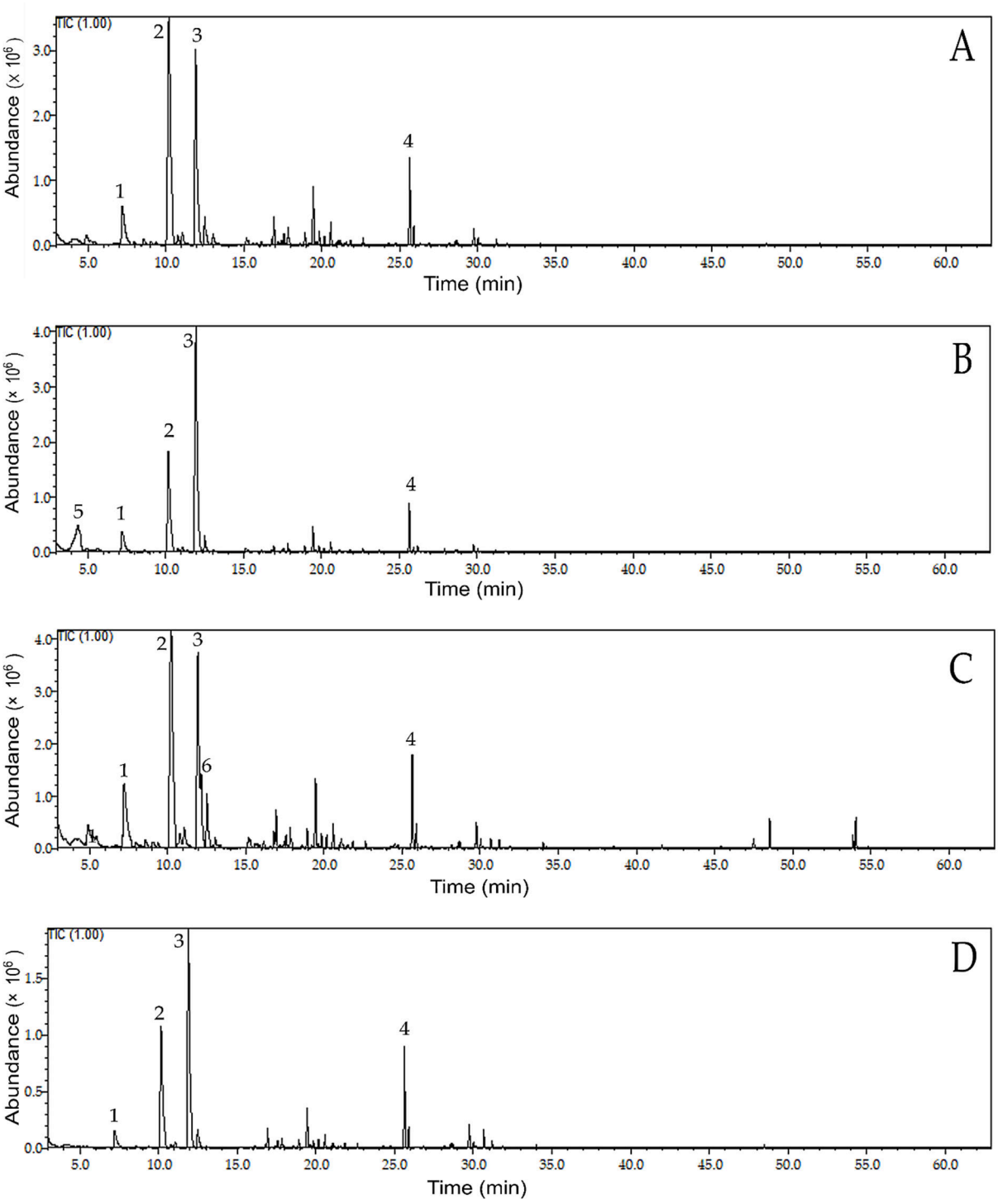
	CT	**ST**	**PT**	**SPT**
Moisture (%)	5.70 ± 0.00 ^{a}	5.65 ± 0.07 ^{ab}	5.45 ± 0.07 ^{b}	5.55 ± 0.07 ^{ab}

* in dry base. Means (± standard deviation) with different letters in the same line are statistically different (Tukey's test, $p \leq 0.05$).

3.2 PROFILE OF VOLATILE COMPOUNDS IN FERMENTED AND DRIED COCOA BEANS

With chromatogram analysis (Figure 1A–D) and comparison with literature data, we identified thirty-six volatile compounds in cocoa beans in this study classified into seven distinct classes: acids, alcohols, aldehydes, ketones, esters, pyrazines and alkanes (Table 2). There was a total of 91.87% of identification in the control treatment (CT), 95.03% in the treatment with *S. cerevisiae* (ST), 93.45% in the treatment with *P. kudriavzevii* (PT), and 94.44% in the treatment with the mixed inoculum (SPT).

Figure 1 – Ion Chromatograms of the GC-MS analysis on fermented and dried cocoa beans in four treatments [1].



Notes. [1]: CT—control treatment (**A**); ST—*Saccharomyces cerevisiae inoculum* (***B***); PT—*Pichia kudriavzevii inoculum* (***C***) and SPT—*S. cerevisiae* and *P. kudriavzevii inoculum (1:1),* (***D***). (1)—Benzaldehyde; (2)—Phenylacetaldehyde; (3)—Tetramethylpyrazine; (4)—Isoamyl benzoate; (5)—Isovaleric acid; (6)—2-Nonanone.

The acids are generally associated with the result of bacterial metabolism (lactic and acetic bacteria) developed during cocoa fermentation. The presence of acids in large quantities in cocoa beans is not desirable due to the attribution of undesirable notes of sweat, vinegar and rancidity to the quality of chocolate as a final product. The production of these compounds is also related to the performance of bacteria of the genus *Bacillus* sp. at the end of the fermentation process (BARIŠIĆ et al., 2019; RODRIGUEZ-CAMPOS et al., 2012; SCHWAN; WHEALS, 2004). The ST treatment showed a higher acid content, with isovaleric acid responsible for 10.77% and acetic 2.09%. On the other hand, the PT treatment presented a greater variety of acidic compounds: octanoic, palmitic and oleic.

Table 2 – Volatile compounds (GC-MS) in fermented and dried cocoa beans with the addition of three different yeast starter cultures [1].

(to be continued)

RI	Compound	CT (%)	ST (%)	PT (%)	SPT (%)	Odour Description [2]
Acids						
841	Isovaleric acid	-	10.77	-	-	Sweat, rancid (off-flavour)
1,173	Octanoic acid	-	-	0.38	-	Sweat, fatty (off-flavour)
1,256	Acetic acid	-	2.09	-	-	Sour, vinegar (off-flavour)
1,964	Palmitic acid	-	-	0.33	-	
2,169	Oleic acid	-	-	1.25	-	
Alcohols						
894	2-Heptanol	-	-	0.32	-	Fruity, citrus, herbal
1,099	2-Nonanol	-	-	3.98	2.91	Fat, green
1,111	2-Phenylethanol	0.93	0.15	0.35	-	Honey, rummy, floral
Aldehydes						
955	Benzaldehyde	6.99	4.68	10.19	3.30	Roasted almonds, candy, burnt sugar
1,040	Phenylacetaldehyde	36.50	21.79	30.78	26.87	Floral, honey
1,271	2-Phenylbut-2-enal	0.45	0.26	0.56	0.70	Floral, honey, powdery, cocoa

Table 2 – Volatile compounds (GC-MS) in fermented and dried cocoa beans with the addition of three different yeast starter cultures [1].

(conclusion)

RI	Compound	CT (%)	ST (%)	PT (%)	SPT (%)	Odour Description [2]
	Dodecanal	-	-	-	0.39	
1,490	5-Methyl-2-phenyl-2-hexenal	1.01	0.46	1.22	1.85	Cocoa
Ketones						
1,062	Acetophenone	1.65	0.56	1.45	-	Flower, almond, pungent, sweet
1,090	2-Nonanone	-	-	5.25	-	Fruity, sweet, waxy, green herbaceous
1,119	Isophorone	0.09	-	-	-	
Esters						
868	Isopenthyl acetate	0.94	-	1.94	-	
1,163	Benzyl acetate	-	-	0.34	-	Floral, jasmine
1,169	Ethyl benzoate	0.16	-	-	-	Camomile, flower, fruity
1,196	Ethyl octanoate	0.38	0.21	-	-	Fruity, floral
1,255	2-Phenethyl acetate	4.36	0.53	4.21	4.23	Fruity, sweet, roses, honey, floral
1,394	Isoamyl benzoate	5.09	4.00	4.51	8.95	Balsam, sweet
1,594	Ethyl dodecanoate	0.06	-	0.15	-	Sweet, floral
1,994	Ethyl hexadecanoate	0.10	-	1.15	0.21	
Pyrazines						
1,000	2,3,5-Trimethylpyrazine	0.59	-	-	-	Roasted cocoa
1,084	Tetramethylpyrazine	24.76	46.77	19.71	41.20	Roasted cocoa, chocolate
Alkanes						
1,055	3-Methyldecane	-	-	1.09	-	
1,200	*n*-Dodecane	1.68	0.48	1.74	1.78	Off-flavour
1,264	2-Methyldodecane	0.78	-	-	-	
1,280	4,6-Dimethyldodecane	1.18	-	-	-	
1,300	Tridecane	0.11	-	0.16	-	Off-flavour
1,400	*n*-Tetradecane	0.93	-	0.93	1.51	Off-flavour
1,490	Pentadecane	0.12	0.25	0.30	0.42	Off-flavour
1,711	Heptadodecane	-	-	-	0.12	Off-flavour
Others						
1,099	Linalool	2.78	2.03	-	-	Floral
1,181	Naphthalene	0.23	-	1.16	-	
	Total (%)	91.87	95.03	93.45	94.44	

Notes. RI: retention index. [1]: CT: control fermentation, without addition of inoculum; ST: fermentation with *Saccharomyces cerevisiae* inoculum; PT: fermentation with *Pichia kudriavzevii* inoculum; SPT: fermentation with the 1:1 addition of both species. [2]: These characteristics were found in the literature (AFOAKWA et al., 2008; APROTOSOAIE; LUCA; MIRON, 2016; BASTOS et al., 2019; RODRIGUEZ-CAMPOS et al., 2011, 2012; TUENTER et al., 2020; UTRILLA-VÁZQUEZ et al., 2020).

Only three different alcoholic compounds were identified in all fermentation treatments: 2-Heptanol, 2-Nonanol and 2-Phenylethanol. These compounds are the result of the metabolism of the yeast population active in the anaerobic phase of fermentation, after using the sugars present in the pulp of the cocoa fruit as a carbon source together with the microbial activity of the process (BARIŠIĆ et al., 2019; DE VUYST; LEROY, 2020; LIMA et al., 2011).

Saccharomyces cerevisiae is one of the yeast species that is most prominent in the production of aromatic compounds, including alcohols, during cocoa fermentation. They manage to produce numerous desirable compounds to chocolate, (the alcoholic compounds of this study, for example), providing floral, fruity and citrus aromas (BATISTA et al., 2016; KONÉ et al., 2016). The PT treatment produced about four times more alcohols than the other treatments, with 2-Nonanol gaining prominence, which gives citrus notes to the almond and has its formation associated with also *S. cerevisiae* (BASTOS et al., 2019).

The amount of 2-Phenylethanol was relatively low in the CT, ST and PT treatments, as reported in the study by Tuenter et al., (2020). The formation of this compound is the result of yeast activity during fermentation thanks to the action of glycosidase in the precursor connections of the fruit pulp aromas, and also by the conversion of the amino acid phenylalanine and it is expected to obtain chocolates with floral notes (CHETSCHIK et al., 2018; TUENTER et al., 2020).

Five aldehydes were identified in our study: 2-Phenylbut-2-enal, Dodecanal, 5-Methyl-2-phenyl-2-hexenal and in greater quantities of Benzaldehyde and Phenylacetaldehyde. These compounds are the results of the metabolism of different yeast species at the beginning of fermentation, such as *Galactomyces geotrichum* (KONÉ et al., 2016).

Control fermentation (CT) and the treatment with *P. kudriavzevii* inoculum (PT), showed the highest total amount of aldehydes, showing that the population of *P. kudriavzevii* present, is capable of producing good quantities of these compounds, which has already been confirmed in laboratory scale fermentations by Pereira et al.,

(2017). However, the excessive amount of Benzaldehyde must be monitored to avoid the production of bitterness notes (CASTRO-ALAYO et al., 2019; CHETSCHIK et al., 2018). The same behaviour was observed in relation to ketones, where the greatest amounts of Acetophenone and 2-Nonanone stand out in the fermentation with the PT inoculum, which can confer almonds with floral and sweet notes (CASTRO-ALAYO et al., 2019; HO; ZHAO; FLEET, 2014).

A greater variety of esters can be seen in the control fermentation (CT); however, the mixed inoculation fermentation (SPT) has the highest concentrations, accounting for 13.39% of its total followed by 11.09% (CT), 7.79% (PT) and 4.74% (ST). The esters class is strongly related to almonds of good sensory quality, as they provide desirable aromatic notes of roses, flowers and fruity notes, as is the case of 2-Phenylethyl acetate and Isoamyl benzoate, which are products of yeast metabolism active in the fermentation (RODRIGUEZ-CAMPOS et al., 2011, 2012). In general, yeasts are relevant producers of esters during cocoa fermentation (MOREIRA et al., 2018).

The metabolic method of production of phenylacetaldehyde was elucidated from the use of aromatic enzymes such as aminotransferase, which can convert amino acids (tyrosine, phenylalanine and tryptophan) in the stage of transamination of these compounds. These activities have been associated with lactic acid bacteria *Lactobacillus brevis* and *Lactobacillus plantarum* (AGYIRIFO et al., 2019), the last of which is widely found in cocoa fermentations performed in the Amazonian region (SERRA et al., 2019).

Only two types of pyrazines were identified in this study: 2,3,5-Trimethylpyrazine (only in CT fermentation) and Tetramethylpyrazine (in all treatments). The ST and SPT treatments showed the highest concentrations of Tetramethylpyrazine in fermented and dried cocoa beans. This factor is of great importance for the food industry, as these compounds confer typical notes of chocolate (AFOAKWA et al., 2008; HAMDOUCHE et al., 2019). On the other hand, it is suggested to monitor the excessive amount of these compounds to avoid unpleasant notes of roasted product.

The formation of pyrazines is directly related to Strecker degradation begins in the drying process of the cocoa beans provided also by the action of the enzymes β-glycosidases, proteases, lipases and amylases, produced by bacteria of the genus *Bacillus* sp. (ASSI-CLAIR et al., 2019; CASTRO-ALAYO et al., 2019; RODRIGUEZ-CAMPOS et al., 2012; SERRA et al., 2019; UTRILLA-VÁZQUEZ et al., 2020). Some

parameters are decisive in the presence or absence of pyrazines in cocoa beans, such as, for example, as techniques and fermentation time, fruit maturation stage, cocoa variety, seed storage and chocolate processing (VISINTIN et al., 2017).

In a recent study (SANTOS et al., 2020), the researchers elucidated the significant influence that starter cultures of yeast species *Candida parapsilosis*, *Torulaspora delbrueckii* and *Pichia kluyveri* had on the formation and increase in flavour precursor amino acids (participants of the Maillard reaction) throughout fermentation. The authors highlight the high capacity of production of extracellular proteolytic enzymes due to the metabolism of these yeast species, with the ability to act inside the cotyledon providing a higher concentration of free amino acids in cocoa beans.

Saccharomyces cerevisiae and species of the genus *Pichia* are frequently identified in cocoa fermentation in several locations (CHAGAS JUNIOR; FERREIRA; LOPES, 2020; FIGUEROA-HERNÁNDEZ et al., 2019). These species and several others are linked to processes that are essential in the first hours of cocoa fermentation, such as: consumption of citric acid present in the pulp; increasing the pH of the medium and favouring the development of the bacterial community; ethanol production at low oxygen concentrations; production of aromatic compounds desirable to chocolate such as ethers, aldehydes and ketones; and production of pectinolytic enzymes that favour the release of fermentable sugars from the pulp, making them available for fermentation (RAMOS et al., 2014; SCHWAN; WHEALS, 2004; TOFALO et al., 2019).

The conversion of pulp carbohydrates into ethanol by the yeast's metabolic activity is essential for the formation of chocolate compounds, since the ethanol formed is oxidized to acetic acid by acetic bacteria, with an increase in temperature causing the embryo to die and, thus, triggering several chemical and structural reactions that allow the formation of other flavour and flavour precursor compounds and chocolate (BATISTA et al., 2016; SCHWAN; WHEALS, 2004).

Saccharomyces cerevisiae is a species of yeast that is widely studied in the application of inoculants in cocoa fermentation, due to its high capacity for assimilation of pulp sugars (mainly glucose and fructose), satisfactory production of volatile compounds desirable to chocolate, production of toxins capable of inhibiting some yeasts not tolerant to ethanol (BATISTA et al., 2016; RAMOS et al., 2014).

The interaction of *S. cerevisiae* and *P. kudriavzevii* was recently reported in the study by Chagas Junior et al., (2021) which proved the good relationship between the two species in foreign cocoa fermentations, providing almonds with higher levels of phenolic compounds, pH and lower levels of acidity, methylxanthines, acidity and putrefactive amines, thus showing itself as an excellent alternative in the production of good quality almonds.

In our study, a good interaction was observed between these two species in relation to the production of desirable volatile compounds for cocoa beans what can highlight the production of good quality almonds for the local production of chocolate, since the production of chocolates in Pará state is still in the beginning, but its recognition is already noticed in several nations that appreciate this product. The city of Medicilândia, for example, is the largest representative of the State abroad, having several higher quality product certificates, placing Pará in international evidence.

3.3 MULTIVARIATE ANALYSIS (PCA AND HCA) OF VOLATILE COMPOUNDS IN COCOA BEANS FERMENTED AND DRIED PRODUCED WITH DIFFERENT YEAST INOCULA

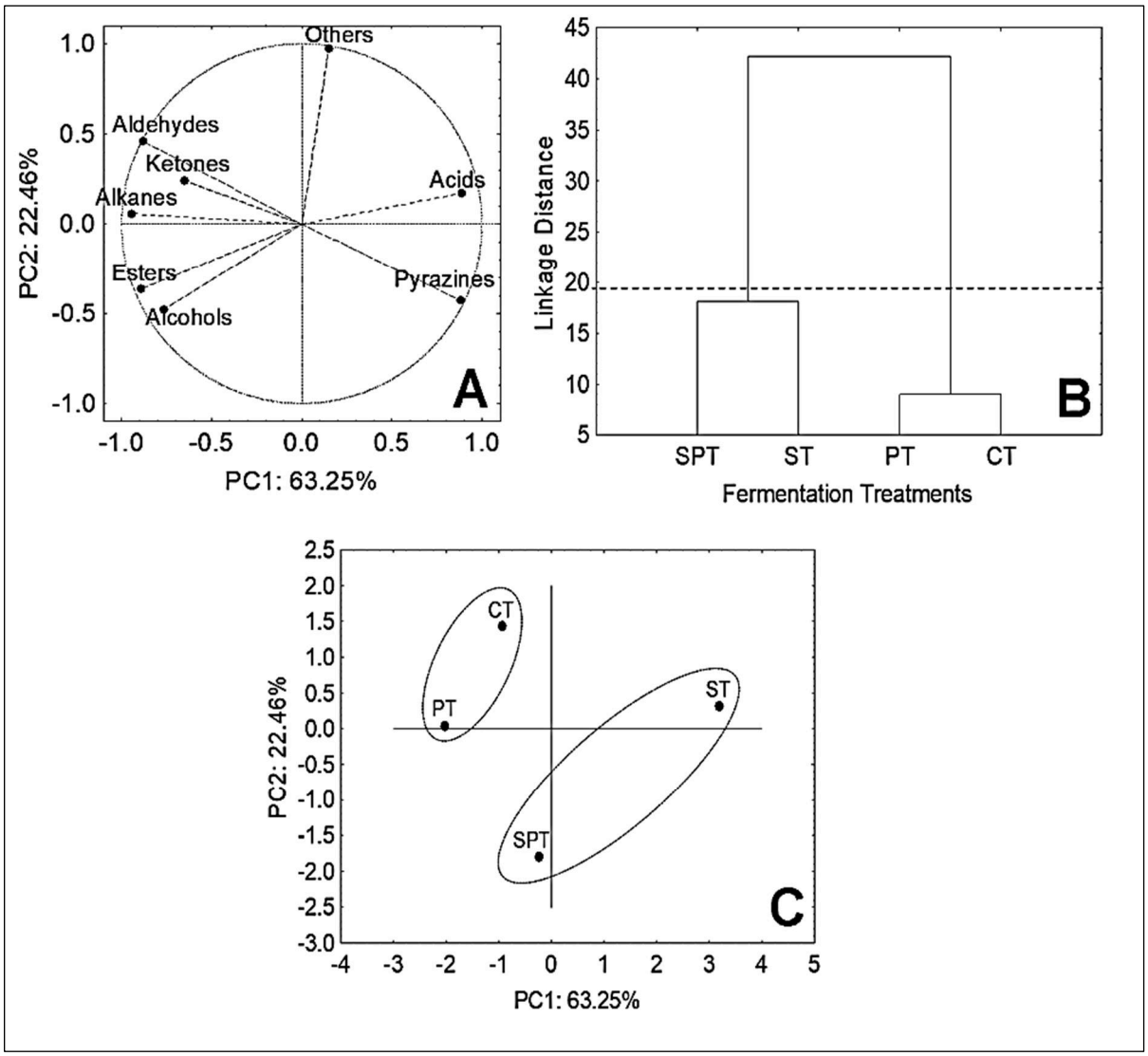
Finally, we performed the PCA and HCA to check and group the fermented and dried almonds in this study according to the amount of the identified volatile compound content. Previous studies have already demonstrated the importance of this analysis in cocoa fermentation in order to establish the identity of different fermentation techniques, fruit storage, drying and roasting at different temperatures and the influence of starter cultures to the process (CHAGAS JUNIOR et al., 2021; KONÉ et al., 2020; RODRIGUEZ-CAMPOS et al., 2011, 2012).

In order to better understand the importance of the four fermentation treatments used, we chose to analyse the volatile compounds in their entirety according to their chemical classes, for a better visualization of the results and better understanding.

The sum of the first two components (PC1 + PC2) accounted for 85.71% of the variance of the main data (Figure 2A). Of all the chemical classes identified, esters and aldehydes were the most affected by the yeast inocula in the four fermentation treatments. The formation of aldehydes was strongly correlated ($r = -0.95$) with

sufficient concentrations of pyrazines, which are also formed by the aldol condensation route in cocoa fermentation as well as the formation of esters from the condensation of alcohols and acids (mainly acetic acid), forming ethyl esters and acetates compounds, respectively (DE VUYST; LEROY, 2020). This correlation was strongly negative between esters and acids (r = −0.97) and strongly positive between esters and alcohols (r = 0.75), elucidating the amounts of these compounds formed in this study.

Figure 2 – Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) of volatile compounds in dried and fermented cocoa beans with different starters [1].



Notes. [1]: CT—control treatment, ST—*Saccharomyces cerevisiae* inoculum, PT—*Pichia kudriavzevii* inoculum; and SPT—*S. cerevisiae* and *P. kudriavzevii* inoculum (1:1). Principal Component Analysis (PCA) (**A**,**C**). Hierarchical Cluster Analysis (HCA) (**B**).

With the analysis of HCA it was possible to classify the fermented and dried cocoa beans in two distinct groups (Figure 2B,C): group 1—characterized by the fermented almonds in the ST and SPT treatments that had higher concentrations of acids and lower alcohols, ketones and esters and higher amounts of pyrazines; and group 2—fermented and dried almonds in CT and PT treatments, which are characterized by having the lowest concentrations of acidic compounds and high concentrations of desirable compounds in chocolate, such as aldehydes, ketones, alcohols and esters. In this group, pyrazine values remain satisfactory. On the other hand, there is a need to monitor the content of alkanes, which are considered off-flavors (BASTOS et al., 2019).

4 CONCLUSIONS

In this research, we observed that inoculations with the species *P. kudriavzevii* (PT, SPT) provided the formation of a greater variety of desirable compounds for cocoa beans intended for chocolate production, such as, for example, a greater variety of aldehydes and esters (fruity floral, sweet notes) and lesser amounts of undesirable compounds (off-flavors) such as alkanes and acids, proving to be an excellent alternative for cocoa almond producers in the region. To better understand the physiological dynamics and synergy of the microbiota responsible for the development of these compounds, they must be carried out and thus establish parameters for the development of starter cultures that can be used by producers and the food industry.

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Capítulo IV

The first report of *Lactobacillus farraginis* and *Lactobacillus parafarraginis* identified on-farm cocoa beans fermentation in Brazilian Amazon

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Artigo submetido ao periódico **International Journal of Agriculture and Biology** (Capes Qualis A4, 2019),

Carta de Submissão (Anexo A)

“The first report of *Lactobacillus farraginis* and *Lactobacillus parafarraginis* identified on-farm cocoa beans fermentation in Brazilian Amazon”

ABSTRACT

The microbiota that participates in the cocoa fermentation process is one of the main factors for the development of fermented almonds with desirable characteristics for chocolate. Several cocoa fermenting locations have a peculiar microbiota, thus generating almonds characteristic of each region. The presence of lactic acid bacteria (LAB) during cocoa fermentation still generates much discussion in the scientific community, because some researches reports that the LAB does not perform positively in the cocoa fermentation. This study aimed to identify the LAB population at the beginning of cocoa fermentation and contribute to the understanding the microbial dynamics of the microbial succession in the cocoa fermentation. With the sequencing of the 16S region was possible to identify five species of LAB: *Lactobacillus farraginis*, *Lactobacillus parafarraginis*, *Lactobacillus zeae*, *Lactobacillus casei,* and *Pediococcus acidilactici*, the possible being the most isolated. The importance that each species identified during the cocoa fermentation in this study is still unknown, but there are reports of conservation and preservation of the quality of the process and can be an excellent option for the production of starter cultures to be added to the fermentation process and obtaining cocoa beans with good quality. The identified species are registered in the GenBank database (accessions from MT117900 to MT117915).

Keywords: *Theobroma cacao*; Chocolate; Bioactive amines; *Pediococcus*.

1 INTRODUCTION

Microbial diversity, composed mainly of three distinct groups of microorganisms: yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB), is an important factor in the cocoa fermentation process (FIGUEROA-HERNÁNDEZ et al., 2019). Moreover, other factors such as the genotype of the fruit, techniques used for fermentation, and

the time of the process, provide the necessary conditions for the formation of aroma and flavor compounds desirable to the chocolate (LIMA et al., 2011).

During the cocoa fermentation process, LABs are identified as important for maintaining the flow of metabolites and providing the growth of AAB (SERRA et al., 2019). However, the diversity of LAB is a determining factor in the final quality of cocoa beans (OUATTARA et al., 2017) and the role of LAB species can be related to these results since they can produce antimicrobial compounds and decrease pH levels, providing stability for the growth control of possible deteriorating microorganisms (GHANBARI et al., 2013; OZOGUL et al., 2015).

Although studies have been reported indicating little benefit from having the action of these bacteria on cocoa fermentation recently, studies have pointed out the effectiveness of this microbial group for the production of agents that inhibit toxic compounds such as mycotoxins and bioactive amines considered to be putrefactive in foods (BARTKIENE et al., 2018; FIGUEROA-HERNÁNDEZ et al., 2019; RUGGIRELLO et al., 2019).

Few studies report the role of the microbiota during cocoa fermentation in the Brazilian Amazon, a biome that concentrates the largest cocoa production in Brazil (ALMEIDA et al., 2018; DE ARAÚJO et al., 2019; SERRA et al., 2019). The identification of LAB species naturally occurring in cocoa fermentation on a farm in the Brazilian Amazon is an initial and relevant step towards a better understanding of the action of these microorganisms in an important fermented cocoa seed producing region.

In this research, we aimed to isolate and identify the initial LAB population on-farm natural cocoa fermentation in Tomé-Açu city (a highly producer of cocoa in the Brazilian Amazon).

2 MATERIAL AND METHODS

2.1 FERMENTATION ASSAY

Natural cocoa fermentation was carried out on a farm in Tomé-Açu city, PA, Brazil (02º28'41.3"S and 48º16'50.7"W) in September 2017 (according to the local methodology) in three wooden boxes with a capacity of 50 kg/each for seven days. Small portions of 20 g were removed from five different points of the trough totaling ≈100 g of samples in the initial fermentation time. Samples of cocoa beans collected were stored aseptically in sterile polyethylene bags and stored under refrigeration (4 ± 2 ºC) (CHAGAS JUNIOR et al., 2021).

2.2 OBTAINING THE LAB CULTURES

Seeds with pulp (20 g) were aseptically macerated in new sterile polyethylene bags and homogenized in 180 mL of peptone water (pH 7.20, Kasvi, São José dos Pinhais, PR, Brazil) obtaining the dilution 10^{-1}, and at the end, the dilution 10^{-8} was obtained. Aliquots of 1 mL of each dilution were transferred to sterile Petri dishes and added De Man, Rogosa & Sharpe (MRS) agar (pH 6.2, Kasvi) with 0.2% nystatin (Prati-Donaduzzi, Toledo, PR, Brazil) to inhibit fungal growth (CHAGAS JUNIOR et al., 2021).

The Petri dishes were incubated at 37 ºC for 4 days (CAMU et al., 2007) and the LAB counting was expressed in $\log_{10}$CFU/g.

After incubation, 35% of the colonies counting (PAPALEXANDRATOU et al., 2011a) were isolated and tested according to the catalase and gram test for prior identification of LAB. Colonies with catalase-negative and gram (+) were isolated and purified (DA SILVA et al., 2010) in Petri dishes containing MRS Agar by the striation technique and incubated at 37 ºC for 48 h. This procedure was repeated twice to obtain pure colonies.

2.3 DNA EXTRACTION, POLYMERASE CHAIN REACTION (PCR) AND SEQUENCING

The genomic DNA was extracted following the procedures of Sambrook; Russell (2001) with subsequent resuspension in 100 µL of TE buffer (pH 6.0) and frozen until further analysis.

For PCR analysis, 2 µL of the extracted DNA was mixed in a microtube containing MilliQ water (Invitrogen, Carlsbad, CA, USA), Q-solution (Qiagen, Maryland, USA), 10× buffer solution (Invitrogen), dNTP mix (Bioron, Römemberg, Germany), $MgCl_2$ (50 mM, Invitrogen), Taq DNA polymerase (5U/µL, Invitrogen) and aliquots (10 pmol) of each primer: 616-f (5'-AGAGTTTGATYMTGGCTCAG-3') and 907-r (5'-CCGTCAATTCMTTTRAGTTT-3') (MORALES; HOLBEN, 2009; ZIMMERMANN et al., 2005). The PCR analysis was carried out in a Labtrace thermocycler (model K960, Hangzhou, Zhejiang, China) programmed with the conditions: initial denaturation at 95 ºC/5 min.; 35 cycles of denaturation (94 ºC/1 min.), annealing (55.5 ºC/2 min.), extension (72 ºC/2 min.) and final extension (72 ºC/10 min.) (DE ARAÚJO et al., 2019). The PCR products were purified with the PCR-clean-up kit (AP-PCR-250, Axygen, USA), following the procedures of manufacturer.

After purification, ~20 ng of purified amplified DNA was prepared for sequencing with the Kit Big Dye Terminator v. 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Bidirectional reactions were sequenced and analyzed using the equipment AB-3500 DNA Analyser (Applied Biosystems, Foster City, CA, USA) with the same pair primer, and the sequences obtained were edited with the BioEdit software (HALL, 1999). The sequences were submitted to the Genbank database (https://www.ncbi.nlm.nih.gov).

3 RESULTS AND DISCUSSION

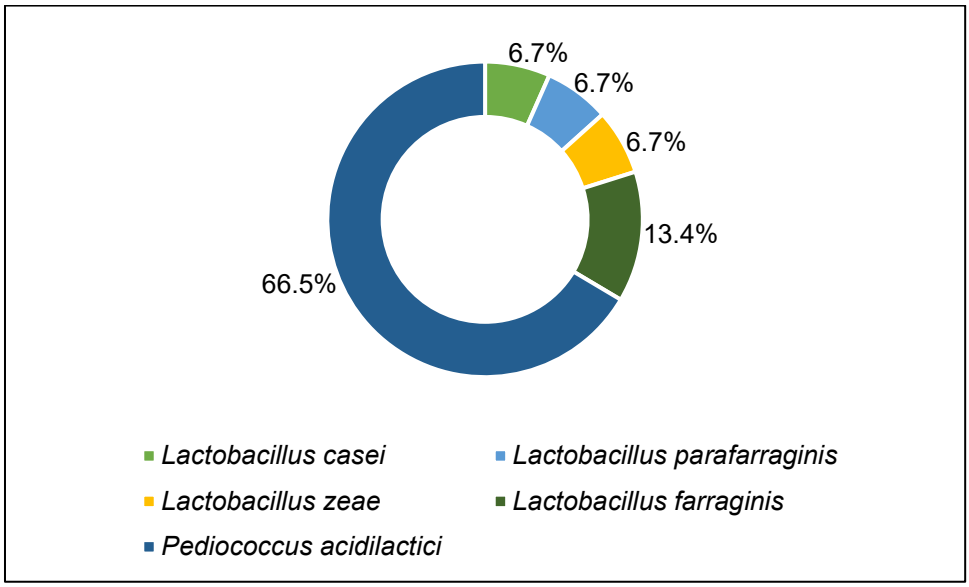
The LAB counting started at 7.60 $\log_{10}$CFU/g. This is according to LAB counting found in a recent study with starter cultures of yeasts in the same city (CHAGAS

JUNIOR et al., 2021). In this study, the authors also report an increase in the LAB population during the fermentation processes.

This is related to the metabolites produced by the other groups active during cocoa fermentation, such as lactic and acetic acids formed by LAB and AAB, respectively, suggesting that they can hydrolyze the glycosidic bonds of sucrose and also the action of invertase releasing more glucose and fructose in the medium. In this way, these available sugars were used for metabolizing these microbial groups, with an increase in the population active in the process (CHAGAS JUNIOR et al., 2021; DE VUYST; WECKX, 2016).

We identified five species of LAB (Figure1) active in cocoa fermentation: *Pediococcus acidilactici* (66.5%), *Lactobacillus farraginis* (13.4%), *Lactobacillus parafarraginis* (6.7%), *Lactobacillus zeae* (6.7%) e *Lactobacillus casei* (6.7%).

Figure 1 – Distribution of the identified LAB at the beginning of natural on-farm cocoa fermentation in Tomé-Açu, Brazil.



The genus *Lactobacillus* was most identified. However, the amount of *P. acidilactici* isolated was higher, showing to be one of the predominant species at the beginning of fermentation. The presence of the genus *Lactobacillus* in the cocoa fermentation is reported in several locations such as Brazil (ILLEGHEMS et al., 2012; MOREIRA et al., 2013, 2017; PAPALEXANDRATOU et al., 2011b), Ghana

(BORTOLINI et al., 2016; NIELSEN et al., 2007) and Ivory Coast (AGYIRIFO et al., 2019; HAMDOUCHE et al., 2015).

For the first time, *L. farraginis* and *L. parafarraginis* were isolated in cocoa fermentation in the Brazilian Amazon. These two species were able to promote aerobic stability, increase acetic acid, and decrease the yeast population in corn silages (LIU et al., 2014; SILVA, 2019) therefore, can be studied as an alternative to starter cultures for cocoa fermentation.

Pediococcus acidilactici has already been reported in cocoa fermentation in Honduras (ROMANENS et al., 2018), Nigeria (KOSTINEK et al., 2008), and in Brazil (BASTOS et al., 2018; PAPALEXANDRATOU et al., 2011b). This being the first time that this species is isolated and identified in the fermentation of Amazonian cocoa.

The species identified in our study, are effective in reducing the production of biogenic amines that impart degradation characteristics in foods, such as cadaverine and putrescine and mycotoxins in cereal fermentations (BARTKIENE et al., 2018) and on a laboratory scale with fermentative broths using known bacterial species for the production of these compounds (OZOGUL et al., 2015). Its influence during cocoa fermentation is still unknown, but the importance of studies on this topic is emphasized since this processing stage affects the levels of amines produced (DO CARMO BRITO et al., 2017; SPIZZIRRI et al., 2019).

Some studies reported the lack of importance of LABs for the cocoa fermentation process (HO; FLEET; ZHAO, 2018; HO; ZHAO; FLEET, 2014, 2015). However, it is important to highlight the strong interaction of the three main microbial groups active in the process (yeasts, LAB and AAB), where there is previously a consumption of fermentable sugars, mainly by the yeasts and after LAB (HO; ZHAO; FLEET, 2015). Recent evidence reinforces the benefits of lactic acid bacteria in cocoa fermentation. In the study by Chagas Junior et al., (2021), after the addition of yeast inoculums, cocoa beans with lower levels of acidity and higher levels of bioactive compounds were obtained, in addition to reduced fermentation time. It is suggested that the yeasts of the inocula found some synergy with the microbiota naturally found in the fermentation of the study site, including with the LAB.

Lactobacillus zeae and *Lactobacillus casei* were identified in this study and may represent a good indication of *natural microbial protection* on cocoa beans fermentation, since these species are recognized for their high antimicrobial activities (inhibition of pathogenic microorganisms, like *Staphylococcus aureus* and *E. coli*, for example) in addition to providing product development foods with great benefits for human ingestion, such as fermented milk and foods with good antioxidant capacity (BURITI; SAAD, 2007; PRAIA, 2020; STANISAVLJEVIĆ et al., 2015).

The presence of these LAB species may explain the low concentration of biogenic amines reported by Chagas Junior et al., (2021) after the addition of yeast starter cultures in cocoa fermentation in Tomé-Açu, suggesting a good microbial interaction during the process, in addition to providing inhibition of the proliferation of pathogenic bacteria that produce these substances (GLORIA, 2005), as previously mentioned in this research.

Glucose and fructose are converted into ethanol, lactic acid and acetic acid, making the necessary conditions for the proliferation of AAB, which will oxidize the ethanol produced in acetic acid and, consequently, raise the temperature of the fermentation and ultimately trigger the innumerable physical-chemical reactions that will give the desirable attributes to chocolate (DE VUYST; LEROY, 2020).

The modulation of the LAB population in cocoa fermentations should be further studied because of the strong correlation of some species such as *Lactobacillus fermentum* in the production of volatile compounds desirable to chocolate as a final product (MOTA-GUTIERREZ et al., 2018). This can be confirmed in a recent review paper, in which the formation of pyruvates, aldehydes (2-methylbutanal, 3-methylbutanal and benzaldehyde) can be obtained through the conversion of amino acids throughout the fermentation process (DE VUYST; LEROY, 2020).

Fermentation techniques adopted by cocoa producers must be taken into account for the success of the process, as is the case with the fermentation time and the revolving practice, where it is proven that its realization provides the ideal aeration for AAB. We also should consider the formation of several desirable volatile compounds (HAMDOUCHE et al., 2019) and other phenomena such as the volatilization of the acids produced and exudation of the phenolic and methylxanthine

compounds of the seeds, which can confer unpleasant tastes of bitterness and astringency (LEITE et al., 2013; NAZARUDDIN et al., 2006).

Another importance worth mentioning for LAB is the ability of some species such as *L. plantarum* and *L. fermentum* to produce good amounts of phenyllactic acid together with yeast species such as *Saccharomyces cerevisiae* and *Candida ethanolica* that are capable of working synergistically providing inhibition of the proliferation of some filamentous fungi. This proliferation is considered undesirable to the cocoa fermentation process, such as *Aspergillus niger*, *A. carbonarius* and *A. ochraceus*, responsible for the production of the mycotoxin ochratoxin (RUGGIRELLO et al., 2019).

In our study, we did not identify the presence of species. *L. fermentum* and *L. plantarum*. However, the genus *Lactobacillus* was present in four different species, which may also suggest its positive influence on the modulation of good quality seeds.

4 CONCLUSIONS

In this study, we isolated and identified, for the first time, four species of lactic acid bacteria (LAB) at the beginning of the cocoa fermentation process in a city in the Brazilian Amazon. The presence of *P. acidilactici*, *L. farraginis* and *L. parafaraginis* may explain the positive balance of substances formed during the process (bioactive amines and acetic acid, for example) reported in some recent studies and serve as an option for future studies on the use of this group of microorganisms in the production of starter cultures.

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2. ALMEIDA, S. F. O.; SILVA, L. R. C.; **CHAGAS JUNIOR, G. C. A.**; OLIVEIRA, G.; SILVA, S. H. M.; VASCONCELOS, S.; LOPES, A. S. Diversity of yeasts during fermentation of cocoa from two sites in the Brazilian Amazon. **Acta Amazonica**, v. 49, n. 1, p. 64–70, 2018. **(ANEXO C)**

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- *Resumos expandidos publicados em anais e apresentações de trabalhos em eventos*

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12. SILVA, L. R. C.; **CHAGAS JUNIOR, G. C. A.**; LOPES, A. S. Yeasts isolated from banana leaves as environmental source for fermentation of cocoa seeds. In: I Simpósio Internacional de Cacau e Chocolate, Ilhéus, Brasil. 2019. **Apresentação oral.**

- *Cursos de Atualização*

1. **"Controle de Qualidade em Produtos Industrializados"**, de 24 a 27/04/18. Sociedade Brasileira de Microbiologia (SP). Carga horária: 32 h.

2. **"Produção de Chocolate: da amêndoa à barra"**, 13/08/18. XXVI Congresso Brasileiro de Ciência e Tecnologia de Alimentos (PA). Carga horária: 4 h.

ANEXOS

ANEXO A

Carta de submissão do Artigo IV

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ANEXO B

Capa do artigo

“Estabilidade físico-química e microbiológica de farinha de mandioca fermentada comercializada em Belém do Pará”

ESTABILIDADE FÍSICO–QUÍMICA E MICROBIOLÓGICA DE FARINHA DE MANDIOCA FERMENTADA COMERCIALIZADA EM BELÉM DO PARÁ.

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RESUMO

A farinha de mandioca fermentada é um alimento típico da região amazônica brasileira e sua produção ocorre com as seguintes etapas: colheita, descascamento, fermentação (tubérculos são imersos em um tanque com água ou córrego), lavagem, trituração, prensagem, moagem e torrefação. A farinha de mandioca é uma fonte importante de carboidratos na Amazônia, especialmente pelas pessoas de baixa renda. Neste trabalho estudou-se o comportamento físico-químico e microbiológico da farinha de mandioca fermentada comercializada em um mercado de rua e em supermercado. Para tal, 36 quilogramas de farinha de mandioca fermentada foram adquiridos e mantidos sob as mesmas condições ambientais do mercado de rua e do supermercado. Durante dois meses, as amostras foram analisadas quanto à umidade, cinzas acidez total titulável, pH e atividade de água. Os resultados mostraram que o pH e a acidez total titulável foram estáveis, mas a umidade e a atividade de água mudaram e aumentaram significativamente (p>0,05).

Palavras-chave: *Segurança alimentar. Manihot esculenta. Vida de prateleira. Alimento tradicional.*

ABSTRACT

Fermented cassava flour is a typical food of Amazonia region and its production occurs with following stages: fermentation (unpeeled cassava tubers are immersed in a water tank or stream), peeling, milling and roasting. The cassava flour is an important source of carbohydrates in Amazonia, especially for low-income people. In this work studied the physical-chemical and microbiological behavior of the fermented cassava flour commercialized in a street market, and at a supermarket. 36 kg of the fermented cassava flour were purchased and maintained under the same environmental conditions of the street market and that of the supermarket. During two months the samples were analyzed for moisture, ash, total titratable acidity, pH and water activity. The results showed that the pH and total titratable acidity were stable, but the moisture and the water activity have changed and increased significantly (p>0.05).

Keywords: *Food safety. Manihot esculenta. Shelf-life. Traditional food.*

ANEXO C

Capa do artigo

“Diversity of yeasts during fermentation of cocoa from two sites in the Brazilian Amazon”

ACTA
AMAZONICA

http://dx.doi.org/10.1590/1809-4392201703712

ORIGINAL ARTICLE

Diversity of yeasts during fermentation of cocoa from two sites in the Brazilian Amazon

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ABSTRACT

The purpose of this study was to identify the yeasts involved in spontaneous fermentation of cocoa from the Brazilian Amazon region. The fermentation process was carried out experimentally with cocoa seeds from two sites (Medicilândia and Tucumã), State of Pará, northern Brazil, during a six-day period. Totals of 44 yeasts were isolated from Medicilândia and 29 from Tucumã. Molecular identification was carried out by sequencing the D1/D2 region fragment of the rRNA 26S gene, expanded with universal primers for the NL1GC and LS2 eukaryotes. *Pichia manshurica* and *Saccharomyces cerevisiae* were identified in Medicilândia and five yeast species (*Pichia fermentans, P. kudriavzevii, P. manshurica, S. cerevisiae* and *Zygosaccharomyces bailii*) were identified in Tucumã. The results showed that *P. manshurica* and *S. cerevisiae* may have potential for use as starter cultures in future studies to improve the quality of cocoa seeds fermented in the Brazilian Amazon region.

KEYWORDS: accession; molecular identification; cocoa beans; *Pichia Manshurica; Saccharomyces cerevisiae*; *Theobroma cacao*

Diversidade de leveduras presentes na fermentação de cacau de duas localidades na Amazônia brasileira

RESUMO

A proposta deste estudo foi identificar as leveduras envolvidas na fermentação espontânea de cacau da Amazônia brasileira. A fermentação foi realizada em Medicilândia e Tucumã, Pará, Brasil, durante 6 dias. Em total foram obtidos 44 isolados de leveduras de Medicilândia e 29 de Tucumã. A identificação molecular foi realizada por sequenciamento do fragmento da região D1/D2 do gene rRNA 26S, amplificado com *primers* universais para eucariotos NL1GC e LS2. Em Medicilândia, foram identificadas *Pichia manshurica* e *Saccharomyces cerevisiae*. Em Tucumã foram identificadas cinco espécies (*Pichia fermentans, P. kudriavzevii, P. manshurica, S. cerevisiae e Zygosaccharomyces bailii*). Os resultados sugerem que *P. manshurica* e *S. cerevisiae* podem ter potencial para uso como culturas *starter* em estudos futuros, para melhorar a qualidade das sementes de cacau fermentadas na Amazônia brasileira.

PALAVRAS-CHAVE: acesso; identificação molecular; *Pichia manshurica; Saccharomyces cerevisiae*; *Theobroma cacao*

INTRODUCTION

Up to the mid-1990s, Brazilian cocoa was cultivated almost exclusively in the northeastern state of Bahia. However, since the onset of the witch's broom disease, which devastated cocoa plantations in Bahia in the 1980's and early 1990's, the state of Pará, in the Brazilian Amazon region, became an increasingly important source of cocoa (Castelo and Almeida 2015). In 2016, Pará produced 115,127 tons of fermented cocoa seeds (CEPLAC 2016), and to date it is the more important cocoa producer state in Brazil (IBGE 2017).

The fermentation of cocoa seeds is a natural and spontaneous process that is carried out by a succession of microorganisms (Afoakwa *et al.* 2013; Kadow *et al.* 2013; Krahmer *et al.* 2015). Cocoa fermentation is carried out by yeasts and by acetic and lactic acid bacteria. Yeasts play an essential role in fermentation, since these microorganisms are responsible for the production of ethanol from the fermentable sugars present in the pulp of the seeds, also facilitating the intake of oxygen through degradation of the pulp (Ho *et al.* 2014). Low quantities of oxygen allow for the growth of lactic and acetic acid bacteria, and for the release

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ANEXO D

Capa do artigo

“Determination of theobromine and caffeine in fermented and unfermented Amazonian cocoa (*Theobroma cacao* L.) beans using square wave voltammetry after chromatographic separation”

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Determination of theobromine and caffeine in fermented and unfermented Amazonian cocoa (*Theobroma cacao* L.) beans using square wave voltammetry after chromatographic separation

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ARTICLE INFO

Keywords:
Fermented Amazonian cocoa
Methylxanthines
Caffeine
Theobromine
Boron doped diamond

ABSTRACT

The concentrations of theobromine (TB) and caffeine (CF) together with other substances such as phenolic compounds, contribute to the perception of the bitter taste characteristic of fermented and unfermented Amazonian cocoa samples and their edible derivatives, such as chocolates. Thus, an analytical method based on square wave voltammetry (SWV) with a boron doped diamond electrode (BDD) was proposed to determine them. The supporting electrolyte, pH buffer solutions and parameters of the SWV technique were studied, and after determining the optimal conditions, analytical curves were constructed and the limits of detection and quantification calculated as 0.027 μmol L^{-1} and 0.093 μmol L^{-1} for CF and 0.025 and 0.085 μmol L^{-1} for TB. Intra-day and inter-day repeatability tests were carried out and the relative standard deviation values were below 2.51% and 6.50%, respectively. Interference studies were carried out and no potential interferents were found, except for high levels of TB in the presence of low CF concentrations, and *vice-versa*. To overcome this drawback, a preparative chromatographic procedure was used and the isolated aliquots of TB and CF quantified using SWV and high performance liquid chromatography (HPLC) with photodiode array detectors (PAD) as the reference method. Due to the low concentration of CF in the Amazonian cocoa sample, a miniaturized electrochemical cell produced in a 3D printer was developed. Based on the results, the relative errors were lower than 10% and the critical values for the F and t tests were lower than those tabulated at the 95% confidence level, with $n = 3$. The recoveries ranged from 80% to 115%. According to the results one can determine the TB and CF contents with acceptable precision and accuracy in fermented and unfermented Amazonian cocoa samples with a reduced amount of residues, representing good analytical tools for the quality control of fermented cocoa and chocolate.

1. Introduction

Cocoa or *Theobroma cacao L.* shows wide genetic diversity that extends along the Amazon and Orinoco rivers (Venezuela, Brazil and Colombia) (Rusconi & Conti, 2010). Of the three main varieties exploited for chocolate production, the *Forastero* variety presents a reduced bitter taste, lower astringency and lower acidity as compared to the *Criollo* and *Trinitario* varieties. In addition, the *Forastero* variety is that responsible for about 95% of the world cocoa production since the trees are more productive and resistant to diseases and pests (Kongor et al., 2016).

Cocoa is one of the main sources of methylxanthines in the human diet, especially TB (Aprotosoaie, Luca, & Miron, 2016). Methylxanthines are naturally occurring alkaloid purines found in some plant species. TB and CF are the major alkaloids in cocoa, TB being the major specie with between 1 and 4% as compared to CF, with less than 0.3% (Zheng, Koyama, Nagai, & Ashihara, 2004) and theophylline (TF) with 0.0075% in medium (Brunetto et al., 2007). In addition, cocoa is a source of proteins, lipids, carbohydrates and phenolic compounds (do Carmo Brito, Campos Chisté, da Silva Pena, Abreu Gloria, & Santos

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ANEXO E

Capa do artigo

“Coconut water - Based probiotic drink proposal: evaluation of microbiological stability and lactic acid estimation”

Praia AB, et al., J Food Sci Nutr 2020, 6: 062
DOI: 10.24966/FSN-1076/100062

HSOA Journal of Food Science and Nutrition

Research Article

Coconut Water-Based Probiotic Drink Proposal: Evaluation of Microbiological Stability and Lactic Acid Estimation

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Abstract

Probiotics are in high demand for their role as a health promoter, with lactose-fermented foods being the main source of getting them. However, the options for probiotics are lower for a group of consumers who are allergic or lactose intolerant. As an alternative to a non-dairy product, this work aims to propose the formulation of a low-cost drink with probiotic characteristics based on coconut water, free from lactose and fermented by *Lactobacillus casei shirota*. Two products were made: one with packaged coconut water and the other with fresh coconut water. The inoculum was obtained from a commercial fermented drink (Yakult®). In the first stage (fermentation), the total cultivation time was 48h; however, the most suitable time was 12h at 36ºC for both cultures, with monitoring of pH, total acidity and cell concentration. Cultivations were carried out in duplicates with a repetition of the process for each product. After the first stage, the second stage (microbiological stability) at refrigeration temperature (5°C to 8°C) was started. The total refrigeration time was 120h; however, the most suitable time was 72h for both drinks. The estimate of lactic acid production was investigated using infrared spectrometry with Attenuated Total Reflectance (FTIR-ATR). It was possible to observe specific bands of carboxylic acids. The results obtained were promising and show potential to produce probiotic non-dairy drinks with inoculum and low-cost substrate.

Keywords: Coconut water; Fermentation; Functional foods; Lactobacillus; Probiotics

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Introduction

Chronic-degenerative diseases have been the main cause of death in the world and controlling the risk factors for these diseases is a way to increase life expectancy. Aspects such as the practice of physical activities and a balanced diet are essential for the treatment and prevention of such diseases, contributing to the maintenance of health [1]. In this context, there is an increase in interest in functional foods, to get the beneficial, metabolic and physiological effects that these can cause.

Probiotics are gram-positive bacteria, defined internationally as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [2]. Probiotics work to maintain healthy intestinal function in the prevention and treatment of diarrhea and food allergies, relief of lactose intolerance and normalization of intestinal transit [3]; decreased serum cholesterol, reduced plasma LDL levels and glucose homeostasis and in the immune function, regulating the defense system [3-5].

Allergies and intolerances related to milk are the main causes of restricting the consumption of dairy products. Allergies and intolerances are manifested through the biochemical incapacity of the organism to digest, absorb or metabolize a specific component. Lactose intolerance is characterized by the inability to digest lactose due to the lack or decrease of the enzyme lactase [6].

Bearing in mind that the most common form of access to probiotics is through fermented milk and yogurts, people who are lactose intolerant cannot consume this food and enjoy its benefits. It is predicted that about 75% of the population, when they reach adulthood, develop this intolerance, because of the loss of the ability to digest lactose [7].

Probiotic foods must meet safety and functionality criteria, which include having no adverse effects and not being associated with infectious diseases, besides containing a microorganism that can survive, maintain metabolic activity and grow at the destination site [5].

Coconut water is the liquid part of the coconut fruit, is low in calories, has a sweet taste and its composition contains mineral salts, vitamins, amino acids and carbohydrates. Coconut water acts as an excellent natural isotonic and among the functional properties attributed is rehydration and electrolyte replacement [8-10].

Some studies show the development of probiotic drinks with coconut water [11-15]. None of these studies show the use of a low-cost

ANEXO F

Capa do artigo

“The microbiota diversity identified during the cocoa fermentation and the benefits of the starter cultures use: an overview”

International Journal of Food Science +Technology

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Review

The microbiota diversity identified during the cocoa fermentation and the benefits of the starter cultures use: an overview

Gilson Celso Albuquerque Chagas Junior, Nelson Rosa Ferreira & Alessandra Santos Lopes

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(Received 19 May 2020; Accepted in revised form 21 July 2020)

Summary Cocoa fermentation is a process that is constantly in evidence in the scientific world, as it is the pillar for the production of one of the most consumed foods in the world: the chocolate. Along with well-applied fermentation techniques, careful handling and variety of fruit, the microbiota (or cocobiota) is responsible for imparting characteristic and desirable aromas to chocolate. In addition to providing the start of essential physical and chemical reactions inside the seed to reduce the astringency and natural bitterness of the seeds, the production of starter crops is an alternative to obtain fermented beans with specific desirable characteristics. In this review, we discuss the latest studies on cocobiota identification and the benefits of using starter cultures and we conclude that advances have been made in recent years with regard to improving the fermentation process after using starter cultures, like reducing time, producing desirable volatile compounds and inhibiting putrefactive compounds.

Keywords Acetic acid bacteria, chocolate, lactic acid bacteria, putrefactive amines, volatile compounds, yeasts.

Introduction

One of the crucial factors to obtain a chocolate of acceptable quality in the market is the fermentative process that occurs in cocoa beans, which when conducted correctly will give the ideal conditions for the development of the sensory properties necessary for the production of the product. Fermentation is the stage where the complex biochemical reactions occur that cause the death of the cocoa seed embryo, hydrolysis of sugars and proteins, release of enzymes and substrates and diffusion of phenolic compounds that come into contact with enzymes (Schwan & Wheals, 2004).

Fermentation is an essential step towards obtaining good quality cocoa beans. For the International Cocoa Organization (ICCO), good quality almonds are considered to be commercialized, those which: (i) are fully fermented and dried; (ii) it is free of smoke notes and/or other abnormal and/or uncharacteristic of beans well-fermented; (iii) is free from any evidence of tampering; (iv) has a uniform size; (v) it is free of broken, fragmented grains and/or with pieces of bark; and (vi) is free of foreign matter (Ferreira, 2017).

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Within biotechnological processes, cocoa fermentation falls into a category of fermentation process called solid-state fermentation (SSF). In this way, we can consider that the wooden boxes (which are normally used in seed fermentations carried out in Brazil) are examples of static bioreactors, and as such, this implies a certain difficulty in aeration, homogeneous distribution of nutrients and difficulty in carrying out the scale-up.

The microbial diversity present in cocoa fermentation has often been associated with different fermentation locations, as well as the conditions of the fermentation process (such as, available nutrients, pH, temperature, oxygen, among others). In fact, several studies reveal a wide diversity of yeasts in cocoa fermentation in different countries (Figueroa-Hernández *et al.*, 2019).

Despite this, cocoa is one of the few fruits that has a complex and defined microbial succession during the fermentation process. Three most reported groups of microorganisms gain prominence during cocoa fermentation, among which they exert influence during the process: yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB), and more recently, the participation of filamentous fungi is increasingly being studied. It is known that some species of filamentous fungi are natural producers of hydrolases

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ANEXO G

Capa do artigo

“Chemical implications and time reduction of on-farm cocoa fermentation by *Saccharomyces cerevisiae* and *Pichia kudriavzevii*”

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Analytical Methods

Chemical implications and time reduction of on-farm cocoa fermentation by *Saccharomyces cerevisiae* and *Pichia kudriavzevii*

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ARTICLE INFO

Keywords:
Starter
Phenylethylamine
PCA
HCA
Putrefactive amines

ABSTRACT

The use of starters during fermentation has been gaining momentum as it can warrant high-quality chocolate. The objective of this study was to investigate the influence of *Saccharomyces cerevisiae* (Sc) and *Pichia kudriavzevii* (Pk) during on-farm fermentation on physico-chemical and microbiological characteristics and levels of methylxanthines and bioactive amines of cocoa. Four treatments were used: ScPk (1:1), only Sc, only Pk, and no starter (control). The starters lead to changes throughout fermentation, but provided fermented cocoa with similar pH, titratable acidity, reducing sugars and phenolic compounds. ScPk shortened fermentation time by 24 h. The ScPk fermented and dried cocoa had higher levels of monomeric phenols, methylxanthines, phenylethylamine and lower levels of the putrefactive amines – putrescine and cadaverine ($p < 0.05$). The results were confirmed by multivariate analysis. Based on these results, the mixture of both yeasts species is a promising starter for cocoa fermentation decreasing duration time and modulating high-quality components.

1. Introduction

Cocoa bean is the main raw material for chocolate production. It undergoes several important processing stages from fruit opening to industrial processing. However, fermentation is outstanding, due to its role in the formation of precursors for high-quality chocolate aroma and flavor (Ho, Zhao, & Fleet, 2015).

Cocoa fermentation is a natural and spontaneous process, involving different microorganisms, including yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Each group develops stepwise according to the environmental conditions in which they are present. Yeasts and LAB hydrolyze fermentable sugars (glucose, fructose and sucrose) transforming them into ethanol and lactic acid, leading to increased temperature (Figueroa-Hernández, Mota-Gutierrez, & Ferrocino, 2019). Yeasts, e.g. *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Kluyveromyces marxianus*, also produce pectinolytic enzymes. These enzymes break down pectin which is responsible for the viscosity and stickiness of cocoa pulp, causing the collapse of the pulp and allowing the formation of void space between the beans, favoring air percolation (Schwan & Wheals, 2004; Ouattara, Koffi, Karou, Sangaré, Niamke, & Diopoh, 2008; Ho, Zhao, & Fleet, 2014; Vuyst & Weckx, 2016). The aeration conditions are needed for the growth of AAB which convert ethanol into acetic acid, increasing the temperature and leading to the death of the seed embryo (Moreira, Vilela, Miguel, Santos, Lima, & Schwan, 2017). The high temperature and low pH are needed for protein breakdown and release of amino acids, which are precursors of

Abbreviations: AAB, Acetic acid bacteria; ANOVA, Analysis of variance; CF, Control fermentation; GYC, Glucose yeast medium with calcium carbonate; HCA, Hierarchical Cluster Analysis; LAB, Lactic acid bacteria; LOD, Limit of detection; LOQ, Limit of quantification; meq. NaOH 0.1N/100 g, milliequivalent sodium hydroxide solution 0.1N per 100 g sample; mg ECE/g, milligram equivalent epicatechin per gram sample; MRS, De Man, Rogosa & Sharpe; PCA, Principal Component Analysis; Pk, Fermentation with *Pichia kudriavzevii*; PPO, polyphenoloxidase; YPD, Yeast extract peptone dextrose; Sc, Fermentation with *Saccharomyces cerevisiae*; ScPk, Fermentation with both species of yeasts (Sc + Pk); TPC, Total phenolic compounds; TRS, Total reducing sugars; TTA, Total titratable acidity

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ANEXO H

Capa do artigo

"Profile of volatile compounds of on-farm fermented and dried cocoa beans inoculated with *Saccharomyces cerevisiae* KY794742 and *Pichia kudriavzevii* KY794725"

molecules

MDPI

Communication

Profile of Volatile Compounds of On-Farm Fermented and Dried Cocoa Beans Inoculated with *Saccharomyces cerevisiae* KY794742 and *Pichia kudriavzevii* KY794725

Gilson Celso Albuquerque Chagas Junior [1,*], Nelson Rosa Ferreira [1,*], Eloisa Helena de Aguiar Andrade [2], Lidiane Diniz do Nascimento [2], Francilia Campos de Siqueira [1] and Alessandra Santos Lopes [1,*]

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Abstract: This study aimed to identify the volatile compounds in the fermented and dried cocoa beans conducted with three distinct inoculants of yeast species due to their high fermentative capacity: *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, the mixture in equal proportions 1:1 of both species, and a control fermentation (with no inoculum application). Three starter cultures of yeasts, previously isolated and identified in cocoa fermentation in the municipality of Tomé-Açu, Pará state, Brazil. The seeds with pulp were removed manually and placed in wooden boxes for the fermentation process that lasted from 6 to 7 days. On the last day of fermentation, the almonds were packaged properly and placed to dry (36 °C), followed by preparation for the analysis of volatile compounds by GC-MS technique. In addition to the control fermentation, a high capacity for the formation of desirable compounds in chocolate by the inoculants with *P. kudriavzevii* was observed, which was confirmed through multivariate analyses, classifying these almonds with the highest content of aldehydes, esters, ketones and alcohols and low concentration of off-flavours. We conclude that the addition of mixed culture starter can be an excellent alternative for cocoa producers, suggesting obtaining cocoa beans with desirable characteristics for chocolate production, as well as creating a product identity for the producing region.

Keywords: chocolate; GC-MS; PCA; HCA

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1. Introduction

Being among the most appreciated products in different locations in the world, chocolate is the result of a complex processing process where different physical, chemical and microbiological reactions occur, from the collection of the cocoa fruit until the final product [1,2].

In 2019, Pará state was the largest Brazilian cocoa producer, with a total of 135 thousand tons of harvested fruit expected, representing a 25% growth in five years [3]. Recently, the city of Tomé-Açu was awarded the Geographical Indication certification, being the only municipality in Pará to have it, thus allowing greater representation in the trade of products related to the fruit [4].

The processing of cocoa beans has well-defined stages: harvesting, breaking and opening the fruit, fermentation, drying, roasting, grinding and refining, conching and tempering. Among the aforementioned stages, fermentation is one of the essential stages, since it is there that the formation of the precursors of chocolate aroma and flavour occurs.

ANEXO I

Capa do artigo

“Yeast isolation and identification during on-farm cocoa natural fermentation in a highly producer region in northern Brazil.”

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Yeast isolation and identification during on-farm cocoa natural fermentation in a highly producer region in northern Brazil

Isolamento e identificação de leveduras durante a fermentação natural de cacau em uma região altamente produtora no norte do Brasil

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The cocoa seeds from Brazilian Amazon are recognized for the high international market value in addition to the desirable aroma and taste. We aimed to identify yeast cultures in a natural cocoa fermentation process in one of the Amazonian regions of great importance in the cocoa beans market. Natural fermentation was carried out for seven days according to the methodologies of the producer and at 24 h intervals, seed samples were collected and physical-chemical and microbiological analyzes were performed. The contents of lipids, proteins, ash and moisture did not differ ($p \geq 0.05$) differently from temperature, pH and total titratable acidity ($p \leq 0.05$). We identified three species in the fermentation: *Pichia kudriavzevii*, *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*, the most common being during the process. We can verify the importance of knowing the microbiota active in cocoa fermentation to propose improvements during this process of great economic importance for the Amazon region.
Keywords: Amazon region, chocolate, *Saccharomyces*

As sementes de cacau da Amazônia brasileira são reconhecidas pelo alto valor no mercado internacional, além do aroma e sabor desejáveis. Nosso objetivo foi identificar culturas de leveduras em um processo de fermentação natural do cacau em uma das regiões amazônicas de grande importância no mercado de grãos de cacau. A fermentação natural foi realizada por sete dias de acordo com as metodologias do produtor e em intervalos de 24 horas foram coletadas amostras de sementes e realizadas análises físico-químicas e microbiológicas. Os teores de lipídios, proteínas, cinzas e umidade não tiveram diferença estatística ($p \geq$ 0,05) diferentemente da temperatura, pH e acidez total titulável ($p \leq 0.05$). Identificamos três espécies na fermentação: *Pichia kudriavzevii*, *Torulaspora delbrueckii* e *Saccharomyces cerevisiae*, sendo a mais comum durante o processo. Podemos verificar a importância de se conhecer a microbiota ativa na fermentação do cacau para propor melhorias durante este processo de grande importância econômica para a Região Amazônica.
Palavras-chave: Região Amazônica, chocolate, *Saccharomyces*

ANEXO J

Capa do capítulo de livro

“Carboxymethyl cellulose-coated polypropylene films containing essential oil for food preservation”

CHAPTER 8

Carboxymethyl cellulose-coated polypropylene films containing essential oil for food preservation

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8.1 Introduction

Cellulose is well known for being the most abundant renewable and biodegradable material found in nature. However, this homopolymer has limited application as it is insoluble in water, as well as in ordinary organic and inorganic solvents. In contrast, carboxymethylcellulose (CMC), which is the modified cellulose obtained through its carboxymethylation (Zhang et al., 2020). It has low toxicity and can be used for the production of biodegradable films, with a strong hydrophilicity and a stable internal network structure. Such properties are very interesting to be used in the production of composite films, which can be applied for numerous purposes (Lan et al., 2018; Peng et al., 2020).

Nowadays, the use of polymers obtained by nonrenewable sources such as oil in packaging has increased widely. Over the past 10 years, extensive researches have been done on biodegradable films and coatings obtained from polymers such as polysaccharides, lipids, and proteins or combinations thereof. Edible films and coatings are biodegradable and protect food products, prevent quality deterioration, and extend shelf life (Dashipour et al., 2014).

Biopolymer film has become an alternative to synthetic materials that can be applied in the food industry, minimizing the environmental impacts related to the use of synthetic plastics. CMC is deemed Generally Recognized as Safe (GRAS) by the US Food and Drug Administration and is predominantly used in the food industry due to its good film-forming ability (Honarvar et al., 2017).

Bio-Polymer-based Nano Films
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