Universidade Federal do Tocantins

Campus Universitário de Gurupi

Programa de Pós-Graduação em Biotecnologia

JONATAN PEREGRINO ALVAREZ

AROMA-PRODUCING YEASTS ASSOCIATED WITH COCOA BEANS FERMENTATION: STARTER CULTURE SELECTION FOR FLAVOR MODULATION OF CHOCOLATE

Gurupi - TO 2017

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Dissertação apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade Federal do Tocantins como parte dos requisitos para a obtenção do título de Mestre em Biotecnologia.

Orientador Porf. Dr. Carlos Ricardo Soccol

Gurupi - TO 2017

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da Universidade Federal do Tocantins

A473a Alvarez , Jonatan .

AROMA-PRODUCING YEASTS ASSOCIATED WITH COCOA BEANS FERMENTATION: STARTER CULTURE SELECTION FOR FLAVOR MODULATION OF CHOCOLATE. / Jonatan Alvarez . – Gurupi, TO, 2017.

59 f.

Monografia Graduação - Universidade Federal do Tocantins – Câmpus Universitário de Gurupi - Curso de Engenharia de Bioprocessos e Biotecnologia, 2017.

Orientador: Carlos Soccol

Coorientador: Gilberto Pereira

1. Levedura . 2. Fermentação . 3. Cultura iniciadora . 4. Cacau . I. Título

CDD 660.6

Elaborado pelo sistema de geração automatica de ficha catalográfica da UFT com os dados fornecidos pelo(a) autor(a).

UNIVERSIDADE FEDERAL DO **TOCANTINS**
PRÓ-REITORIA DE **PESQUISA E PÓS-GRADUAÇÃO**
PROGRAMA **DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA**
Rua Badejós, Chácaras 69 e 72 - CEP: 77402-970 - Caixa Postal 66 | Gurupi/TO
(63) 3311-3549 | www.uft.edu.br/biotecnologia | ppgbiotec@uft.edu.br

Ata de Defesa nº 23/2017

ATA DA DEFESA PÚBLICA DA DISSERTAÇÃO DE MESTRADO DE JONATAN PEREGRINO ALVAREZ DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA DA UNIVERSIDADE FEDERAL DO TOCANTINS.

Aos 22 dias do mês de março do ano de 2017, às 09 horas da manhã, na sala 03, edifício Planta Piloto CEMBAPAR, da Universidade Federal do Paraná, reuniu-se a Comissão Examinadora da Defesa Pública, composta pelos seguintes membros: Prof. Orientador Dr. CARLOS RICARDO SOCCOL da Universidade Federal do Paraná, Profª. Drª. MARIA GIOVANA BINDER PAGNONCELLI da Universidade Federal do Paraná, Profª. Drª. VALCINEIDE OLIVEIRA DE ANDRADE TANOBE da Universidade Federal do Paraná Prof. Dr. GILBERTO VINICIUS DE MELO PEREIRA da Universidade Federal do Paraná e Prof Dr. GESSIEL NEWTON SCHEIDT da Universidade Federal do Tocantins, sob a presidência do primeiro, a fim de proceder a arguição pública da DISSERTAÇÃO DE MESTRADO de JONATAN PEREGRINO ALVAREZ, intitulada **"Aroma-producing yeasts associated with cocoa beans fermentation: starter culture selection for flavor modulation of chocolate"**. Após a exposição, o discente foi arguido oralmente pelos membros da Comissão Examinadora, tendo parecer favorável à aprovação, com as devidas correções apontadas pela banca, habilitando-o ao título de Mestre em Biotecnologia. Nada mais havendo a tratar, foi lavrada a presente ata, que, após lida e aprovada, foi assinada pelos membros da Comissão Examinadora.

Prof. Dr. Carlos Ricardo Soccol
Universidade Federal do Paraná
Orientador

Profª Drª Maria Giovana Binder Pagnoncelli
Universidade Federal do Paraná
1ª Examinadora

Prof. Dr. Gilberto Vinicius de Melo Pereira
Universidade Federal do Paraná
Coorientador e 2º Examinador

Prof. Dr. Gessiel Newton Scheidt
Universidade Federal do Tocantins
3º Examinador

Profª Drª Valcineide Oliveira De Andrade Tanobe
Universidade Federal do Paraná
4ª Examinadora

Gurupi, 22 de março de 2017.

Prof. Dr. Gessiel Newton Scheidt
Coordenador do Programa de Pós-graduação em Biotecnologia

AGRADECIMIENTOS

A Dios por darme la capacidad de llegar hasta donde estoy hoy.

A mis padres por darme la vida y la educación, por ayudarme a ser la persona de la que están orgullosos.

A mi asesor Profesor Carlos Ricardo Soccol por la oportunidad de participar en este gran proyecto.

Un agradecimiento muy grande para todos los profesores y funcionarios del programa de Bioprocesos y Biotecnología por darme la bienvenida, por las clases, por los consejos y las enseñanzas.

Al Profesor Dr. Gilberto Vinícius de Melo Pereira y a Dra. Valcineide Tanobe por la ayuda y la orientación proporcionada sin la cual no habría sido posible este logro.

A todos mis compañeros de laboratorio Extranjeros y Brasileños por su apoyo y amistad.

A CONACYT por el apoyo económico de mi beca sin el cual este logro no sería posible.

A Brasil por recibirme con los brazos abiertos, por la relación de amistad con México que siga creciendo día a día, por toda la maravillosa gente que tuve la oportunidad de conocer. C

Al proyecto Biocau por la oportunidad de participar en este proyecto y al CAPS por el apoyo económico otorgado a este proyecto.

ABSTRACT

Chocolate is one of the most important products for the food industry, being of economic interest all over the world. The cocoa quality depends directly on the post-harvest processing, being the cocoa-pulp fermentation a crucial step for chocolate quality development. The aim of this work was to study the diversity of aroma-producing yeasts associated with cocoa beans fermentation and to select suitable yeast starter culture to cocoa flavor modulation. A total of 39 cocoa-derived yeast isolates were screened for their capacity to produce volatile aroma compounds in a cocoa pulp simulation medium. The seven highest aroma-producing yeasts were identified by ITS-rRNA gene sequencing as belonging to *Pichia kudriavzevii*, in spite of exhibiting different metabolic profiles. A computer-assisted analysis of rep-PCR genomic fingerprints of *Pichia kudriavzevii* strains clearly differentiated the upper aroma-forming yeast strains (G1 group; P. kudriavzevii LPB06 and P. kudriavzevii LPB07) from the other strains (G2 group). This demonstrates the potential of rep-PCR technique as a promising genotypic tool for rapid and reliable speciation of aromatic yeast strains. In the second stage of this study, two strains with superior aroma production, namely *P. kudriavzevii* LPB06 and *P. kudriavzevii* LPB07, were used in cocoa beans fermentation at laboratory scale. They were able to establish an accelerated fermentation process with efficient yeast growth, sugars consumption and ethanol formation compared to the spontaneous process. The resulting cocoa beans were analyzed by diverse chemical analysis methods, including SPME-GC/MS, FTIR spectroscopy and metal and colorimetric analysis. All together, the results indicated that inoculated fermentations generated cocoa beans with better color development and richer aroma composition, suggesting that cocoa-associated yeast diversity at strain level can be exploited for flavor modulation of cocoa beans.

Key words: Yeasts, Cocoa Beans Fermentation, Aroma compounds, starter culture.

RESUMO

Atualmente, o chocolate é um dos produtos mais importantes para a indústria de alimentos, sendo de interesse econômico em todo o mundo. A qualidade do cacau depende diretamente do processamento pós-colheita, sendo a fermentação da polpa um passo crucial para o desenvolvimento da qualidade do chocolate. O objetivo deste trabalho foi estudar a diversidade de leveduras aromáticas associadas à fermentação de cacau e selecionar uma cultura iniciadora com potencial para modular o *flavor* de chocolate. Um total de 39 leveduras foram isoladas e caracterizadas quanto à formação de compostos aromáticos. As sete melhores produtoras foram identificadas através do sequenciamento do gene ITS-rRNA como *Pichia kudriavzevii*, apesar de apresentarem diferentes perfis metabólicos. Análise de impressões digitais (*fingerprints*) dos isolados pela técnica de rep-PCR claramente distinguiu as cepas com maior produção de compostos aromáticos, demonstrando o potencial desta técnica como uma ferramenta para rápida e confiável seleção de leveduras. Na segunda etapa deste estudo, duas cepas com superior formação de aroma (*P. kudriavzevii* LPB06 e *P. kudriavzevii* LPB07) foram testadas como culturas iniciadoras para fermentações de cacau em escala laboratorial. Estas duas cepas foram capazes de estabelecer um acelerado processo fermentativo, com eficiente consumo de açúcares e formação de etanol, em comparação ao método natural. As amêndoas de cacau resultantes destes processos foram analisadas por diferentes métodos químicos, incluindo SPME-GC/MS, espectroscopia FTIR e análises de metal e calorimetria. Os resultados indicaram que as fermentações inoculadas desenvolveram amêndoas de cacau com melhor cor e composição de aroma, sugerindo que a diversidade de levedura em fermentações de cacau pode ser explorada para a modulação do *flavor* de chocolate.

Palavras-chave: Leveduras, Fermentação de Cacau, Compostos aromáticos, cultura iniciadora.

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1. INTRODUÇÃO

A matéria-prima para a produção do chocolate é a semente derivada do fruto do cacau (*Theobroma cacao* L.,), que após passar pelos processos de fermentação, secagem, quebra e separação da casca recebe o nome de *nibs*. O processo fermentativo ocorre por ação de uma sucessão de diferentes grupos microbianos na polpa envoltória das sementes, que é rica em carboidratos (principalmente sacarose, frutose e glicose) e ácido orgânicos (principalmente ácido cítrico). Os álcoois (principalmente etanol) e os ácidos orgânicos (principalmente ácido lático e ácido acético) produzidos pelo metabolismo microbiano, associados à alta temperatura da massa de cacau em fermentação, são fatores que contribuem para a morte do gérmen e desencadeiam uma série de alterações químicas, essenciais para o desenvolvimento do "flavor" do chocolate (ARDHANA; FLEET, 2003; BIEHL et al., 1993; LEHRIAN; PATTERSON).

O processo fermentativo das sementes de cacau é conduzido por microrganismos nativos do meio ambiente, que convertem os açucares da polpa em diferentes metabólitos, dando passo a uma secessão microbiana complexa e a diversas reações bioquímicas no interior das sementes. Leveduras, bactérias do ácido lático, bactérias do ácido acético, e em alguns casos, bactérias formadoras de esporos, são os principais grupos microbianos presentes durante o processo de fermentação do cacau (Pereira et al., 2013).

O tempo requerido para a fermentação é variável, geralmente 3 a 7 dias (SCHWAN; WHEALS, 2004). Inicialmente, o alto conteúdo de açúcares e baixo pH, associados com a baixa tensão de oxigênio, favorecem o desenvolvimento de leveduras que dominam o processo fermentativo até geralmente 48 horas a partir de seu início. As leveduras

hidrolisam e fermentam os açúcares produzindo etanol e CO_2. As condições ocasionadas pelo crescimento das leveduras estimulam o desenvolvimento de bactérias do ácido lático. Estas bactérias também fermentam os açúcares e utilizam o ácido cítrico presente na polpa, apresentando crescimento máximo em torno de 16 às 48 horas. Com a desintegração da polpa que envolve as amêndoas e o revolvimento da massa em fermentação, a aeração se torna maior, favorecendo o crescimento das bactérias do ácido acético. Tais bactérias promovem a oxidação do etanol - produzidos inicialmente pelas leveduras a ácido acético, em uma reação extremamente exotérmica, elevando a temperatura da massa fermentativa para patamares de 45 a 50ºC (ARDHANA; FLEET, 2003; CAMU et al., 2007; SCHWAN; WHEALS, 2004; THOMPSON; MILLER; LOPEZ, 2007; VUYST et al., 2010).

O uso de culturas iniciadoras na elaboração de produtos fermentados é uma prática comum na indústria de alimentos estando diretamente relacionada às características de flavor, textura, cor e vida-de-prateleira do produto final. Processadoras de cacau requerem um fornecimento constante de amêndoas de cacau, que deve obedecer a critérios de qualidade. Entretanto, o processo fermentativo de cacau é ainda realizado de forma rudimentar, principalmente sob folhas de bananeira ou em caixas de madeira. A inconstância ou a falta de padronização destes processos geram amêndoas de qualidades variadas (LAGUNES-GÁLVEZ et al., 2007). O objetivo deste estudo foi investigar a diversidade metabólica e genética de leveduras aromáticas presentes durante a etapa de fermentação de cacau. Em uma segunda etapa, o potencial de leveduras aromáticas, selecionadas como culturas iniciadoras, foi investigado, abrangendo aspectos relativos ao processo fermentativo (eficiência de crescimento e produção de metabólitos) e à qualidade

das sementes produzidas (prova de corte, desenvolvimento de cor e análises físico-químicas).

2.LITERATURE REVIEW

2.1 Characteristics of the cocoa pod and cocoa beans

From the twenty-two species that constitute the genus *Theobroma* (family Sterculiaceae*), Theobroma cacao* L., is commercially the most important, due to the value of its seeds (BARTLEy, 2005; WOOD, 1975). Its natural habitat is the lower storey of the evergreen rain forest in the Amazon basin and other tropical areas of South and Central America (Fowler, 1999; Lass, 1999). The seeds, commonly known as cocoa beans, are the principal raw material for chocolate production (LIMA et al., 2011). Globally, the major cocoa producing countries in descending order are Cote d'Ivory, Ghana, Indonesia, Brazil, Nigeria, Cameroon and Ecuador, where it provides both economic and socio-economic benefit for farmers and the countries at large (E. TEYE et al. 2013).

The cocoa tree is a perennial tree, 8 to 15 m in height, which under a more intensive cultivation is limited to 2.5 to 3 m bypruning, for better phytosanitary control (Fowler, 1999; Wood, 1975). Commercial cocoa is derived from the seeds (beans) of the ripe fruit (pods) of the plant *Theobroma cacao* (Fig. 1). Each cocoa bean consists of two cotyledons and a small embryo, all enclosed in a skin ("shell"). The cotyledons comprise of two types of cells—storage or parenchyma cells, containing fat globules, protein bodies and starch granules, and bigger pigmented cells, containing polyphenols and methylxanthines (BIEHL ET AL., 1977; DEL BOCA, 1962; FERRÃO, 2002).

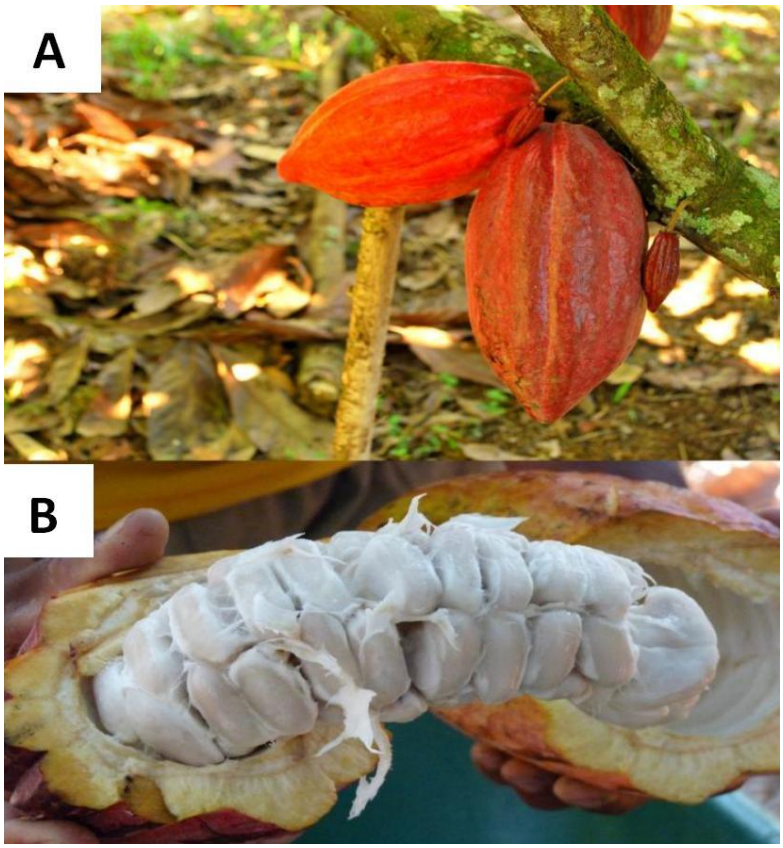

Figure 1 – (A) Cocoa fruit pod growing directly out of the bark on a trunk of a cocoa tree; (B) Opened cocoa pod with beans embedded in a mucilaginous pulp. Source: Pereira, GVM Master's thesis). Federal University of Lavras. Reprinted with permission.

The cotyledons are referred to as the *nibs* in the cured beans. In the cotyledons, the fat is the most important component, representing about half the weight of the dry seed (LIMA et al., 2011). The total amount of polyphenols in dried fresh cocoa beans may vary between 12 and 20% (w/w) and these are responsible for its high astringency, contributing to their bitterness as well (FORSYTH; QUESNEL, 1957; KIM; KEENEY, 1984).

2.2 Cocoa beans fermentation

For the technological transformation of cocoa beans in chocolate, four steps are essences for the development of the typical chocolate flavor: fermentation, drying, roasting and conching (AFOAKWA et al., 2008). The fermentation and drying are taken place in local farms where cocoa is grown and harvested by the people who live there; these steps are performed empirically, which results in a process that becomes diverse and not standardized.

Unfermented cocoa beans lack full chocolate flavor and are highly bitter and astringent. Fermentation is the first step in the chocolate making chain and consists of a 5- to 7-day process during which microorganisms grow within the pulp material that surrounds the seeds (beans) of the cocoa fruit (SCHWAN, PEREIRA, FLEET. 2016).

During fermentation, a diversity of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) grow in a somewhat successional profile. Various species of Bacillus, other bacteria, and filamentous fungi may also grow throughout fermentation and can affect bean quality and cocoa flavor. Although the physiological and biochemical roles of the predominant microorganisms of fermentation are now reasonably well understood, and the importance of a well-ordered microbial succession in the development of the cocoa aroma has been established, the process remains as an intriguing complex of microbiological, biochemical, and chemical interactions that, to date, have defied industrialized control and management (SCHWAN, PEREIRA, FLEET. 2016).

Cocoa pods are harvested from the trees, the pods are broken open, and the beans are manually extracted. The beans (50–500 kg) are assembled into heaps on the ground, or placed into boxes, trays, or baskets; and the fermentation is allowed to develop spontaneously. The microorganisms responsible for the fermentation are indigenous species that originate as natural contaminants of the process. After fermentation, the beans are placed on the ground or drying platforms and dried in the sun until a moisture content of less than 11% is achieved (Amoa-Awua, W. 2014).

The cocoa bean mucilage, also called pulp, representing 10% of seed weight and is composed of water (84.5%), pentosans (pectin 2.7%), sucrose (0.7%), glucose and fructose (10%), proteins (0.6%) acid (0.7%) and salts (0.8%), being a rich source of carbon and nitrogen for the microorganisms (SCHWAN; wheals, 2004). The pulp that wraps the beans becomes the medium in which the microorganisms will unfold and replayed anaerobically in the first few hours due to the lack of oxygen caused by the thick layer of pulp present in the seeds.

It was also observed that the temperature of the cocoa bean mass increased significantly during fermentation, from initial values of 25–30°C to about 50°C by the end of the process. Such increases were important in the development of chocolate character. It was concluded that metabolites produced by microbial growth in the pulp could diffuse across the testa into the seed and, conversely, substances normally found in the seed, including polyphenols, peptides, alkaloids, sugars, and some proteins, could diffuse back into the pulp during fermentation (Knapp 1937; Roelofsen 1958; Quesnel 1966). Such movements were determined by the permeability and integrity of the testa, which changes as the fermentation progresses (Lehrian and Patterson 1983; Wood and Lass 1985).

The fermentation of cocoa beans occurs at two levels. The first involves reactions that take place in the pulp, in the outer part of the beans, and the second involves several hydrolytic reactions that occur within the cotyledons (SCHWAN, WHEALS. 2004). Changes in the pH, temperature, sugar content, and fermentation products exert selection pressure on the already existing natural biotypes, favoring those strains that are better adapted to this environment (VANDENBERGH, 1993). In sum, fermentation is an essential step to obtain good quality almonds due to complex biochemical reactions that lead to the death of the embryo, hydrolysis of sugars and proteins, release of enzymes and substrates, diffusion of phenolic compounds that come in contact with the enzymes, among others (FORSYTH, QUESNEL, 1957, BECKETT, 1994, BRITO, 2000, EFRAIM, 2004).

The actual methods of fermentation vary in different cocoa-producing countries and regions, and even from one cocoa grower to another within a region or country (SCHWAN; WHEALS, 2004). Fermentations may be performed in heaps (e.g., Ghana and Ivory Coast), boxes (e.g., Brazil and Malaysia), baskets (e.g., Nigeria and Ghana), trays (e.g., Ghana), sacks (e.g., Ecuador) and platforms fermentation (e.g., Ecuador).

The box fermentation system is widely used in Brazil, Indonesia and Malaysia (WOOD; LASS, 1985). A number of boxes are arranged, each having a capacity of generally 1,000 kg cocoa beans, which are turned to another box after 1-2 days, and finally after 2-3 days of further fermentation into the next box. The box fermentation systems facilitates turning, as the movement of the beans is aided by gravity and cocoa of good quality can be produced (SCHWAN et al., 1990; THOMPSON; MILLER; LOPEZ, 2007; WOOD; LASS, 1985).

However, a problem occasionally encountered during box fermentation is uneven temperature and oxygen distribution through the fermenting mass with the corners and areas around aeration holes being better aerated and occasionally colder and less acidic than the rest of the fermenting mass enabling moulds to grow abundantly (THOMPSON; MILLER; LOPEZ, 2007).

Ghanaian farmers traditionally ferment the cocoa beans using the heap method in the field among the trees close to where the pods were harvested (TOMLINS; BAKER; DAPLYN, 1993). Heap fermentation is carried out on banana leaves on the ground, and the heap of cocoa beans is covered with banana leaves and sticks. The duration of the heap process fermentation is around from 4 to 7 days (BAKER; TOMLINS; GRAY, 1994).

Once the fermentation is finished, the drying stage must begin, and if it is slow or poorly conducted, the growth of fungi may be promoted, which will confer an unpleasant taste on the final product or produce harmful toxins for health Human (CRESPO 1985). On the other hand, it should not be done too quickly, using high temperatures, to avoid the migration of cocoa butter to the forehead (film surrounding the almond) and affect the development of the the characteristic chocolate flavor (Efraim et al. Al., 2008).

2.3 Microbiology of cocoa beans fermentation

Early in the fermentation, several species of yeasts proliferate, leading to production of ethanol and secretion of pectinolytic enzymes. This is followed by a phase in which bacteria appear, principally lactic-acid bacteria and acetic-acid bacteria. The starter organisms such as yeast, lactic acid bacteria (LAB) and Acetic acid bacteria (AAB) were

envisaged based on the earlier studies and also their functional role in the cocoa fermentation process (CAMU et al., 2007).

The initial acidity of the pulp (pH 3.6), due to citric acid, together with low oxygen levels, favor colonization by yeasts (SCHWAN, LOPEZ, 1990). The yeasts are able to utilize pulp carbohydrates under both aerobic and anaerobic conditions. The size of the yeast population increases from 10^7 CFU/g of pulp to 10^8 CFU/g of pulp during the first 12 h, then remains almost constant for the next 12 h after, which there is a dramatic decline of four orders of magnitude over the next day followed by a slower decrease leading to a final population of only 10 viable cells per gram of pulp (SCHWAN, ROSE, BOARD, 1995).

Schwan (1998) reports some important roles of yeast: (i) breakdown of citric acid in the pulp, leading to an increase in pH from 3.5 to 4.2, which allows growth of bacteria; (ii) production of ethanol under low-oxygen and high-sugar conditions, which is eventually consumed oxidative; (iii) production of organic acids (oxalic, phosphoric, succinic, malic, and acetic acids), which permeabilize and kill the bean cotyledons; (iv) production of some volatile organic compounds which may contribute either to chocolate flavor or, more likely, to precursors of chocolate flavor; and (v) secretion of pectinases which reduces the viscosity of the pulp, allowing aeration of the pulp mass.

The qualitative and quantitative profile of these metabolites will vary with the species and strain of yeasts that predominate (PEREIRA et al. 2012a, b). The ethanol concentration will determine the ecology of the microbiota that, subsequently, grows in the fermenting bean mass, and it becomes a substrate for the growth of AAB. However, it also passes into the inner tissues of the bean, thereby contributing to its intracellular disorganization and death, along with acetic acid later produced by the AAB (QUESNEL

1965; LEHRIAN and PATTERSON 1983). The carbon dioxide that is produced will contribute to the microaerobic conditions of the bean mass, thereby encouraging the growth of LAB.

Several yeast species isolated from cocoa bean fermentations are pectinolytic and are considered to have an important role in degradation and solubilization of the pulp, especially during the first 24 h (CARR 1982; CASCANTE et al. 1994; SCHWAN et al. 1997). Hydrolysis of pectin requires several enzymes, including polygalacturonase, pectin methylesterase, and pectin lyase, as described previously (ROELOFSEN 1953; SCHWAN et al. 1996; SILVA et al. 2005). This activity decreases the viscous nature of the bean mass and facilitates its mixing and penetration of air (oxygen) that encourages the growth of AAB. *S. cerevisiae*, *Candida saitoana*, *K. marxianus*, and *Pichia norvegensis* are pectinolytic yeasts that were isolated from cocoa fermentations in the Ivory Coast (SANCHEZ et al. 1984). In Brazil, pectinolytic yeasts included *K. marxianus, S. cerevisiae*, *Candida rugopelliculosa*, and *Kluyveromyces thermotolerans* (SCHWAN and ROSE 1994; SCHWAN et al. 1997).

Metabolism of organic acids is another mechanism by which yeasts are considered to impact on cocoa bean composition and quality. Utilization of citric acid in the pulp and lactic acid produced by the LAB would decrease the acidity of the beans. Of the main species associated with cocoa fermentations, *P. kudriazevii* (*C. krusei*) has the ability to assimilate citric acid and lactic acid (Jespersen et al. 2005; Lagunes-Galvez et al. 2007; Daniel et al. 2009). Yeasts also produce organic acids such as succinic and acetic acids, the levels of which depend on the species and strain (Radler 1993; Pereira et al. 2012a, b).

After yeast growth, the amended conditions favor the development of LAB. The number of these organisms reaches a peak around 36 hours after the fermentation process begins and the bacterial population reached 6.4×10^7 CFU/g of pulp (Schwan, Wheals, 2010). This period of time is coincident with the decline of the yeast population (Schwan, 1998). The LAB exhibit the fastest growth rate during the 16–48 h period of fermentation and are present in greater numbers, but not necessarily in biomass, than yeasts for a short period of time (Schwan, 1995).

The great majority of LAB use glucose via the Embden-Meyerhof-Parnas pathway, yielding more than 85% lactic acid. However, some species use glucose via the hexose monophosphate shunt, forming 50% lactic acid, as well as combinations of ethanol, acetic acid, glycerol, mannitol, and carbon dioxide (Schwan, 1998).

The LAB phase in the microbial sequence of the cocoa fermentations was earlier considered insignificant because it is brief and releases by-products which served as substrates for others bacteria (ROELOFSEN, 1958). Studying cocoa fermentations in Trinidad, Forsyth and Rombouts (1952) incubated plates anaerobically, resulting in improved recovery of LAB isolates. Between 24-36 h of fermentation, the authors observed isolates reached populations of $10^7 – 10^8$ CFU/g of cocoa pulp, which represented only 20% of the total microflora at that time. This, and their apparent transience, meant that isolates were considered unimportant and remained until some years later (ROMBOUTS, 1952).

The functional roles of LAB during cocoa fermentation have been discussed by several researchers (Passos et al. 1984 a, b; Schwan and Wheals 2004; De Vuyst et al. 2010; Lima et al. 2011a; Thompson et al. 2013). The sugary, acidic, microaerobic environment of the pulp is conducive to the growth of LAB. Moreover, some species

within this group are tolerant of the higher ethanol and temperature conditions that develop during fermentation (e.g., *L. plantarum* and *L. fermentum*) (Pereira et al. 2012a, b, 2013). Metabolically, LAB conduct three major reactions during bean fermentation: (i) they ferment pulp sugars, (ii) they utilize citric acid, and (iii) some may reduce fructose to mannitol. These reactions contribute to changes in the composition of the pulp that impact on cocoa bean and chocolate quality and, possibly, the microbial ecology of the fermentation. However, not all the lactic acid produced by LAB will end up in the bean as some of it may be utilized by yeasts and AAB (Schwan and Wheals 2004; Lefeber et al. 2010, 2011; Pereira et al. 2012a,b). The metabolism of fructose into mannitol varies with the strain, with a stronger tendency being found in some strains of *L. fermentum* (Lefeber et al. 2010, 2011).

The characteristic vinegar-like aroma of cocoa bean fermentations that follows the "alcoholic" phase of fermentation led early investigators (Bainbridge and Davies 1912; Loew 1913; Nicholls 1913; Knapp 1937). Roelofsen and Giesberger (1947) reported the first significant study of the species associated with these fermentations. They followed the growth of these bacteria during the fermentation of beans in Java, Indonesia, and observed a dominance of *Acetobacter rancens* (now *Acetobacter pasteurianus*) and *Acetobacter melanogenum* (now *Gluconobacter oxydans*). The spaces formed between the beans, due to collapse of the parenchyma cells in the pulp between beans, allow air to enter; aeration of the pulp mass is important for the growth of AAB. To speed up the fermentation process and enhance the quality of the final product, pectinases may be added to the pulp or strains over-producing pectinolytic enzymes may be used (SCHWAN; WHEALS, 2004).

Metabolically, these bacteria conduct two main reactions: oxidation of pulp sugars and acids, and oxidation of ethanol. Although AAB may be present in pulp at the commencement of fermentation (Ardhana and Fleet 2003), their potential to grow and utilize the pulp sugars would be limited by the absence of oxygen at this stage

Their main growth and activity generally occur after yeast growth and bean mixing, when oxygen has had a greater opportunity to permeate the bean mass. At this time, they rapidly utilize the ethanol produced by the yeasts, oxidizing it to acetic acid and producing concentrations as great as 20 mg/g pulp (Lima et al. 2011a).

This oxidation reaction is quite exothermic (De Vuyst et al. 2010), and is considered to be a main factor in causing the temperature of the bean mass to increase to 50°C or more. This acid has several implications for cocoa bean and chocolate quality. First, it diffuses into the bean where, in combination with ethanol and the elevated temperature, it brings about cellular disorganization and bean death.

2.4 Use of yeast starter cultures in cocoa beans fermentation

When fermentation is carried out without selected microorganisms, it can result in a process with different desired efficiency and, therefore, a product of varying quality. However, cocoa fermentation remains an empirical process that does not give rise to beans of consistent quality, which obliges processors to alter their formulations continually (Laguna 2007).

Former research on starter culture development for controlled cocoa bean fermentations mainly focussed on the introduction of pectinolytic yeasts for enhanced cocoa pulp juice production (BUAMAH; DZOGBEFIA; OLDHAM, 1997; DZOGBEFIA; BUAMAH;

OLDHAM, 1999; LEAL JÚNIOR et al., 2008; SAMAH; PTIH; SELAMAT, 1992). Buamah, Dzogbefia and Oldham (1997) and Dzogbefia, Buamah and Oldham (1999) used pectinolytic yeast *Kluyveromyces fragilis*, *C. norvegensis* (now classified as *P. norvegensis*), *T. candida* (now classified as *C. saitoana*), and *S. chevalieri* in controlled fermentation of cocoa beans in sterile funnels under laboratory conditions. *S. chevalieri* and *K. fragilis* both significantly increased the yields of cocoa sweatings, which could be commercialized as jam, marmalade, or syrup (BUAMAH; DZOGBEFIA; OLDHAM, 1997). Furthermore, the inoculation did not alter the physic-chemical properties of the degraded pulp. The exclusion of AAB from the fermentation also resulted in low levels of acetic acid (DZOGBEFIA; BUAMAH; OLDHAM, 1999).

More recently, a controlled inoculation of cacao bean fermentation using a *Kluyveromyces marxianus* hybrid yeast strain, with an increased pectinolytic activity, would improve an earlier liquid drainage from the fermentation mass, developing a superior final product quality (LEAL JÚNIOR et al., 2008). Introduction of the hybrid yeast affected the profile of total seed protein degradation evaluated by polyacrylamide gel electrophoresis, with improved seed protein degradation, and reduction of titrable acidity. The increase in mass aeration during the first 24 h seemed to be fundamental for the improvement of fermentation quality, demonstrating the potential application of this improved hybrid yeast strain with superior exogenous pectinolytic activity (LEAL JÚNIOR et al., 2008).

Although these preliminary experiments of the application of defined starter cultures show satisfying results, they have not been introduced in the field. The major difficultly associated with inoculated cocoa fermentations is the removal of natural

microbiota. In addition, studies reporting the effect of selected microorganisms on the final quality of the product are still scarce.

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3.RESEARCH RESULTS

Great intraspecies diversity of *Pichia kudriavzevii* in cocoa fermentation highlights the importance of yeast strain selection for flavor modulation of cocoa beans

(Artigo submetido ao periódico indexado: LWT-Food Science and Technology)

ABSTRACT

The use of selected yeasts has recently been proposed as an interesting strategy to improve cocoa bean fermentation and chocolate quality. In this study, a total of 39 cocoa-derived yeast isolates were screened for their capacity to produce volatile aroma compounds in a cocoa pulp simulation medium. The seven highest aroma-producing yeasts were identified by ITS-rRNA gene sequencing as belonging to *Pichia kudriavzevii*, in spite of exhibiting different metabolic profiles. $(GTG)^5$-rep-PCR genomic fingerprinting approach confirmed this high intraspecies diversity of *P. kudriavzevii* in cocoa fermentation. Two strains with superior aroma production, namely *P. kudriavzevii* LPB06 and *P. kudriavzevii* LPB07, were used in cocoa beans fermentation at laboratory scale. They were able to establish an accelerated fermentation process with efficient yeast growth, sugars consumption and ethanol formation compared to the spontaneous process. The resulting cocoa beans were analyzed by diverse chemical analysis methods, including SPME-GC/MS, FTIR spectroscopy and metal and colorimetric analysis. All together, the results indicated that inoculated fermentations generated cocoa beans with better color development and richer aroma composition, suggesting that cocoa-associated yeast diversity at strain level can be exploited for flavor modulation of cocoa beans.

Key-words: cocoa beans fermentation, chocolate, rep-PCR, aroma-producing yeast, *Pichia kudriavzevii*

3.2. Introduction

Microbial fermentation plays an important role in the chocolate production chain with major impacts on product quality and value (Pereira, Soccol & Soccol, 2016a). The fermentation process consists of an on-farm, 5- to 7-day process during which microorganisms grow in the pulp material that surrounds the seeds (beans) of the cocoa fruit (*Theobroma cacao*). This involves the action of complex microbial interactions, led mainly by yeasts, lactic acid and acetic acid bacteria. The microbial activity and pH increasing generates metabolites and conditions that kill the beans, triggering an array of biochemical reactions essentials for cocoa quality development (Schwan & Wheals, 2004a).

Yeasts are among the most frequently isolated microorganisms from fermenting cocoa beans. The chemical composition of the cocoa pulp, especially its acidity and high sugar content, is likely to encourage the initial growth of different yeast genera, such as *Saccharomyces, Pichia, Hanseniaspora, Candida* and *Debaryomyces* (Schwan, Pereira & Fleet, 2014b). The main metabolic function of yeasts is to perform an alcoholic fermentation of pulp sugars to produce mainly ethanol and carbon dioxide, and a vast array of secondary metabolites, such as higher alcohols, organic acids, esters, aldehydes, ketones, sulfur, and nitrogen volatiles. These secondary metabolites generally have high flavor impact and will also diffuse into the bean to affect chocolate quality, although little research has been done on this topic (Rodriguez-Campos et al., 2011; 2012). In addition,

others function are attributed to yeast metabolism, such as acceleration of carbohydrate consumption, pectinolytic activity and inhibition of growth of pathogenic microorganisms (Batista et al., 2015a; Lefeber et al., 2012; Mahazar et al., 2015).

Due to all these aspects, the use of yeast starter cultures has been proposed as a powerful tool to conduct controlled cocoa fermentations. Some species already tested include *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Pichia kluyveri*, *Hanseniaspora uvarum* and *Candida* sp. (Annan et al., 2003; Batista et al., 2015b; Crafack et al., 2013; Leal Jr et al., 2008; Lefeber et al., 2012; Sandhya et al., 2016).

Microbial strains are subdivisions within a species which are distinguished by genotypic and/or phenotypic features. The differentiation occurs when the species undergo significant mutations generated by natural processes, such as sexual reproduction, changes in ploidy, transposons, genetic recombination or horizontal gene transfer (Steensels et al., 2014). The natural diversity of yeast strains has become very clear with the advent of next-generation sequencing technologies that enable in-depth characterization of the genetic variation (Borneman et al., 2011; Illeghems, Weckx, & De Vuyst, 2015; Illeghems, Pelicaen, De Vuyst, & Weckx, 2016). This implies that natural mutations can form strains with important fermentative characteristics, which can be exploited for selecting appropriate starter cultures.

The aim of this work was to: (i) study the diversity of aroma-producing yeast strains in cocoa bean fermentation, (ii) select suitable yeast starter cultures and (iii) evaluate their influence for cocoa flavor modulation.

3.3. Material and Methods

3.3.1. Yeast isolation

Yeast strains were isolated from 24-h spontaneously fermenting cocoa beans. A total of 200 g of cocoa beans originated from Bahia State, Brazil, were deposited in 500 mL Erlenmeyer flasks and incubated at 30 ºC for 24 hours. At the end of the process, 25 g of cocoa beans with their pulp were added to 225 mL saline-peptone water (0.1% [v/v] bacteriological peptone [Himedia], 0.8% [v/v] NaCl [Merck, Whitehouse Station, NJ]) and was homogenized in a stomacher at normal speed for 5 min, followed by serial dilutions. Yeasts were isolated by surface inoculation on YEPG agar containing 0.01% (w/v) chloramphenicol incubated at 30 °C for 48 hours. Following incubation, the morphological characterization and counts of each colony type obtained were recorded. Individual colonies of each morphotype were subcultured and purified. The purified isolates were stored at -80 °C in YEPG broth containing 20% (v/v) glycerol until use.

3.3.2. Selection of aroma-producing yeasts

The isolated yeasts were evaluated for their ability to produce volatile organic compounds in a cocoa pulp simulation medium according to Pereira, Miguel, Ramos and Schwan (2012). The inoculum was grown in 150 mL Erlenmeyer flasks containing 20 mL of the formulated medium at 30 °C during 48 h, and they were subsequently transferred to the fermentations when reaching 10^6 CFU.mL^{-1}. The fermentations were carried out in triplicate for each yeast strain in 500 mL Erlenmeyer flasks containing 200 mL of the cocoa pulp simulation medium at 30 °C under static conditions. The volatile compounds, produced after 24 h of fermentation in the headspace of the Erlenmeyer flasks, were

analyzed by gas chromatography. A carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) type 75 μm SPME fiber (Supelco, Bellefonte, PA) was used to extract volatile constituents from the headspace of the cocoa pulp simulation medium. The volatile compounds were identified by comparing the peak retention times against those of authentic standards purchased from Sigma in a gas chromatograph (Shimadzu model 17A) equipped with a flame ionization detector at 230 °C (Pereira et al., 2015).

3.3.3. Identification of selected yeasts

Seven selected aroma-producing yeast strains were identified by sequencing the rDNA internal transcribed spacer (ITS) region. Yeast cultures were grown under appropriate conditions, collected from agar plates with a sterile pipette tip and resuspended in 50 µL of ultra-pure water. The suspension was heated for 15 min at 95 °C, and 1 µL of this suspension was used as a DNA template in PCR experiments. The 5.8S ITS rRNA gene region of yeast strains was amplified using the primers ITS1 and ITS4 and sequenced by an ABI3730 XL automatic DNA sequencer. The sequences obtained were compared with sequences available in the GenBank database through a basic local alignment search tool (BLAST). The nucleotide sequences were deposited in the GenBank database under access numbers KY305205 to KY305211.

3.3.4. Rep-PCR analysis

The seven selected yeasts were typed at strain level using repetitive extragenic palindromic (Rep)-PCR technique using a $(GTG)^5$ primer (5‘-GTG GTG GTG GTG GTG-3‘) (Versalonic et al., 1994). Amplifications were performed in a final volume of 12.5 µL containing 1.25 µL of 10x PCR buffer (Invitrogen), 0.75 µL of MgCl2 (50mM), 0.25 µL of dNTP Mix (10mM), 1.25 µL of $(GTG)^5$ primer (20 pmol), 0.2 µL of 5U.µL-1 Platinum® Taq DNA polymerase (Invitrogen). The PCR reactions were repeated three times to confirm the generated fingerprints. For each PCR reaction, an initial denaturation step at 94°C for 5 min was performed, followed by 35 cycles of denaturation at 94° C for 15 sec, annealing at 55° C for 45 sec and extension at 72° C for 90 sec. Final extension step was performed at 72 °C for 6 min. PCR amplifications were performed using a Veriti Thermo Cycler (ThermoFisher Scientific, Waltham, MA). Amplicons (10 µL) were loaded and

separated by electrophoresis in 1.8% (w/v) agarose gels in 1x TBE at 10 $V.cm^{-1}$. DNA markers GeneRuler 100bp DNA Ladder (Invitrogen) were run along with the samples as a reference. After electrophoresis, PCR products were stained with ethidium bromide (10 $mg.mL^{-1}$) and scanned using FLA-5000 laser-based imaging system.

3.3.5. Computer-assisted analysis of genomic fingerprints

The genomic fingerprints obtained were converted to a two-dimensional binary matrix (1 = presence of a band; 0 = absence of a band). Similarity matrices were calculated with the Dice coefficient using the unweighted pair group method with the arithmetic averages clustering algorithm (UPGMA). Computer-assisted analysis was performed with the SYSTAT® program for Windows.

3.3.6. Fermentations of cocoa beans with selected yeast strains

Cocoa beans were fermented with two superior aroma-producing yeast strains, *Pichia kudriavzevii* LPB06 and *Pichia kudriavzevii* LPB07. The fermentation experiments were performed at bench scale disposing 400 g of cocoa beans and the surrounding pulp into 1 L Erlenmeyer flasks and incubated at 30 ºC for 166 h. As a control, spontaneous process was allowed to ferment with indigenous microorganisms present in the cocoa fruit. The Erlenmeyer flasks were kept in incubators, and the temperature was adjusted every 12 h to simulate the temperature of large-scale fermentation according the method describe in Pereira et al. (2012). Samples were collected at each 24 h for microbiological and physicochemical analyses. The growth of the yeasts was calculated by colony-forming unit (CFU) through plating of tenfold serial dilution onto YEPG agar containing 0.01% (w/v) chloramphenicol.

3.3.7. High performance liquid chromatography (HPLC) analysis

The concentration of the reducing sugars (glucose and fructose) and ethanol formation of the fermenting pulp mass was monitored during the course of fermentation. About 200 mg of the surrounding pulp was diluted in 5 mL of saline-peptone water and homogenized. Then were centrifuged at 6000 *g* and filtered through 0.22-µm pore size filter (Sartorius Stedim, Goettingen, Germany). The samples were analyzed through a HPLC apparatus (Aglient Technologies 1260 Infinity Series; Aglient Technologies, Santa Clara, California) equipped with a Hi-Plex H column (300 x 7.7 mm; Aglient Technologies, Santa Clara, California) connected to a refractive index (RI) detector (Aglient Technologies, Santa Clara, California). The column was eluated with a mobile phase containing 5mM H_2SO_4, at 60 ºC and a flow rate of 0.6 $mL.min^{-1}$.

3.4. Cocoa bean quality determination

3.4.1. Fermentation index

The Fermentation Index (FI) was performed according to Gourieva & Tserrevitinov (1979). About 0.1 g of crushed cocoa beans was extracted with 50 mL of a methanol:HCl (97:3, v/v) solution . The homogenate was refrigerated (8 ºC) during 20 h and then filtered under vacuum. The filtrate was read in a spectrophotometer (Konica Minolta CM-5) at 460 and 530 nm absorbance. FI was estimated through the ratio between absorbance reads in the 460 and 530 nm ranges. The readings were performed in triplicate, and the mean of each assay was reported.

3.4.2. Instrumental color parameters analysis

Color measurements of fermented and unfermented cocoa beans were recorded with a spectrophotometer (Konica Minolta CM-5, Tokyo, Japan). Readings were performed with the adjusted equipment in Reflectance with specular included, using the standard white (No. C6299 from 03/96) and black (No. C6299G from 03/96) calibrations. The configuration included illuminant D65 with an angle of incidence of 45^0. The images were converted into Cielab system using the pixel to pixel color reading application obtaining the values L* (luminosity), a* (red-green component) and b* (yellow-blue component).

Other parameters were obtained according to the calculation reported in Misnawi, Jinap, Jamilah, & Nazamid (2003):

$$C^*(chroma\ or\ saturation) = (a^{*^2} + b^{*^2})^{1/2}$$

$$h^{s^*}(hue\ angle, dominant\ colour) = tan^{-1}\, b^*/_{a^*}$$

$$TCD^* = \left(\left(L_f{}^* - L_i{}^*\right) + \left(a_f{}^* - a_i{}^*\right) + (b_f{}^* - b_i{}^*)^2\right)^{1/2}$$

(Note: subscripts 'i' and 'f' correspond to the unfermented and fermented assays traits, respectively).

3.4.3. Fourier transform of infrared (FTIR) spectroscopy

Functional groups in samples of grounded fermented and unfermented cocoa beans were determined by FTIR on a VERTEX 70 (Bruker) containing a DRIFT accessory with 64 scans and a 4 cm^{-1} resolution at the 4000 to 400 cm^{-1} wave length region. The samples

were crushed, pulverized and oven dried. Before determination, about 20 mg of the samples were mixed with 100 mg of potassium bromide (KBr), homogenized, and the reads were recorded.

3.4.4. Metal analysis

For sample preparation, fermented and unfermented cocoa beans were transferred to a 250 mL volumetric flask and acidified with 5 mL of concentrated P.A. HNO_3 and H_2O_2 30%. Subsequently, the system was allowed to heating for 40 min at 80 ºC. The extract was filtered at a 0.45 µm pore size filter and the volume was completed to 100 mL. Reference solutions were prepared using deionized water with resistivity of 18.2 $M\Omega.cm^{-1}$ through a Milli-Q water purification system linked to a water distillatory Fisatom (Model 534, Brazil). Glassware used in this procedure was subjected to a decontamination treatment with HNO_3 10% (w/v) for 24 hours prior to use.

For metal content determination, an ICP-OES (Varian, Model ES 720, Palo Alto, CA) was used simultaneously with axial arrangement and solid-state detector. The torch was aligned horizontally and vertically with a Mn^{+2} standard solution concentration of 5.0 $mg.L^{-1}$. The optical system of the ICP OES was calibrated with multi-element stock solution of scanned patterns. Spectral lines were selected considering the absence of interferences and appropriate sensitivity for determining elements in high and low concentrations. The operation conditions were as follows: power of 1.10 kW, plasma gas flow of 15 $L.min^{-1}$, auxiliary gas flow of 1.5 $L.min^{-1}$, nebulizer pressure of 180 kPa, triplicate time read of 3 s, stabilization time of 15 s, sample delay of 30 s, pump speed of 15 rpm and sample washing time of 3 s.

3.4.5. Volatile organic compounds determination of cocoa beans by Gas Chromatography coupled to mass spectrophotometry (GC-MS

The extraction of volatile compounds from the fermented cocoa bean samples were performed using a headspace (HS) vial coupled to a SPME fiber (CAR / PDMS df75µm partially crosslinked, Supelco). For each determination, 1 g of sample was stored in a 20 mL HS vial. The flask was heated at 70 °C for 10 min without shaking, followed by 15 min of fiber exposure in COMBI-PAL system for balancing the volume within the vial.

The compounds adsorbed by the fiber were desorbed into the gas chromatograph injection system gas phase (CGMS-gun TQ Series 8040 and 2010 Plus GC-MS Shimadzu, Tokyo, Japan) to 250 ° C. The compounds were separated on a column 95% PDMS/5% PHENYL (30 m x 0.25 mm, 0.25 mm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard Enterprise, Palo Alto, CA). Helium was used as carrier gas at a rate of 1.0 $mL.min^{-1}$. Mass spectra were obtained by electron impact at 70 eV. The compounds were identified by comparison to the mass spectra from the library database (Nist'98 and Wiley7n).

3.4.6. Statistical analysis

The data were analyzed in a completely randomized design with three replicates. A Tukey's test was performed using Statistica 7.5 (StatSoft, Tulsa, OK). Level of significance was established in a two-sided P-value < 0.05.

3.5. Results and Discussion

3.5.1. Yeast isolation and screening

A total of 39 yeasts were isolated from 24-h fermenting cocoa beans and screened for their ability to produce volatile aroma compounds in a cocoa pulp simulation medium (data not shown). The seven best aroma-producing yeasts were identified by ITS-rRNA gene sequencing as belonging to the species *Pichia kudriavzevii*, in spite of exhibiting different metabolic profiles (Table 1). The yeast *P. kudriavzevii* (synonymous *Issatchenkia orientalis*) is widely distributed, often occurring in soil, fruits and various natural fermentations (Kurtzman, Fell, Boekhout, & Robert, 2011).

Table 1
Volatile aroma compounds formation of isolated yeast strains in a cocoa pulp simulation medium

Isolates	Molecular Identification	Compounds (µL.L^{-1})					
		Ethanol	Acetaldehyde	Ethyl acetate	2,3-Butadione	n-Buthyl acetate	Hexyl acetate
LPB01	*P. kudriavzevii*	90.36 ± 12.1^{a}	ND^{a}	27.83 ± 5.23^{a}	ND^{a}	ND^{a}	ND^{a}
LPB02	*P. kudriavzevii*	207.12 ± 22.4^{b}	3.63 ± 0.5^{b}	12.37 ± 3.1^{a,b}	ND^{a}	ND^{a}	ND^{a}
LPB03	*P. kudriavzevii*	52.1 ± 7.3^{a,c}	ND^{a}	9.03 ± 3.5^{b}	ND^{a}	ND^{a}	ND^{a}
LPB04	*P. kudriavzevii*	165.92 ± 23.5^{b}	ND^{a}	26.87 ± 6.8^{a}	ND^{a}	ND^{a}	ND^{a}
LPB05	*P. kudriavzevii*	39.34 ± 8.5^{c}	4.49 ± 1.6^{b}	18.18 ± 8.5^{a,b}	ND^{a}	ND^{a}	ND^{a}
LPB06	*P. kudriavzevii*	161.81 ± 22.4^{b}	59.57 ± 9.7^{c}	22.32 ± 5.5^{a}	0.56 ± 0.1^{b}	2.49 ± 0.1^{b}	0.64 ± 0.1^{b}
LPB07	*P. kudriavzevii*	80.44 ± 9.54^{a}	78.52 ± 12.5^{c}	62.48 ± 9.4^{c}	ND^{a}	ND^{a}	ND^{a}

*Means of triplicate fermentations in each column bearing the same letters are not significantly different ($p > 0.05$) from one another using Tukey's test (mean ± standard deviation). ND.: not detected

It was reported as being the most well adapted and relevant yeast species involved in fermentations of Ghanaian and Ivorian cocoa beans, representing over 80% of total isolates (Daniel et al., 2009; Samagaci, Ouattara, Niamké, & Lemaire, 2016). This dominance can be explained due to its ability to withstand the stressful conditions imposed by cocoa fermentation, including high temperature and ethanol concentration and ability to metabolize pulp citric acid (Daniel et al., 2009; De Vuyst, Lefeber, Papalexandratou, & Camu, 2010; Jespersen, Nielsen, Hønholt, Jakobsen, 2005; Pereira et al., 2012; Samagaci et al., 2016). The facility to metabolize citric acid might be considered an advantage of this species, which causes the decrease of the acidity of cocoa pulp, and equilibration of the bean pH to values around 5.0–5.5 that are considered optimal for endogenous proteolytic and other enzymatic activities (De Vuyst et al., 2010). Thus, *P. kudriavzevii* growth during cocoa fermentations can contribute to changes in the composition of the pulp that impact on cocoa bean and chocolate quality and possibly on the whole microbial ecology of the fermentation (Schwan, Pereira & Fleet, 2014b).

There is increasing evidence from molecular characterization of isolates that a set of strains within each species may evolve throughout the fermentation (Daniel et al., 2009; Illeghems et al., 2016; Jespersen et al., 2005; Nielsen, Hønholt, Tano-Debrah, & Jespersen, 2005) and, thereby, adding a further complexity to the ecology and its potential impact on cocoa flavor and quality. Such strain complexity is well known for other food and beverage fermentations, such as wines where as many as eight or more strains of *S. cerevisiae* may contribute to the wine fermentation (Fleet, 2008). In this study, the variation in the formation of volatile compounds by cocoa-derived yeast strains indicated a high intraspecies diversity of *P. kudriavzevii*. To confirm this hypothesis, *P. kudriavzevii* strains

were typed at strain level through rep-PCR analysis. $(GTG)^5$-rep-PCR genomic fingerprinting consisted usually of 11 to 24 fragments ranging between 250 to 2072 bp (Fig. 1A). A computer-assisted analysis of genomic fingerprints (Fig. 1B) clearly differentiated the superior aroma-forming yeast strains (G1 group; *P. kudriavzevii* LPB06 and *P. kudriavzevii* LP07) from the other strains (G2 group). This demonstrates the potential of $(GTG)^5$-rep-PCR technique as a promising genotypic tool for rapid and reliable speciation of aromatic yeast strains.

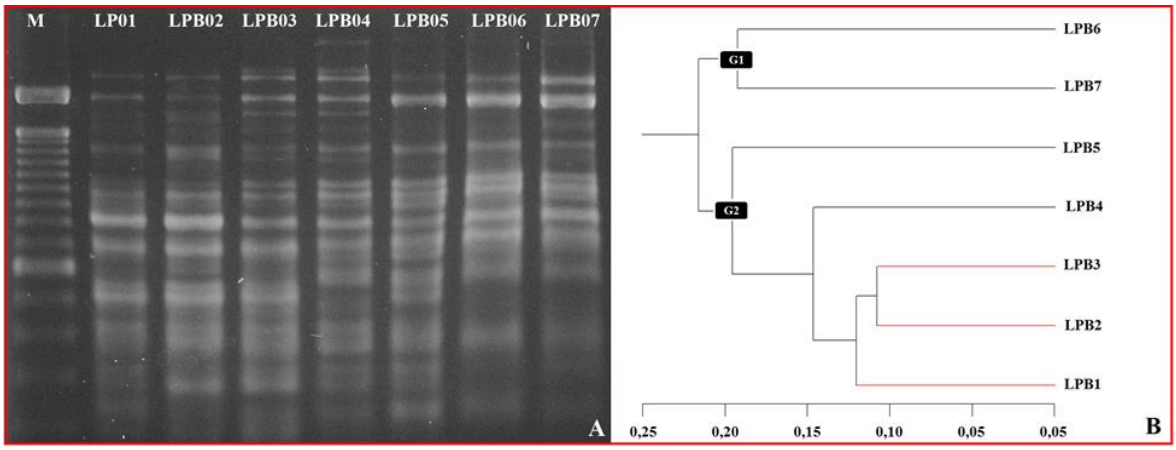

Fig. 2. $(GTG)^5$-rep-PCR fingerprinting of *Pichia kudriavzevii* strains (A) and cluster analysis showing the intraspecies relationship of the isolates (B). The cluster analysis evidences the segregation of *P. kudriavzevii* strains into two main groups: high aromatic (G1) and less aromatic (G2)

3.5.2. Fermentation assay

Recent studies have highlighted the great potential of *P. kudriavzevii* to improve cocoa beans fermentation (Koné et al., 2016; Samagaci et al., 2016), but it has not yet been tested as a starter culture. In this study, the strains *P. kudriavzevii* LPB06 and *P. kudriavzevii* LPB07 were selected to perform cocoa beans fermentations trials at laboratory scale due to their high yields of acetaldehyde (59.57 and 78.52 $\mu L.L^{-1}$, respectively) and

ethyl acetate (22.32 and 62.48 μL.L^{-1}, respectively) (Table 1). These flavor-active esters could attribute distinct fruity sensory notes to the cocoa bean through diffusion during the fermentation process, enriching the flavor of the final product (Camu et al., 2008; Rodriguez-Campos et al., 2011; Schwan & Wheals, 2004).

Total yeast count, sugar consumption and ethanol production of spontaneous (control) and inoculated processes are showed in Fig. 2. A continuous microbial growth with maximum value after 96 h was observed for all fermentation processes, followed by an expressive decrease until the end of the fermentation. However, *P. kudriavzevii* inoculations achieved counts 2 log superior (over 8.9 after 96 h fermentation) when compared to the spontaneous fermentation (6.845 log CFU.mL^{-1} at 96 h fermentation). Ho, Zhao and Fleet (2015) outlined the growth of different yeast species during spontaneous cocoa beans fermentations carried out in Australia and reported a similar *P. kudriavzevii* growth pattern as the our work.

P. kudriavzevii inoculation resulted in an efficient sugar conversion (glucose and fructose) into ethanol. Ethanol concentration had a constant increase until 120 h, achieving concentrations of 8.96 and 9.37 mg.g^{-1} when cocoa beans were inoculated with the strains LPB 06 and LPB 07, respectively (Fig. 2B, C). On the other hand, low ethanol content was observed in the spontaneous process (maximum of 0.875 mg.g^{-1} pulp at 120 h). Ethanol formation has a crucial role for cocoa fermentation process since it becomes a carbon source for the acetic acid bacteria and, along with the acetic acid produced by this microorganism, diffuses into the cotyledons triggering several biochemical reactions essential for the aroma formation during the roasting process (Schwan & Wheals, 2004).

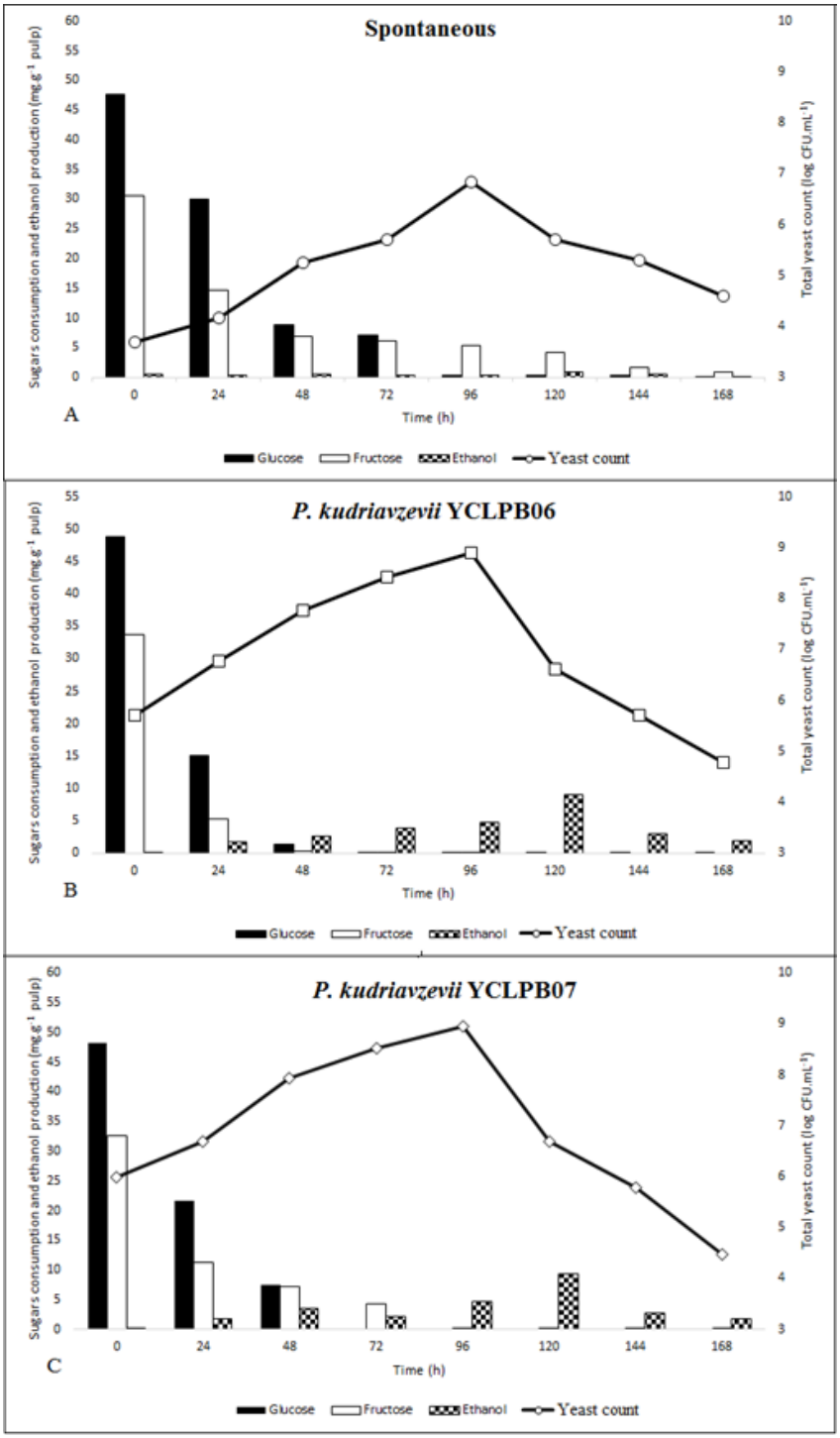

Fig. 3. Monitoring of total yeast population (○, □, ◊), consumption of glucose and fructose, and production of ethanol during spontaneous (A) and inoculated cocoa beans fermentation with *P. kudriavzevii* strains LPB 06 (B) and LPB 07 (C)

3.5.3. Determination of cocoa bean quality

Yeast fermentation of pulp sugars produces a vast array of volatile metabolites that are well known for their aromatic and flavorant properties (Swiegers, Bartowsky, Henschke, & Pretorius, 2005; Pereira et al., 2015). These volatiles diffuse into the cocoa beans, which may influence in their chemical composition. In this study, a total of 34 volatile organic compounds were identified in the fermented cocoa beans by SPME-GC-MS analysis, with predominance of esters (8 compounds) and acetates (6 compounds) (Table 2). Interestingly, cocoa samples from inoculated processes showed a richer aroma composition compared to control (spontaneous process). Some compounds found in the inoculated samples are typically reported in the literature as attributable to *Pichia* metabolism (i.e., benzaldehyde, ethyl acetate, 1-butanol, phenylethyl alcohol, pentatonic acid, and 2-hydroxy-4-methyl-ethyl ester) (Koné et al., 2016; Vestner et al., 2011), while others may have been generated by biochemical reactions within cocoa beans (i.e., linalool and β-myrcene). Although no experimental evidence has been given, it is often mentioned in the literature that such metabolites might contribute to unique fruity, floral, sweet and other notes to food products' character (Owusu, Petersen, & Heimdal, 2012; Pereira et al., 2014; Pereira et al., 2016b).

Table 2 Composition of volatile organic compounds of cocoa beans derived from inoculated (LPB 06 and LPB 07) and control (spontaneous) treatments by SPME-GC/MS.

Volatile aroma compounds	Fermentation assay		
	Spontaneous	LPB 06	LPB 07
Furans (3)			
Butyrolactone	+	+	+
Furan, tetrahydro-2-methyl-	+	+	+
Furfuryl Alcohol	-	+	-
Esters (8)			
Oxalic acid isobutyl nonyl ester	+	+	+
Pentatonic acid, 2-hydroxy-4-methyl-ethyl ester	-	-	+
Acetic acid, 2-phenilethyl ester	+	+	+
Hexanoic acid, ethyl ester	-	+	+
n-Butiryc acid 2-ethylhexyl ester	-	-	+
Benzoic acid, pent-2-yl ester	-	-	+
Octanoic acid, ethyl ester	-	-	+
Isoamyl Propanoate	-	+	+
Acetates (6)			
1-Butanol, 3-methyl-.acetate	+	+	+
1-Methoxy- 2, propyl acetate	+	+	+
Ethanol, 2(2-butoxyethoxy)-acetate	-	-	+
1-Ethylpentyl acetate	-	-	+
2-Heptanol, acetate	-	-	+
Ethyl acetate	-	+	+
Monoterpene (3)			
Linalool	+	+	+
β-Myrcene	+	-	-
β-Ocimene	-	-	+
Hydrocarbons (5)			
2-Dodecane	-	-	+
Tidecane	-	+	-
Hexadecane	-	-	+
Tetradecane	-	-	+
Heptane, 5-ethyl-2,2,3,trimethyl-	-	-	+
Alcohol (4)			
Phenilethyl alcohol	+	+	+
2-Propanol, 1-(2-methylpropoxy)	+	-	-
1-Pentanol, 2.3-dimthyl-	-	-	+
1-Phenylethanol	-	-	+
Aldehyde (2)			
Benzaldehyde	-	+	+
Acetaldehyde	+	+	+
Ether (1)			
1-Isobutoxy-1-methoxypropane	-	+	+
Other compounds (2)			
2-Cyclopropyl-2-nitro-1-ephyl-ethanol	-	+	-
3-Benzyloxy-1-nitro-butan-2-ol	-	-	+

+ = present; - = absent.

In quality control applications, color development has been considered as a good marker when determining the degree of fermented cocoa beans. During the cocoa fermentation, polyphenols and anthocyanins are subjected to biochemical modifications, which allow the formation of brown colour (Gourieva & Tserevinov, 1979; Pettipher, 1986). In this study, Hue angle (h*) of unfermented cocoa bean powders (Fig. 3) showed dominance of red colour compared with yellow (h* < 45). On the other hand, yellowness became dominant in the fermented samples (i.e., higher correlation between C* and b* than between C* and a*) with no statistical difference between treatments. Misnawi et al. (2003) reported that yellowness is a stronger determinant of cocoa bean color changes during fermentation than redness, which indicates an efficient polyphenol oxidase activity and formation of well fermented cocoa beans.

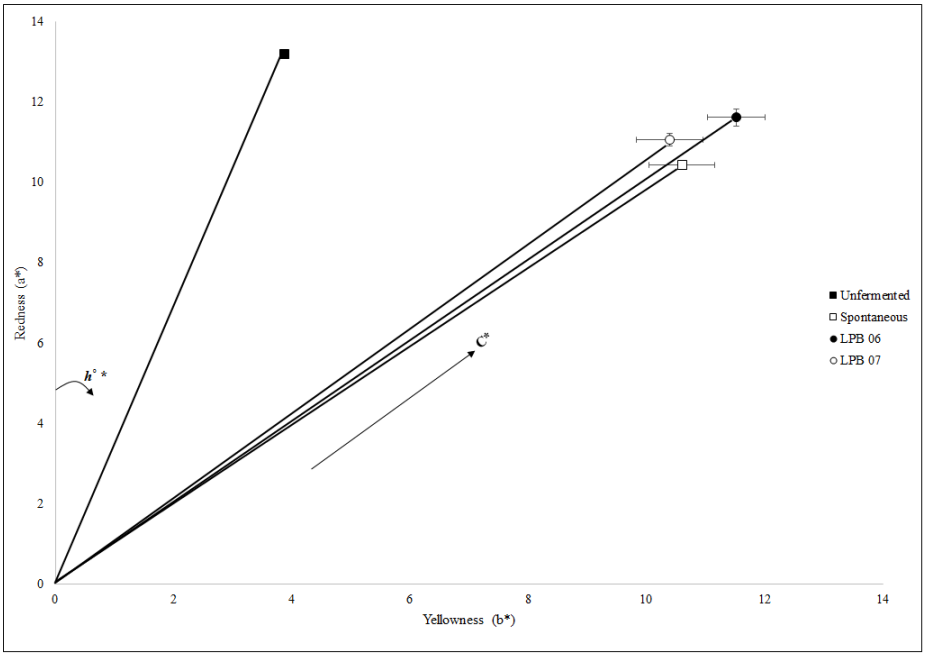

Fig. 4. Changes in hue angle (h*) and chroma (C*) (mean ± standard deviation) of unfermented and fermented cocoa beans in 10 mM acetate buffer at pH 5.5

Thus, although the inoculation process influenced on the bean volatile composition (Table 2), it did not interfere in the hydrolysis and oxidation of polyphenols and color development of cocoa beans. Fermentation index measurements, which are based on the color changes in cotyledons during fermentation, also did not change due to the inoculation processes (value of 1.087, 1.068 and 1.083 for spontaneous, LPB06 and LPB07 fermentations, respectively). Thus, the results show that pigments themselves did not possess any marked for flavor potential in cocoa beans. Furthermore, FTIR spectroscopy analysis also corroborated to this hypothesis, showing a quite similar spectrum for cocoa beans from both inoculated and spontaneous processes (Fig. 4). It was possible to verify the presence of the main organic functions associated with the cocoa aroma, such as alcohol (1257 cm^{-1}), aldehydes (3320 cm^{-1}) and esters (1730 cm^{-1}).

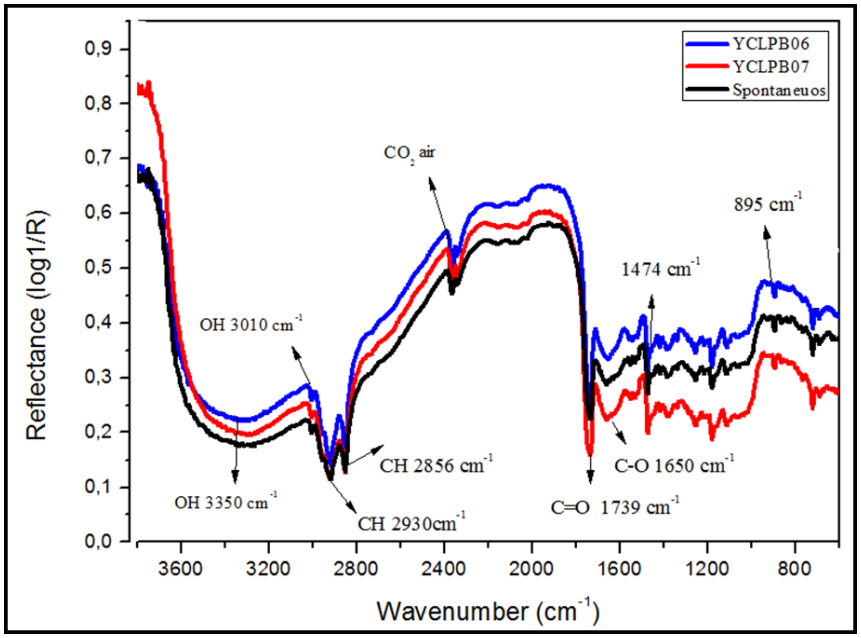

Fig. 5. Chemical functional groups in samples of unfermented and fermented cocoa beans determined by Fourier transform of infrared (FTIR) spectroscopy. The main bands and organic functions associated are: O-H (3350 and 3010 cm^{-1}; alcohols); C-H (2930 and 2856

cm^{-1}; alkenes); C=O (1739 cm^{-1}; carboxylic acids and esters); C-O (1650 cm^{-1}; alcohols or phenols).

The study of the composition and concentration of heavy metals in food products is of great importance due to the variation between essential and toxic constituents. For example, Fe, Z, Cu and Mg are essential, while Li, Cd and Ni are toxic at certain levels (Schroeder, 1973; Somer, 1974). In this study (Table 3), potassium displayed the higher amount among the metals analyzed in the unfermented cocoa beans (10853.48 $mg.kg^{-1}$), followed by phosphorus (6440.43 $mg.kg^{-1}$), magnesium (3080.27 $mg.kg^{-1}$) and calcium (1139.22 $mg.kg^{-1}$). The fermentation processes did not statistically affected the metal composition and concentration of cocoa beans. Several suggestions could be raised as to the origins of these metals in cocoa products but it is widely believed to be from the raw cocoa beans (Amankwaah, Nnuro, Awudza, & Afful, 2015).

In addition, the high content of potassium, phosphorus and calcium is probably correlated with the presence of these macronutrients in the soil through the dispersion of the cocoa pod husk as a field fertilizer by the farmers, which promotes the recycling and bioavailability of these metals for the next crop.

Table 3
Content of metals from the different treatments (spontaneous, inoculated with LPB06 and with LPB07) of unfermented and fermented cocoa beans.

Metals (mg.kg^{-1})	Fermentation assay			
	Unfermented	Spontaneous	LPB 06	LPB 07
Al	302.37 ± 77.54^{a}	211.52 ± 32.75^{a}	295.74 ± 2.24^{a}	269.58 ± 1.925^{a}
Ba	2.21 ± 0.15^{a}	0.92 ± 0.03^{b}	1.49 ± 0.05^{c}	2.34 ± 0.03^{a}
B	11.59 ± 0.16^{a}	16.88 ± 0.14^{b}	10.07 ± 0.34^{c}	22.05 ± 0.22^{d}
Cd	ND^{a}	ND^{a}	ND^{a}	ND^{a}
Ca	1139.22 ± 10.47^{a}	1074.19 ± 14.84^{a}	1039.08 ± 22.96^{a}	858.69 ± 0.72^{b}
Co	ND^{a}	ND^{a}	ND^{a}	ND^{a}
Cu	24.46 ± 0.45^{a}	23.25 ± 0.38^{ab}	20.16 ± 1.11^{b}	20.90 ± 0.26^{ab}
Fe	38.83 ± 0.38^{a}	36.88 ± 0.54^{a}	36.04 ± 1.33^{a}	37.94 ± 0.52^{a}
P	6440.43 ± 41.51^{a}	5084.155 ± 11.76^{b}	6093.68 ± 294.22^{a}	5986.02 ± 85.19^{a}
Li	ND^{a}	ND^{a}	ND^{a}	ND^{a}
Mg	3080.27 ± 34.65^{a}	2370.77 ± 9.87^{b}	2888.40 ± 150.16^{ac}	2557.44 ± 31.00^{bc}
Mn	19.74 ± 0.64^{a}	14.06 ± 0.89^{b}	18.46 ± 0.46^{a}	18.02 ± 0.03^{a}
Mo	0.65 ± 0.01^{a}	0.55 ± 0.01^{a}	0.99 ± 0.04^{b}	0.65 ± 0.03^{a}
Ni	3.58 ± 0.38^{a}	2.21 ± 0.09^{b}	1.84 ± 0.12^{b}	1.93 ± 0.21^{b}
K	10853.48 ± 191.06^{a}	6406.77 ± 9.24^{b}	6961.01 ± 269.01^{b}	7275.95 ± 29.26^{b}
Se	ND^{a}	ND^{a}	ND^{a}	ND^{a}
Na	381.71 ± 9.97^{a}	378.14 ± 14.31^{ab}	461.19 ± 20.98^{c}	391.68 ± 5.00^{a}
V	0.5 ± 0.06^{a}	0.45 ± 0.04^{a}	0.95 ± 0.10^{b}	0.52 ± 0.09^{a}
Zn	75.15 ± 1.01^{a}	56.68 ± 0.98^{b}	51.69 ± 0.69^{c}	61.17 ± 0.41^{b}

*Means of triplicate in each row bearing the same letters are not significantly different ($p > 0.05$) from one another using Duncan's Test (mean ± standard variation). ND: not detectable.Al = Aluminum; Ba = Barium; B = Boron; Cd = Cadmium; Ca = Calcium; Co = Cobalt; Cu = Copper; Fe = Iron; P = Phosphorus; Li = Lithium; Mg = Magnesium; Mn = Manganese; Mo = Molybdenum; Ni = Nickel; K = Potassium; Se = Selenium; Na = Sodium; V = Vanadium; Z = Zinc.

3.5.4. Conclusion

The results of the present study indicated that *P. kudriavzevii* is a dominant, aroma-forming yeast species in cocoa bean fermentation. This species display a great genetic and metabolic diversity, which can be exploited to select strains with suitable characteristics to conduct controlled cocoa beans fermentation. Two selected yeast strains, *P. kudriavzevii* LPB 06 and *P. kudriavzevii* LPB 07, successfully carried out the fermentation process, with efficient sugar metabolism and ethanol formation. Their metabolic activity during the fermentation process influenced the final volatile fraction of fermented cocoa beans. These findings pointed out the need for further investigations to better understand how strain-level yeast diversity can impact the fermentation process and cocoa bean quality. Future studies are needed to prove the efficiency of these inoculants in real, large-scale field conditions.

3.5.5. Acknowledgments

This work was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) through Project PROCAD/BIOCAU grant number 2983/2014. The authors thank CAPES and Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq) for the research scholarship.

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