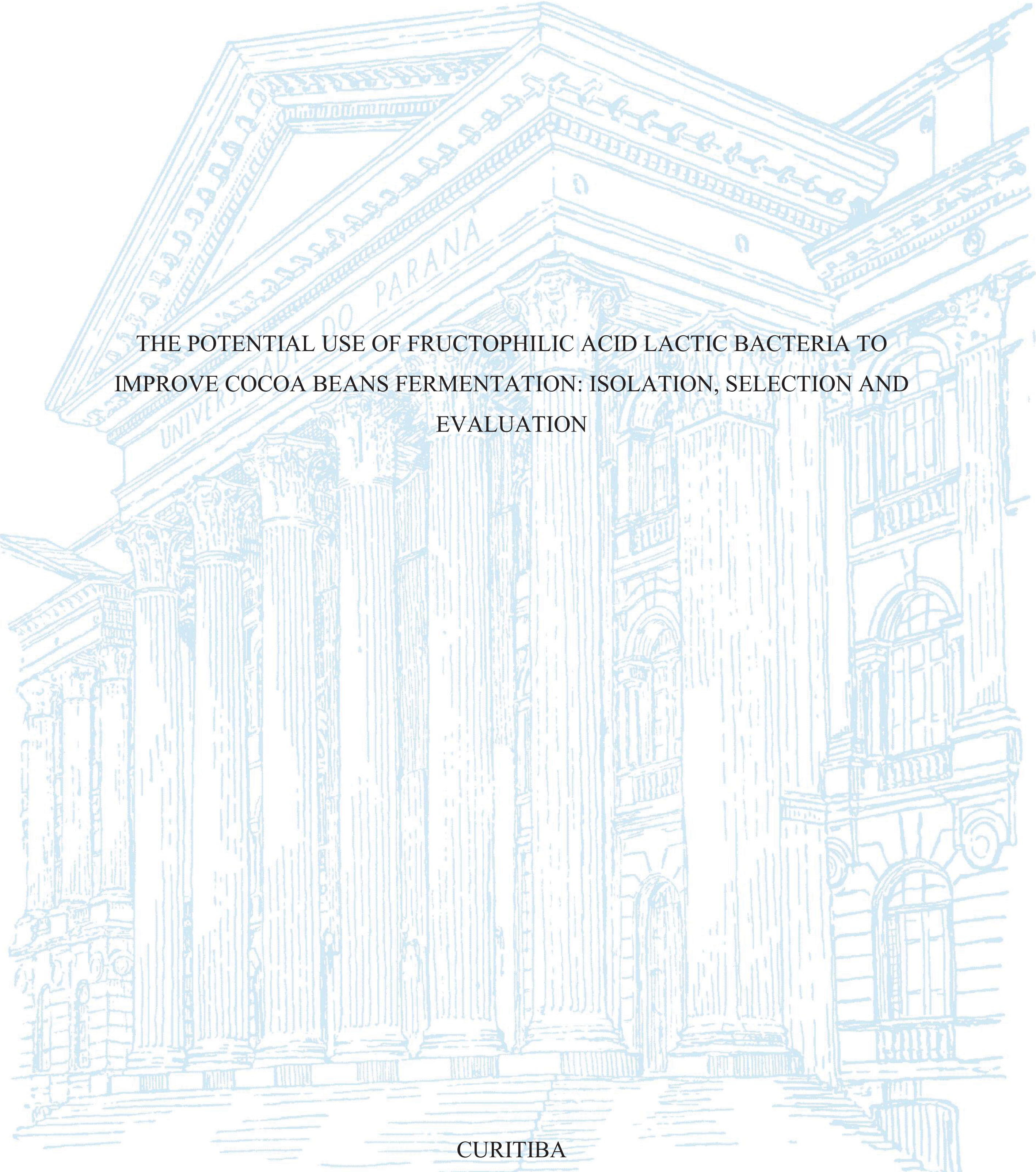
UNIVERSIDADE FEDERAL DO PARANÁ

JÉSSICA APARECIDA VIESSER



THE POTENTIAL USE OF FRUCTOPHILIC ACID LACTIC BACTERIA TO IMPROVE COCOA BEANS FERMENTATION: ISOLATION, SELECTION AND EVALUATION

CURITIBA
2021

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Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do grau de Doutora em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Gilberto Vinícius de Melo Pereira

Coorientador: Prof. Dr. Carlos Ricardo Soccol

CURITIBA
2021

CATALOGAÇÃO NA FONTE – SIBI/UFPR

V665t Viesser, Jéssica Aparecida

The potential use of fructophilic acid lactic bacteria to improve cocoa beans fermentation: isolation, selection and evaluation [recurso eletrônico]/ Jéssica Aparecida Viesser - Curitiba, 2021.

Tese apresentada ao Programa de Pós-graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia da Universidade Federal do Paraná.
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Coorientador: Prof. Dr. Carlos Ricardo Soccol

1. Cacau - processamento. 2. Frutose. I. Pereira, Gilberto Vinícius de Melo. II. Soccol, Carlos Ricardo. III. Título. IV. Universidade Federal do Paraná.

CDD 633.74

Bibliotecária: Vilma Machado CRB9/1563

MINISTÉRIO DA EDUCAÇÃO
SETOR DE TECNOLOGIA
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO ENGENHARIA DE
BIOPROCESSOS E BIOTECNOLOGIA - 40001016036P8

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **JESSICA APARECIDA VIESSER** intitulada: **The potential use of frutophilic acid lactic bacteria to improve cocoa beans fermentation: isolation, selection and evaluation**, sob orientação do Prof. Dr. GILBERTO VINICIUS DE MELO PEREIRA, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
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À minha mãe Ana Maria, com todo meu amor e gratidão.

AGRADECIMENTOS

À Deus por me dar forças para enfrentar as dificuldades e me guiar em todas as decisões.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo suporte financeiro para realização deste trabalho e concessão da bolsa de estudo.

À Universidade Federal do Paraná e ao Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, em especial aos professores Dr. Carlos Ricardo Soccol e Dr. Júlio de Carvalho, pela oportunidade concedida para a realização do doutorado.

Ao meu orientador, professor Dr. Gilberto Vinícius de Melo Pereira, por sua orientação dedicada, disponibilidade, ensinamentos, paciência, amizade e incentivo em realizar este trabalho.

Aos meus pais, Ana Maria e João Antônio, que sempre me incentivaram e apoiaram a lutar pelos meus sonhos. Obrigada pelo amor e carinho incondicional de vocês.

Aos meus irmãos, Marcel e Renan, pela amizade, risadas, conversas e por sempre estarem dispostos a me ajudar.

À minha querida família, em especial à minha vó Silvia, que sempre me apoiou e entendeu quando minha ausência era necessária.

À professora Dra. Leila Teresinha Maranho por acreditar no meu trabalho e me incentivar a fazer o doutorado.

Aos técnicos integrantes do núcleo da Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, especialmente à Mitiyo e ao Ronei, pela amizade, apoio, ensinamentos e aprendizados compartilhados.

Ao professor Dr. Dão Pedro de Carvalho Neto por toda a sua ajuda, disponibilidade e contribuição para a realização deste trabalho. Obrigada pelas discussões científicas, ensinamentos, paciência e amizade.

Ao professor Dr. Hervé Rogez e ao aluno de graduação André Freitas pela parceria e auxílio para a realização das fermentações de cacau na fazenda.

Ao professor Dr. Aristóteles Goés-Neto, Dra. Flávia Aburjaile e Dr. Rodrigo Dias pela parceria, cordialidade e contribuição com as análises de sequenciamento e bioinformática.

Ao professor Dr. Bertram Brenig pela disponibilidade em realizar o sequenciamento do genoma total da bactéria frutofílica.

Ao técnico Thiago, do Departamento de Química, pela sua disponibilidade, cordialidade e auxílio com as análises de cromatografia gasosa.

Ao técnico Vanderlei, do Departamento de Engenharia de Alimentos, pelo auxílio com as análises de colorimetria.

Aos meus amigos e ex-colegas dos Laboratórios de Processos Biotecnológicos (LPB) que me acompanharam no início desta jornada: Anderson, Débora, Matheus, Fernandinha, Pedro, Jair, Natânia, Caroline, Ana Caroline e Adonay. Obrigada pela amizade, risadas, apoio e companheirismo.

Aos meus amigos e colegas dos Laboratórios de Processos Biotecnológicos (LPB) que percorreram toda esta caminhada comigo: Rafaela, Denisse, Zulma, Patrícia, Luis Z., Alex, Diego, Leonardo, Paulo, Brigitte, Bruna, Ana Beatriz, Eliza, Walter, Antônio, Manuel, Raphael, Marcelino, Gisela, Sabrina e Gabriel. Obrigada por serem tão gentis e generosos comigo. Vocês fizeram os dias no laboratório se tornarem mais leves e felizes.

Meu mais sincero obrigada!

RESUMO

No processamento pós-colheita de cacau, a fermentação é uma etapa essencial na formação de cor, sabor e aroma das amêndoas. Durante este processo, microrganismos presentes no ambiente consomem os açúcares da polpa de cacau, facilitando o processo de secagem das sementes. Devido à natureza glucofílica dos microrganismos, a glicose é consumida rapidamente e altos níveis de frutose residual são geralmente observados. Isso acarreta um processo de secagem ineficiente, além de servir como açúcar residual para o crescimento de microrganismos indesejáveis. As bactérias ácido-láticas frutofílicas são metabolicamente caracterizadas por preferirem frutose à glicose como fonte de carbono. Neste contexto, este trabalho teve como objetivo isolar, selecionar e avaliar o potencial de bactérias ácido-láticas frutofílicas para otimizar o consumo de açúcares e formação de metabólitos durante a etapa de fermentação de cacau. Na primeira etapa do estudo, fermentações de cacau em escala laboratorial foram avaliadas quanto à presença e diversidade de bactérias ácido-láticas por sequenciamento de nova geração (Illumina MiSeq), indicando a presença de potenciais bactérias frutofílicas pertencentes aos gêneros *Fructobacillus* e *Lactobacillus*. Um total de 80 cepas microbianas com rápido consumo de frutose foram isoladas e identificadas por métodos moleculares. Dez cepas identificadas como *Lactobacillus plantarum* foram caracterizadas como “bactérias frutofílicas facultativas” a partir do perfil de consumo de diferentes açúcares. *Lactobacillus plantarum* LPBF35 foi finalmente selecionada por produzir moléculas aromáticas, tais como acetato de etila e nonanal. A confirmação da classificação de *L. plantarum* LPBF35 como bactéria frutofílica facultativa ocorreu com base na avaliação do sequenciamento de seu genoma, indicando a presença dos genes *adhE1* e *adhE2* responsáveis pela expressão da enzima álcool/acetaldeído desidrogenase. A introdução de *L. plantarum* LPBF35 como cultura iniciadora otimizou significativamente o consumo de frutose da polpa durante a fermentação de cacau quando comparada aos processos utilizando uma cepa glucofílica (*Pediococcus acidilactici* LPBF66) e à fermentação espontânea (sem inoculação). Na segunda etapa, o metabolismo de *L. plantarum* LPBF35 foi estudado em cultura mista com *Ped. acidilactici* LPBF66 ou *Pichia fermentans* YC5.2. *L. plantarum* LPBF35 dominou todas as co-inoculações avaliadas e, quando inoculada em cultura mista com a levedura *P. fermentans* YC5.2, aumentou a eficiência do consumo de açúcares da polpa e a formação de compostos aromáticos durante a fermentação (2-metil-1-butanol, acetato de isoamila e acetato de etila) e das amêndoas após a secagem (acetato de etila, 2,3-butanodiol, benzaldeído e 2,3-butanodiona). Estes resultados demonstram que o consórcio microbiano entre bactérias frutofílicas e levedura é ideal para melhorar o metabolismo de açúcares e a formação de metabólitos durante a fermentação de cacau.

Palavras-chave: Processamento de cacau. Frutose. Illumina. *Lactobacillus plantarum*.

ABSTRACT

Fermentation is an essential step in the cocoa-processing for color, flavor, and aroma development. During this process, microorganisms in the environment consume sugars present in the cocoa pulp, favoring the drying of the beans. Due to the glucophilic character of microorganisms, glucose is consumed quickly, and high levels of residual fructose are often observed. It results in an inefficient drying process besides being a residual sugar for the growth of undesirable microorganisms. Fructophilic lactic acid bacteria are metabolically characterized by preferring fructose over glucose as a carbon source. In this context, this study aimed to isolate, select, and evaluate the potential of fructophilic lactic acid bacteria to optimize the sugar consumption and metabolites formation during cocoa fermentation. In the first stage of this study, laboratory cocoa beans fermentation was evaluated for the presence and diversity of lactic acid bacteria by new generation sequencing (Illumina MiSeq), indicating the presence of potential fructophilic bacteria of the genera *Fructobacillus* and *Lactobacillus*. A total of 80 microbial strains with fast fructose consumption were isolated and identified by molecular methods. Ten strains identified as *Lactobacillus plantarum* were characterized as facultative fructophilic bacteria due to the consumption profile of different sugars. *Lactobacillus plantarum* LPBF35 was finally selected due to its ability to produce aromatic molecules, such as ethyl acetate and nonanal. The classification of *L. plantarum* LPBF35 was confirmed as facultative fructophilic bacteria based on the evaluation of the sequencing of its genome, indicating the presence of the *adhE1* and *adhE2* genes that express the alcohol/acetaldehyde dehydrogenase enzyme. The introduction of *L. plantarum* LPBF35 as a starter culture significantly optimized the fructose consumption of the pulp during cocoa fermentation when compared to the process using a glucophilic strain (*Pediococcus acidilactici* LPBF66) and the spontaneous fermentation (non-inoculation). In the second stage of this study, the metabolism of *L. plantarum* LPBF35 was studied in a mixed culture with *Ped. acidilactici* LPBF66 or *Pichia fermentans* YC5.2. *L. plantarum* LPBF35 dominated all evaluated co-inoculations and, when inoculated in a mixed culture with the yeast *P. fermentans* YC5.2, it increased the efficiency of the consumption of pulp sugars and formation of aromatic compounds during fermentation (2-methyl-1-butanol, isoamyl acetate, and ethyl acetate) and in the beans after drying (ethyl acetate, 2,3-butanediol, benzaldehyde, and 2,3-butanedione). The results of this study suggest that FLAB and yeast is a microbial consortium that can improve sugar metabolism and aroma formation during cocoa beans fermentation.

Keywords: Cocoa processing. Fructose. Illumina. *Lactobacillus plantarum*.

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SUMMARY

1 INTRODUCTION

Chocolate is the main product obtained from the fermentation and drying of cocoa beans (AFOAKWA et al., 2008; BADRIE et al., 2015; DE VUYST; WECKX, 2016; OZTURK; YOUNG, 2017; SCHWAN; WHEALS, 2004). The consumption of chocolate has been increasing year after year, reaching 7.7 million tons in 2019 (STATISTA, 2021). Consequently, the demand for cocoa beans has also expanded with approximately 5.4 million tons produced between 2017 to 2019 (FAO, 2020). Brazil is the sixth largest cocoa beans producer worldwide, with a 5% share in world production (FAO, 2020).

Fermentation is the first process to which cocoa beans are subjected to produce chocolate. During this process, cocoa pulp is hydrolyzed by microbial growth; this aids the drying process by allowing the pulp to be drained. In addition, fermentation triggers an array of chemical changes within the beans that are precursors of volatile compounds formed during roasting (BEG et al., 2017; DE VUYST; WECKX, 2016). The main microorganisms associated with cocoa fermentation include yeasts, lactic acid bacteria, acetic acid bacteria and *Bacillus* species. This microbiota has preferentially a glucophilic metabolism, *i.e.,* they consume glucose more quickly than fructose. Studies show that residual fructose is still observed after 7 days of cocoa fermentation (LIMA et al., 2011; PAPALEXANDRATOU et al., 2011a; PEREIRA et al., 2012). Fructose may, thus, be one of the causes of long periods of cocoa beans fermentation, lasting up to 7 days (LIMA et al., 2011). In addition, the residual fructose can be metabolized by undesirable fungi and spoilage bacteria that proliferate when the cocoa bean fermentation actually comes to a finish (MOENS; LEFEBER; DE VUYST, 2014). This overfermentation process favors unwanted production of microbial compounds, especially C3–C5 free fatty acids and extracellular proteases and lipases that might have the potential to access and degrade bean proteins and lipids. Whether a causal relation between cocoa fermentation and fructose metabolization exists, it remains unclear.

Although LAB usually have a glucophilic metabolism, recently, a group called fructophilic lactic acid bacteria (FLAB) has been described by Endo and Okada (2008). It includes all species of the genus *Fructobacillus* and some *Lactobacillus* (*L. kunkeei*, *L. brevis*, *L. apinorum*, *L. florum* and *L. plantarum*) for presenting limited or delayed growth on glucose when compared to fructose (ENDO et al., 2012; ENDO; OKADA, 2008; GUSTAW et al., 2018; MAENO et al., 2017; NEVELING; ENDO; DICKS, 2012). Papalexandratou et al. (2011a) reported the presence of *Fructobacillus* sp. by culture-independent approach (PCR-DGGE) during the initial phase of cocoa beans fermentation. Then, *Fructobacillus tropaeoli* was found in Ecuadorian cocoa beans fermentation (PAPALEXANDRATOU et al., 2011b) and *F. pseudofilculneus* occurring occasionally in cocoa fermentation in vessels (LEFEBER et al., 2011a). However, the role of FLAB during cocoa fermentation is not understood. Thus, the aims of this work were to: (i) assess the diversity of fructophilic bacteria in cocoa fermentation, (ii) isolate and characterize fructophilic bacteria from cocoa fermentation process, and (iii) evaluate fructophilic bacteria as cocoa starter cultures, in pure or mixed cultures, to improve fructose consumption and aroma formation during cocoa fermentation process.

This thesis is divided into three chapters. The first chapter consists of an overview of the use of NGS methodologies to study microbial diversity in cocoa beans fermentation and how they can assist in the prospection of microorganisms with biotechnological applications. The second chapter includes the data obtained from the analyses of diversity, isolation, characterization, and evaluation of a frutophilic bacterium as starter culture in laboratory cocoa beans fermentation. The third chapter show the results obtained from implementation of a fructophilic bacterium, in pure and mixed starter cultures, in the field conditions.

2 OBJECTIVES

2.1. MAIN OBJECTIVE

To isolate, identify, and characterize fructophilic lactic acid bacteria (FLAB) associated to cocoa beans fermentation with the objective of improving sugar metabolism and metabolite formation during the process.

2.2. SECONDARY OBJECTIVES

- To evaluate the diversity of FLAB during cocoa beans fermentation by new generation sequencing;
- To isolate FLAB from cocoa beans fermentation using conventional microbiology techniques;
- To select microbial isolates with faster fructose consumption through biochemical assays;
- To verify the fructophilic capacity and aroma molecule production of selected isolates;
- To evaluate the whole genome of FLAB recovered from cocoa fermentation;
- To evaluate the potential of FLAB as a starter culture for cocoa fermentation;
- To evaluate the effects of the inoculation of FLAB on sugar metabolism and aroma formation during cocoa fermentation;
- To evaluate the effects of the inoculation of FLAB on cocoa beans quality development;
- To evaluate the metabolism behavior of FLAB with other important microbial groups from cocoa fermentation;

3 CHAPTER I (LITERATURE REVIEW) - GLOBAL COCOA FERMENTATION MICROBIOME: REVEALING NEW MICROBIAL FUNCTIONS AND BIOTECHNOLOGICAL APPLICATIONS BY NEXT GENERATION SEQUENCING TECHNOLOGIES

Manuscript submitted for publication in the *World Journal of Microbiology and Biotechnology.*

ABSTRACT

This review provides an overview of the application of next generation sequencing (NGS) technologies for microbiome analysis of cocoa bean fermentation, and how this new knowledge can be applied to advances in other sectors of biotechnology. The cocoa-producing regions where NGS has been applied include Brazil, Ghana, Ivory Coast, Cameroon, Nicaragua, and Colombia. These studies have confirmed the dominance of three major microbial groups revealed by culture-dependent approaches, *i.e.,* lactic acid bacteria, acetic acid bacteria, and yeasts. NGS studies have also revealed a more complex microbial diversity, comprising uncultivable microorganisms, sub-dominant populations, and late-growing species. *Lactobacillus* - mainly represented by *L. plantarum* and *L. fermentum* - is the most important and frequent genus with 22 reported species. A total of 83 species were for the first time reported by NGS approaches. Examples include species of *Paenibacillus, Brevibacillus, Methylobacterium, Novosphingobium*, and *Halomonas*. The discovery of these new taxa evidences the potential of cocoa fermentation as a source for prospecting microorganisms with biotechnological potentials. This review addresses how the new insights generated by NGS can be exploited for enzyme production, bioremediation, biofuels and the development of new probiotic and oleaginous microorganisms.

Keywords: Microbial diversity; pyrosequencing; Illumina; yeasts; lactic acid bacteria; acetic acid bacteria.

3.1 INTRODUCTION

Fermented cocoa beans are the main material for the production of chocolate and cocoa-derivative foods, including cocoa paste, butter, powder, and liquor (DE VUYST; WECKX, 2016; VÁSQUEZ et al., 2019). The cocoa beans quality depends on several factors related to agricultural aspects (cocoa plant cultivar, geographic localities, and weather conditions) and on-farm postharvest processing (harvest, fermentation, drying, and storage) (KONGOR et al., 2016; LIMA et al., 2011; MEERSMAN et al., 2013; WOOD; LASS, 1985). Cocoa bean fermentation is the first stage in the production of the desired chocolate taste and aroma (SCHWAN; WHEALS, 2004; WOOD; LASS, 1985). During spontaneous fermentation, the metabolic activities of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) produce mostly ethanol, lactic acid, and acetic acid, respectively. These metabolites diffuse to the cocoa beans and combined with pH and temperature variations cause biochemical reactions inside the beans responsible for the color development and flavor formation (AFOAKWA et al., 2008; CASTRO-ALAYO et al., 2019; DE VUYST; WECKX, 2016; MUÑOZ et al., 2020; SCHWAN; WHEALS, 2004).

Understanding the complex relationships between microbial groups during cocoa bean fermentation allows greater control of the process and its impact on product quality (PEREIRA et al., 2013). The diversity of the microbial community during cocoa fermentation has been assessed through the use of culture-dependent and -independent approaches. Initially, the culture-dependent techniques based on morphological and/or biochemical characteristics were applied for enumeration and identification of cocobiota (ARDHANA; FLEET, 2003; OSTOVAR; KEENEY, 1973; PASSOS et al., 1984; ROELOFSEN, 1958; ROMBOUTS, 1952; SCHWAN, 1998; SCHWAN; VANETTI; SILVA, 1986). These studies identified cultivable microorganisms, including some yeasts (*Candida*, *Kloeckera*, *Pichia*, *Rhodotorula*, and *Saccharomyces*), LAB (*Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*), AAB (*Acetobacter*), other bacteria (*Bacillus*, *Micrococcus*, and *Staphylococcus*), and filamentous fungi (*Aspergillus*, *Mucor*, *Penicillium*, and *Rhizopus*) by Roelofsen (1958), Ostovar and Keeney (1973), Passos et al. (1984), Schwan et al. (1986), and Ardhana and Fleet (2003). However, total microbial diversity has been underestimated due to the limitations of the culture-dependent methods. The cultivation of microorganisms may require growth factors and specific conditions existing in natural habitats that cannot be reproduced in the laboratory (MAYO et al., 2014; PHAM; KIM, 2012). Besides, microorganisms in low abundance are often outcompeted by numerically more abundant microbial species and, therefore, they are not detected (HUGENHOLTZ; GOEBEL; PACE, 1998; JANY; BARBIER, 2008).

In recent decades, the development of culture-independent approaches has been crucial to overcome the limitations of conventional culture-dependent methods through DNA (or RNA) analysis directly extracted from the substrate without any culturing step (GIRAFFA; NEVIANI, 2001; JANY; BARBIER, 2008; MACORI; COTTER, 2018; MAYO et al., 2014). The majority of the studies on microbial diversity during cocoa fermentation have used culture-independent approaches to complement the traditional techniques. Among them, denaturing gradient gel electrophoresis (PCR-DGGE) has been largely applied to access microbial community dynamics and species diversity during cocoa fermentation (ARANA-SÁNCHEZ et al., 2015; BATISTA et al., 2016; CAMU et al., 2007, 2008a; CRAFACK et al., 2013; GARCIA-ARMISEN et al., 2010; HAMDOUCHE et al., 2015, 2019; KONÉ et al., 2016; LEAL JUNIOR et al., 2008; LEFEBER et al., 2011a, 2012; MENEZES et al., 2016; MOREIRA et al., 2013; NIELSEN et al., 2007; NIELSEN; SNITKJAER; VAN DEN BERG, 2008; PAPALEXANDRATOU et al., 2011a, 2011b, 2011c, 2013; PAPALEXANDRATOU; DE VUYST, 2011; PEREIRA et al., 2012; PEREIRA; MAGALHÃES-GUEDES; SCHWAN, 2013; RAMOS et al., 2014). PCR-DGGE is a relatively fast molecular method to identify both cultivable and uncultivable microorganisms using whole microbial community DNA; however it has a low throughput based on the amplification of several, rather small, variable regions of mostly the 16S (bacteria) or 26S (yeasts) rRNA genes, of which the resolution within some genera is limited (ERCOLINI, 2004; ILLEGHEMS et al., 2012)

Recently, high-throughput sequencing (HTS) has permitted to achieve a greater resolution and detection sensitivity than PCR-DGGE gel banding patterns (BORTOLINI et al., 2016; ILLEGHEMS et al., 2012; MAYO et al., 2014). The emergence of next-generation sequencing (NGS) technologies has allowed an in-depth characterization of microbiological diversity and functional in cocoa fermentation with the application of metataxonomics (massive and parallel marker gene sequencing – 16S rRNA/ ITS /18S rRNA) (ALMEIDA et al., 2020; BORTOLINI et al., 2016; ILLEGHEMS et al., 2012; LIMA et al., 2021; MOTA-GUTIERREZ et al., 2018; PACHECO-MONTEALEGRE et al., 2020; PAPALEXANDRATOU et al., 2019; SERRA et al., 2019; VIESSER et al., 2020, 2021) and metagenomics (AGYIRIFO et al., 2019; ILLEGHEMS; WECKX; DE

VUYST, 2015). To date, 454 pyrosequencing (ILLEGHEMS et al., 2012; ILLEGHEMS; WECKX; DE VUYST, 2015) and Illumina platforms (AGYIRIFO et al., 2019; ALMEIDA et al., 2020; BORTOLINI et al., 2016; LIMA et al., 2021; MOTA-GUTIERREZ et al., 2018; PACHECO-MONTEALEGRE et al., 2020; PAPALEXANDRATOU et al., 2019; SERRA et al., 2019; VIESSER et al., 2020, 2021) revealed a diverse and complex microbial community associated with cocoa fermentation in Brazil (ALMEIDA et al., 2020; ILLEGHEMS et al., 2012; LIMA et al., 2021; SERRA et al., 2019; VIESSER et al., 2020, 2021), Ghana (AGYIRIFO et al., 2019; BORTOLINI et al., 2016), Cameroon (BORTOLINI et al., 2016; MOTA-GUTIERREZ et al., 2018), Ivory Coast (BORTOLINI et al., 2016), Nicaragua (PAPALEXANDRATOU et al., 2019), and Colombia (PACHECO-MONTEALEGRE et al., 2020). The aim of this article review is to provide an update on the current knowledge of the microbial composition of cocoa bean fermentations across the globe and how it can modulate the fermentation process. Besides, this review shows the cocoa beans fermentation as a source for prospecting microorganisms with biotechnological potential.

3.2 RESEARCH METHODOLOGY

This review was divided into four parts: (i) a review of global cocoa production, (ii) a review of cocoa beans fermentation process (i.e., main microorganisms involved and their metabolic activities), (iii) an analysis of the microbiome and diversity reported by NGS studies in cocoa beans fermentations from different global regions, and (iv) a discussion on the biotechnological applications of cocobiota. The communities were analyzed from data contained in articles published using NGS platforms as research methodology for cocoa beans fermentation performed in Brazil (ALMEIDA et al., 2020; ILLEGHEMS et al., 2012; LIMA et al., 2021; SERRA et al., 2019; VIESSER et al., 2020), Ghana (AGYIRIFO et al., 2019; BORTOLINI et al., 2016), Cameroon (BORTOLINI et al., 2016; MOTA-GUTIERREZ et al., 2018), Ivory Coast (BORTOLINI et al., 2016), Nicaragua (PAPALEXANDRATOU et al., 2019), and Colombia (PACHECO-MONTEALEGRE et al., 2020). The microbiomes of these regions were analyzed and compared with the aid of a presence/absence table (TABLES 1 and 2) and Venn diagrams (FIGURES 3 and 4) constructed according to Heberle et al. (2015). The microorganisms were separated into two main groups: bacteria and fungi. The bacteria were classified into four subgroups: AAB, LAB, enterobacteria, and other bacteria. In turn, the fungi were classified into three subgroups: *Candida* species, *Pichia* species, and other fungi genera.

3.3 GLOBAL COCOA PRODUCTION AND POSTHARVEST PRACTICES

Cocoa beans are derived from mature fruit of the perennial tree *Theobroma cacao* L., which is native to the Amazon basin located in the South American tropical region (MOTAMAYOR et al., 2008). The cocoa tree is commercially cultivated throughout the equatorial zone between latitudes 20 °N and 20 °S of the Equator, known as the "cocoa belt" (LALIBERTÉ, 2012). This geographic region is characterized by a tropical climate, with temperatures between 18 and 32 °C, altitudes below 400 m, rainfall well-distributed during the year, and high humidity (around 70-90%) (AFOAKWA, 2010). The commercial categorization includes four main cocoa genotypes - *Forastero*, *Criollo*, *Trinitario*, and *Nacional* - based on morphological characteristics, sensory attributes, and geographical origin (BADRIE et al., 2015; OZTURK; YOUNG, 2017). About 95% of the world's cocoa production is related to *Forastero* type cultivation, which is defined as bulk/ordinary cocoa, because it grows faster and with a higher yield than other cocoa types, especially in West Africa (LALIBERTÉ, 2012). *Criollo* and *Trinitario* have a fine flavor, being the latter a hybrid between *Forastero* and *Criollo*; both are used in the production of special chocolate, growing mainly in Central and South America, the Caribbean, and Asia (BADRIE et al., 2015; KONGOR et al., 2016; LALIBERTÉ, 2012). *Nacional* is a native genotype from Ecuador and produces cocoa beans distinguished by their strong floral flavor, which are known as Arriba beans (AFOAKWA, 2010; BADRIE et al., 2015).

Nowadays, Africa, South America, and Asia are the main cocoa bean-producing and -exporting regions (ICCO, 2018). The total worldwide production of cocoa beans increased from 3.3 million tons in 1998 to 5.2 million tons in 2018 (FAO, 2020). Ivory Coast in West Africa is one of the leading cocoa producers, supplying about a third of the total world production, followed by Ghana, Indonesia, Nigeria, Brazil, Cameroon, Ecuador, Dominican Republic, Peru, and Togo. Besides the most important cocoa-producing countries, the cocoa beans are also produced in Malaysia, India, Mexico, Venezuela, Colombia, and 45 other countries (FAO, 2020). Approximately 80-90% of global cocoa production comes from smallholders family farmers (BEG et al., 2017).

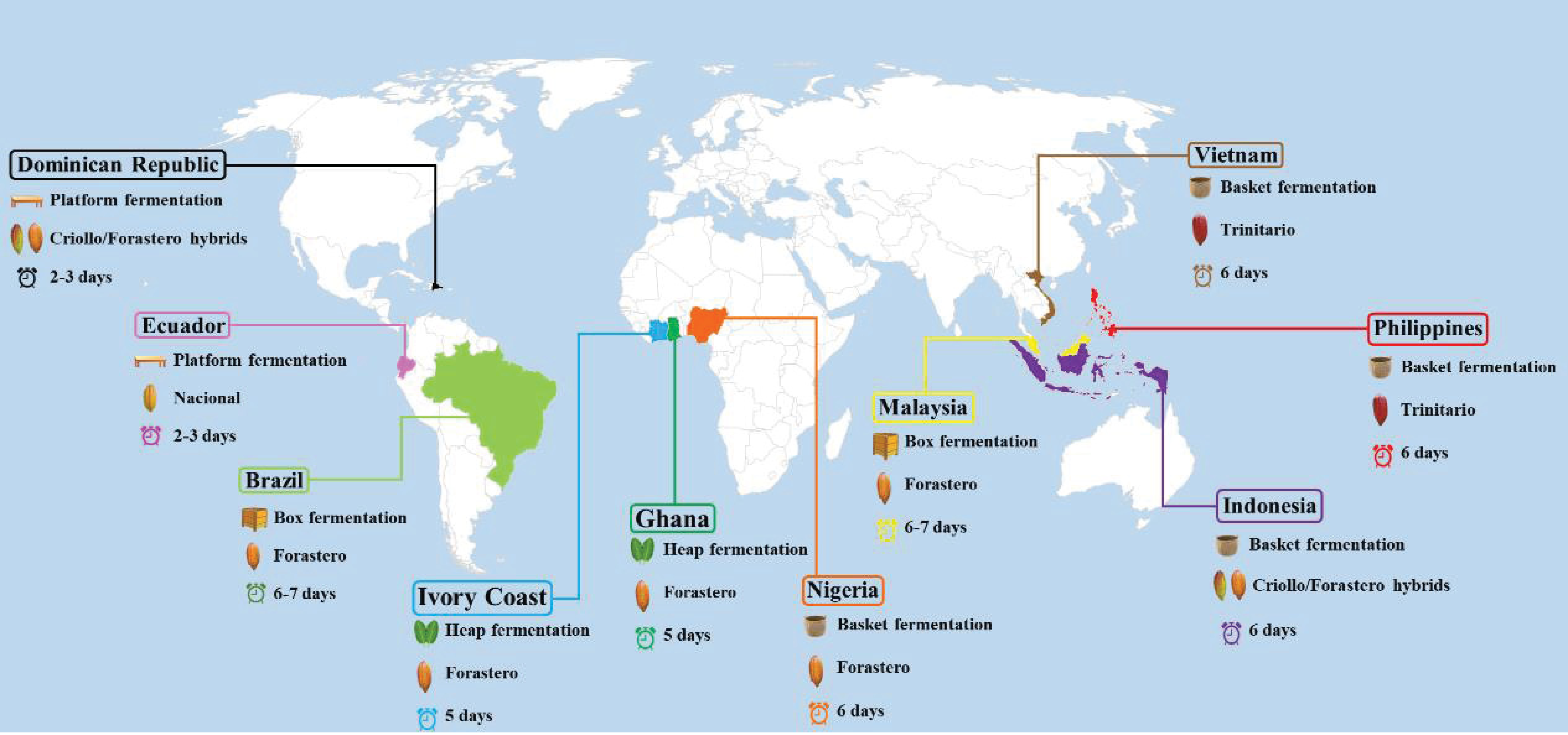
Cocoa processing involves the same traditional operations in the last 150 years (BEG et al., 2017). Postharvest practices are all the primary processes after harvesting cocoa pods until dry beans are obtained, including mature pods harvesting, cocoa beans fermentation, drying, and storage, which are usually carried out on cocoa farms in the country of origin in a non-industrialized way (KONGOR et al., 2016; MUÑOZ et al., 2020). During the postharvest processing, the formation of cocoa aroma and flavor is a combined result of microbial degradation of the cocoa pulp and biochemical reactions inside the beans (LIMA et al., 2011; CASTRO-ALAYO et al., 2019).

Fruit harvesting is the first step in postharvest cocoa processing. When mature, the cocoa pods change the color – usually from green or red to yellow or orange (FOWLER, 2009; AFOAKWA, 2010). The simultaneous presence of different stages of maturation in the same cocoa tree makes the harvest to be carried out over several months (FOWLER, 2009). Harvesting is performed manually using cutlasses or long knives to remove the fruit from the cocoa tree (BEG et al., 2017; WOOD; LASS, 1985).

After harvesting, cocoa fruits are opened immediately using machetes or stored for a few days before opening, a technique known as “pod storage” (AFOAKWA, 2010). The choice of method employed will interfere directly in the quality of cocoa beans used for the next steps of on-farm processing. Pod storage duration has been reported to have a significant impact on acidity, flavor, and composition (fermentable sugars and minerals) of cocoa pulp, as well as volatile composition of dried beans (AFOAKWA et al., 2013; HAMDOUCHE et al., 2019). The mature cocoa fruits contain between 20-40 oval seeds surrounded by a white mucilaginous pulp and attached to a central structure called placenta. Once the fruits are opened, the seeds are removed manually and separated from the placenta to start the fermentation process (FOWLER, 2009; WOOD; LASS, 1985).

There are several methodologies for conducting the fermentation of cocoa beans (FIGURE 1). One of the most popular and traditional methodologies in West Africa, mainly in Ghana and Ivory Coast, consists of placing fresh cocoa seeds forming heaps (approx. 25-2500 kg) and covering them with banana leaves to keep heat and protect them from the rains for around five days, a process known as heap fermentation (FIGURE 1) (FOWLER, 2009; AFOAKWA, 2010; AMOA-AWUA, 2015). The seeds are turned two or three days after the start of fermentation, creating a uniform fermentation and providing a higher quality to the beans (APROTOSOAIE; LUCA; MIRON, 2016).

FIGURE 1 - MAIN POSTHARVEST PRACTICES EMPLOYED AND TYPE COCOA CULTIVATED IN COCA-PRODUCING COUNTRIES.



Source: The author (2020). Adapted from Fowler (2009), Afoakwa (2010), and Amoa-Awua (2015).

In Brazil and Malaysia, the cocoa beans fermentation takes place inside wooden boxes containing small holes to drain the liquid resulting from the degradation of the cocoa pulp and facilitate aeration, called box fermentation (FIGURE 1) (SCHWAN; WHEALS, 2004; NIELSEN et al., 2007; AMOA-AWUA, 2015). Approximately 100-700 kg of cocoa seeds are deposited in each wooden box, which are covered on the surface with banana leaves or jute bags to preserve the heat generated during the fermentation. Once a day, for six or seven days, cocoa mass is turned and transferred to other wooden boxes to allow the aeration of the cocoa mass (FOWLER, 2009; SCHWAN; WHEALS, 2004). The adequate turning of the cocoa mass allows those cocoa seeds that are deposited more deeply in the wooden boxes can be fully fermented (AMOA-AWUA, 2015).

The basket fermentation has been employed on small farms located in Nigeria, Indonesia, the Philippines, and Vietnam (FIGURE 1). This process is very is similar to box fermentation, where baskets made by hand with vegetable material and lined with banana leaves are used to ferment smaller amounts of cocoa seeds (15-50 kg) in an average fermentation time of six days (AMOA-AWUA, 2015; HATMI; KOBARSIH; CAHYANINGRUM, 2015). Two baskets are required for reversal of cocoa beans and facilitate the aeration (HATMI; KOBARSIH; CAHYANINGRUM, 2015).

Drying platforms are the most used for fermentation of cocoa beans (platform fermentation) on farms in countries as Ecuador and Dominican Republic (FIGURE 1). According to this method, cocoa seeds are spread on the platforms during the day to conduct fermentation and stacked at night to maintain the heat, lasting 2-3 days (PAPALEXANDRATOU et al., 2011c; AMOA-AWUA, 2015). This procedure can be applied properly for *Criollo* cocoa, which requires a short period of fermentation (2-3 days), but it is not suitable for *Forastero* cocoa that needs 5 to 8 days of fermentation (APROTOSOAIE; LUCA; MIRON, 2016).

When fermentation is finished, the cocoa beans are recovered from heaps, boxes, baskets, or platforms for drying. This is an important procedure to reduce the moisture content of beans to less than 8% (from about 60% to between 6 to 8%), preventing its deterioration during storage (AFOAKWA et al., 2008). Besides, the drying process improves the flavor development due to reduced acidity (loss of volatile acids) and induces the brown color formation (polyphenol oxidation) in the fermented cocoa beans (ENGESETH; AC PANGAN, 2018; KONGOR et al., 2016). The cocoa beans are generally sun-dried in areas where the weather is dry at harvest time, being spread on raised platforms covered with mats or on the ground until they are fully dried within 7-8 days (FOWLER, 2009; AFOAKWA, 2010). On the order hand, where the weather is less dry and sunny at harvest time, improved methods of solar drying (e.g., using solar energy collectors) or artificial drying (e.g., with fire chambers) have been performed (FOWLER, 2009). The sun-drying is preferentially method required due to the low cost and production of good quality cocoa beans, while during the artificial drying the beans may be dried very quickly, resulting in acidic beans caused by the shell becoming hard and trapping the volatile organic acids inside the beans (FOWLER, 2009).

Finally, dried cocoa beans are stored in warehouses in plastic, jute or sisal bags containing approximately 60-65 kg of dry beans and exported to different countries (BEG et al., 2017; FOWLER, 2009).

3.4 FERMENTATION

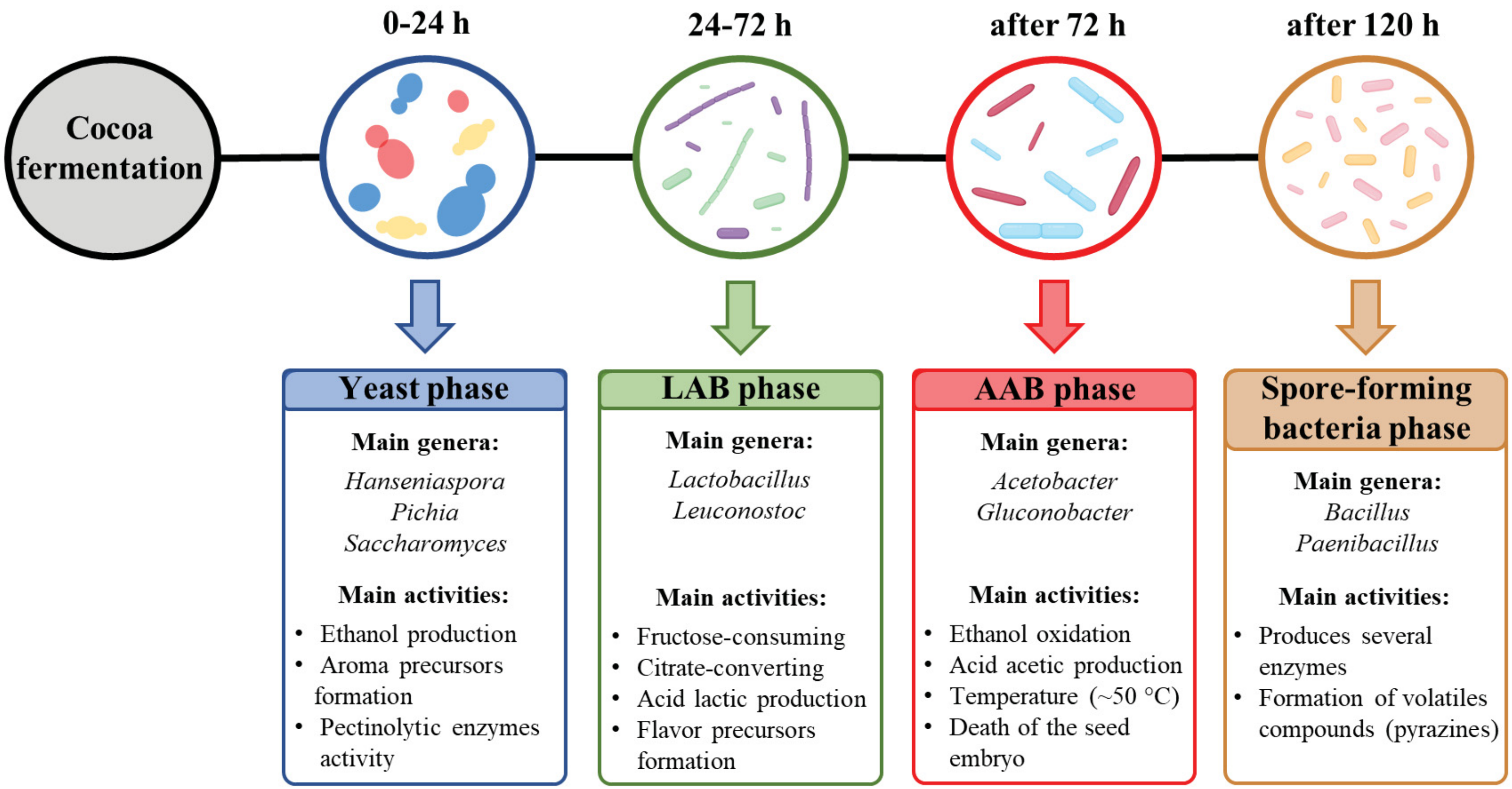
Fermentation is a natural and essential process for 5- to 7-day in the postharvest cocoa-processing, considered as the first stage in the development of the aroma, flavor and color of cocoa beans (DE VUYST; WECKX, 2016; SCHWAN; WHEALS, 2004). This process involves the degradation of mucilaginous pulp that surrounds the beans through complex microbial interactions, mainly by yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Other microorganisms are also present during cocoa fermentation such as enterobacteria, spore-forming bacteria and filamentous fungi; however, its role remains unclear (CAMU et al., 2008b; DE VUYST; LEROY, 2020; FIGUEROA-HERNÁNDEZ et al., 2019; LIMA et al., 2011; SCHWAN; WHEALS, 2004).

Cocoa bean is composed of two cotyledons and an embryo enclosed by a seed coat, covered by a sweet and white mucilaginous pulp (FIGUEROA-HERNÁNDEZ et al., 2019). Cocoa pulp is a rich medium for microbial growth. When cocoa fruit is ripe, pulp contains: water (80-90%), sugars (10-15% - mainly sucrose, glucose, and fructose), citric acid (1-3%), pectin (1-1.5%), proteins (0.5-0.7%), amino acids, minerals (Ca^{+2}, K^{+}, Na^{+}, Mg^{+2}, Fe^{+3}, and Zn), and vitamins (mainly vitamin C) (CAMU et al., 2008b; SARBU; CSUTAK, 2019; SCHWAN; WHEALS, 2004). Within the ripe cocoa fruits, pulp and beans are microbiologically sterile; however, when these are removed from the opened fruits, the pulp is exposed to a variety of microorganisms derived from the surrounding environment (e.g., pods surfaces, soil, and banana leaves), tools used to open them (knives or machetes), worker's hands, and containers where the fermentation process is carried out (e.g., wooden boxes, baskets or platforms), which contribute to subsequent fermentation of cocoa beans (FIGUEROA-HERNÁNDEZ et al., 2019; LEFEBER et al., 2010; SCHWAN; WHEALS, 2004).

Three main phases are distinguished during the fermentation of cocoa beans, characterized by a temporal succession of microorganisms (mainly yeasts, LAB, and AAB) associated with environmental factors such as temperature, pH, and oxygen availability (FIGURE 2). Initially, cocoa pulp is colonized by yeasts due to its acidity (pH 3.0-4.0), high concentrations of fermentable sugars (sucrose, glucose, and fructose), and high viscosity (CAMU et al., 2008a; PAPALEXANDRATOU; DE VUYST, 2011; SCHWAN; WHEALS, 2004). Therefore, this initial phase is defined by the absence of available oxygen, known as the anaerobic fermentation stage (DE VUYST; WECKX, 2016; FIGUEROA-HERNÁNDEZ et al., 2019). Yeasts belonging to genera *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, and *Saccharomyces* are frequently associated with cocoa beans fermentation (AGYIRIFO et al., 2019; HAMDOUCHE et al., 2019; ILLEGHEMS et al., 2012; LIMA et al., 2011; MENEZES et al., 2016; MOREIRA et al., 2013; NIELSEN et al., 2007; PAPALEXANDRATOU et al., 2011b, 2013, 2019; PEREIRA et al., 2012; SERRA et al., 2019). In the first 24 h of fermentation, yeast population increases from 10^5-10^7 CFU/g to 10^8-10^9 CFU/g and declines progressively until reaching small populations of 10-10^2 CFU/g or less at the end of process (SCHWAN; PEREIRA; FLEET, 2015).

The main metabolic function of yeasts during cocoa beans fermentation is to produce ethanol from mainly glucose, producing carbon dioxide and glycerol as side-products, as well as a vast array of precursor compounds (e.g., higher alcohols, esters, organic acids, aldehydes, and ketones) that contribute significantly to the development of flavor and aroma (DE VUYST; WECKX, 2016; PEREIRA et al., 2017; RODRIGUEZ-CAMPOS et al., 2011). Yeast activity also promotes the release of glucose and fructose molecules from the hydrolysis of sucrose, main carbohydrate in the mature cocoa pulp, through the activity of invertase enzymes (DE VUYST; LEROY, 2020; PEREIRA et al., 2013b). Besides, the production of pectinolytic enzymes by yeasts allows the degradation and solubilization of the cocoa pulp, release of sweating, reducing its viscosity and enabling oxygen ingress in the cocoa pulp-bean mass (CRAFACK et al., 2013; HO; ZHAO; FLEET, 2014).

FIGURE 2 - COCOA BEANS FERMENTATION: MAIN GROUPS INVOLVED AND THEIR ACTIVITIES.



Source: The author (2020).

During the anaerobic phase of the cocoa fermentation, yeasts and LAB grow simultaneously due to their non-competitive interactions (DE VUYST; LEROY, 2020; LIMA et al., 2011; SCHWAN; WHEALS, 2004). The yeasts preferentially consume glucose (glucophilic metabolism) as a carbon source and produce ethanol and carbon dioxide under anaerobic conditions through alcoholic fermentation. Alternatively, LAB species that first grow in the cocoa pulp-bean mass does not require oxygen for their growth, presenting a fructophilic metabolism (consuming fructose) with or without citric acid conversion, generating lactic acid, acetic acid, carbon dioxide, and/or ethanol (ADLER et al., 2013; DE VUYST; LEROY, 2020; DE VUYST; WECKX, 2016).

Enterobacteria (e.g., *Erwinia*, *Pantoea*, and *Tatumella*) have been reported in the beginning of the cocoa beans fermentation (DE VUYST; WECKX, 2016; GARCIA-ARMISEN et al., 2010; HAMDOUCHE et al., 2015, 2019; ILLEGHEMS et al., 2012; ILLEGHEMS; WECKX; DE VUYST, 2015; PAPALEXANDRATOU et al., 2011b, 2013, 2019; PEREIRA et al., 2013b). According to Papalexandratou et al. (2011b), these microorganisms may assist in the yeast-mediated pulp depectinization and citric acid assimilation, as well as to producing gluconic acid from glucose. The presence of Enterobacteriaceae family is considered undesirable and impacts the glucose-dependent growth of yeasts and LAB during cocoa fermentation, although gluconic acid may diffuse into the cocoa beans and give it a mild herbal flavor, reducing the bitterness (DE VUYST; LEROY, 2020; PAPALEXANDRATOU et al., 2019).

The concentration of ethanol and carbon dioxide produced by yeasts during the cocoa beans fermentation are factors that determine the composition of the subsequent microbiota (SCHWAN; PEREIRA; FLEET, 2015). Ethanol stimulates the growth of AAB and the carbon dioxide, in turn, contributes to the creation of microaerophilic conditions that favor the growth of LAB (CAMU et al., 2008b; SCHWAN; WHEALS, 2004). Other factors that contribute to the increase in the LAB population include the rise in pH and temperature (from 25-30 to 35-40 °C) due to the consumption of citric cocoa pulp and the heat generated by the exothermic reaction of alcoholic fermentation, respectively (CAMU et al., 2007; DE VUYST; WECKX, 2016; SCHWAN; PEREIRA; FLEET, 2015). Also, the death and autolysis of the yeast cells release nutrients, such as amino acids, vitamins, and polysaccharides, which are favorable for LAB growth (FLEET, 2007; PEREIRA et al., 2012).

The second stage of cocoa bean fermentation (24-72 h) is characterized by the dominance of LAB as consequence of the decline in the yeast population (FIGURE 2)

(DE VUYST; WECKX, 2016; FIGUEROA-HERNÁNDEZ et al., 2019). The LAB populations can reach concentrations of 10^7-10^8 CFU/g after 36-48 h of fermentation (HO et al., 2015; SCHWAN; PEREIRA; FLEET, 2015). At this stage, the cocoa pulp is a favorable substrate for the growth of LAB, since many species are microaerophilic, fructose-consuming, citrate-converting, and ethanol-, acid- and heat-tolerant (ADLER et al., 2013; ARDHANA; FLEET, 2003; CAMU et al., 2007; DE VUYST; WECKX, 2016; LIMA et al., 2011). During cocoa beans fermentation, LAB are metabolically able to: (i) ferment pulp sugars, principally glucose and fructose, to lactic acid and smaller amounts of ethanol and acetic acid, (ii) metabolize citric acid to produce organic acids (lactic and acetic acid) and flavors precursors (acetaldehyde, diacetyl, acetoin, and 2,3-butanediol), and (iii) some species can reduce fructose to mannitol (CAMU et al., 2007; DE VUYST; WECKX, 2016; LAËTITIA; PASCAL; YANN, 2014; LEFEBER et al., 2010; MOENS; LEFEBER; DE VUYST, 2014; SMID; KLEEREBEZEM, 2014). The lactic acid produced by LAB diffuses into the seeds and, because it is not volatile, remains even after drying and roasting, influencing the acidity of the seeds (LEFEBER et al., 2010; PEREIRA et al., 2012; SCHWAN; WHEALS, 2004). This process is also important to activate endogenous enzymes that contribute to the generation of chocolate flavor and aroma; however, the overproduction of lactic acid during cocoa fermentation is not always seen as a desirable property, leading to the excessive acidity in cocoa seeds and chocolate (DE VUYST; WECKX, 2016; SCHWAN; PEREIRA; FLEET, 2015).

The LAB species diversity is limited in the fermenting cocoa pulp-bean mass (ARDHANA; FLEET, 2003; CAMU et al., 2007; ILLEGHEMS; WECKX; DE VUYST, 2015; KOSTINEK et al., 2008; LAGUNES GÁLVEZ et al., 2007; LEFEBER et al., 2011; MEERSMAN et al., 2013; MOTA-GUTIERREZ et al., 2018; NIELSEN et al., 2007; PAPALEXANDRATOU et al., 2011a, 2011b, 2011c, 2019; PEREIRA et al., 2012, 2013; ROMANENS et al., 2018; SERRA et al., 2019; VISINTIN et al., 2016). *Lactobacillus* and *Leuconostoc* are ubiquitous LAB genera reported in cocoa beans fermentation (AGYIRIFO et al., 2019; ALMEIDA et al., 2020; CAMU et al., 2007; ILLEGHEMS et al., 2012; MEERSMAN et al., 2013; MOREIRA et al., 2013; NIELSEN et al., 2007; NIELSEN; SNITKJAER; VAN DEN BERG, 2008; OUATTARA et al., 2017; PAPALEXANDRATOU et al., 2011a, 2011b, 2011c, 2013; SERRA et al., 2019; VIESSER et al., 2020; VISINTIN et al., 2016). *Lactobacillus plantarum* (facultative heterofermentative) is related at the beginning of the cocoa fermentation, while *Lactobacillus fermentum* (strictly heterofermentative) is present at a higher abundance at the end of the process, with both species being the most frequently dominant (BORTOLINI et al., 2016; CAMU et al., 2007, 2008b; FIGUEROA-HERNÁNDEZ et al., 2019; ILLEGHEMS et al., 2012; KOSTINEK et al., 2008; LEFEBER et al., 2011a; MEERSMAN et al., 2013; MOREIRA et al., 2013; MOTA-GUTIERREZ et al., 2018; OUATTARA et al., 2017; PAPALEXANDRATOU et al., 2011b, 2011c, 2011a, 2013; PEREIRA et al., 2012, 2013; VISINTIN et al., 2016). Additionally, other LAB genera such as *Enterococcus*, *Weissella*, *Lactococcus*, *Fructobacillus*, and *Pediococcus* have been also detected during cocoa fermentation, but usually in low abundance (AGYIRIFO et al., 2019; CAMU et al., 2007, 2008b; GARCIA-ARMISEN et al., 2010; KOSTINEK et al., 2008; LEFEBER et al., 2011a; LIMA et al., 2021; OUATTARA et al., 2017; PAPALEXANDRATOU et al., 2011c, 2013, 2011b, 2011a; PEREIRA et al., 2012; ROMANENS et al., 2018; VIESSER et al., 2020).

After 48-72 h of fermentation, LAB populations are reduced due to nutrient depletion. On the other hand, AAB populations increase to concentrations of 10^7-10^8 CFU/g after 72-96 h because of the high levels of ethanol previously produced by yeasts and oxygen ingress in the cocoa pulp-bean mass caused by reduction in the viscosity of the cocoa pulp and regular mixing of the cocoa pulp-bean mass during fermentation, characterizing the third stage of cocoa beans fermentation (FIGURE 2) (CAMU et al., 2008a; DE VUYST; WECKX, 2016; FIGUEROA-HERNÁNDEZ et al., 2019; SCHWAN; WHEALS, 2004). AAB, mainly *Acetobacter* spp., oxidize ethanol (exothermic reaction) produced by the yeasts to acetic acid, although the lactic acid produced by LAB is also converted mainly into acetoin and, in minor amounts, to acetic acid (ADLER et al., 2014; MOENS; LEFEBER; DE VUYST, 2014). Therefore, the growth of AAB is necessary to balance the concentrations of ethanol and lactic acid during cocoa beans fermentation; in addition, to producing acetic acid for bean curing, impacting in the acidity and flavor of fermented cocoa beans (ADLER et al., 2014; DE VUYST; LEROY, 2020). The entry of acetic acid and ethanol in cocoa beans, the decrease in pH (from 6.5 to 4.8) and the increase in temperature (around 50 °C) are the causes of the death of the seed embryo and, consequently, the end-fermentation, triggering several endogenous reactions responsible for the production of flavor, aroma and color precursors (DE VUYST; WECKX, 2016; FIGUEROA-HERNÁNDEZ et al., 2019; LEFEBER et al., 2012; SCHWAN; WHEALS, 2004).

The populations of yeasts, LAB, and AAB are not tolerant to temperatures around 50 °C, so their populations decline until the end of the process (LIMA et al., 2011;

SCHWAN; PEREIRA; FLEET, 2015). On the other hand, in later stages of cocoa beans fermentation (after 120 h) aerobic spore-forming bacteria, such as *Bacillus*, can be detected in concentrations close to 10^7 CFU/g (FIGURE 2) (SCHWAN; PEREIRA; FLEET, 2015). *Bacillus* spp. (*B. subtilis*, *B. pumilus*, and *B. fusiformis*) isolated from cocoa beans fermentation are producers of pectinolytic enzymes, principally pectin lyase and polygalacturonase, which may play an important role during degradation of pectin present in cocoa pulp (OUATTARA et al., 2011; OUATTARA; ELIAS; DUDLEY, 2020). In addition, the enzyme production such as β-glycosidases, proteases, lipases, and amylases, increases the formation of several volatile compounds, including pyrazines, aldehydes, ketones, and alcohols; however, the *Bacillus* genus can also release off-flavors on fermented cocoa beans due to the production of short-chain fatty acids (C3-C5) (ARDHANA; FLEET, 2003; SCHWAN; WHEALS, 2004; SCHWAN; PEREIRA; FLEET, 2015; SERRA et al., 2019).

Filamentous fungi can be found in initial populations of 10^2-10^3 CFU/g and grow to 10^6-10^7 CFU/g during the first 24-36 h of fermentation, although some fungi species have been isolated when the temperature of cocoa pulp-bean mass is around 50 °C at the end of fermentation (ARDHANA; FLEET, 2003; SCHWAN; WHEALS, 2004). The growth of filamentous fungi (e.g., *Aspergillus*, *Fusarium* and *Penicillium*) at levels greater than about 10^6 CFU/g during cocoa beans fermentation is undesired and often associated to off-flavors and mycotoxin formation, especially ochratoxin A and aflatoxins, becoming a significant public health risk factor (MOUNJOUENPOU et al., 2008; SCHWAN; PEREIRA; FLEET, 2015). On the order hand, Ardhana and Fleet (2003) isolated *Penicillium citrinum* from cocoa bean fermentation with high polygalacturonase activity and production of extracelulular proteases and lipases, which possibly contributing to the degradation of cocoa pulp.

The end of cocoa fermentation is characterized by the death of the seed embryo. Increasing the environmental temperature (around 50 °C) and reducing of the internal pH (from 7.0 to 4.0-5.5) of cocoa beans due the diffusion of metabolites such as ethanol, lactic acid and acetic acid cause the destruction of cell structures, release of cellular components, and activation of endogenous enzymes (APROTOSOAIE; LUCA; MIRON, 2016; HANSEN; OLMO; BURRI, 1998). These physicals and biochemicals modifications are essential for the formation of precursors of flavor, aroma and color characteristic of fermented cocoa beans (DE VUYST; WECKX, 2016; MUÑOZ et al., 2020). Several biochemicals reactions are triggered by endogenous enzymes within the cocoa beans, including proteolysis, hydrolysis and oxidation of proteins, carbohydrates and polyphenols, considered as biomarkers for process control (MUÑOZ et al., 2020).

Albumin and vicilin (7S)-class globulin are the two main constituent proteins of cocoa beans and their proteolysis involves the activity of two described enzymes: aspartic endopeptidase and carboxypeptidase (HUE et al., 2016; MUÑOZ et al., 2020; VOIGT et al., 1994). Proteolysis is considered as one of the most important reactions after two or three days of fermentation for the generation of flavor and aroma precursors such as peptides and free amino acids (CALIGIANI et al., 2016; HUE et al., 2016). Amino acids, for example, leucine, alanine, valine, isoleucine, phenylalanine, and tyrosine are precursor molecules in the formation of aromatic compounds (aldehydes) and confer notes described as sweet, floral and fruity to chocolate (CASTRO-ALAYO et al., 2019).

The hydrolysis enzymatic of carbohydrates, specifically sucrose, by invertase enzyme releases reducing sugars (glucose and fructose), which are important flavor and aroma precursors involved in Maillard reactions during the roasting stage (MUÑOZ et al., 2020). For example, 2,5-dimethyl-3-ethyl-pyrazine is a compound obtained from the breakdown of reducing sugars and associated with notes of "roasted" and "nutty" flavor in chocolate (MUÑOZ et al., 2020).

Other components that contribute to flavor profile and are responsible for the formation of the brown color of cocoa beans are the polyphenols (AFOAKWA et al., 2008; MUÑOZ et al., 2020). Three main groups of polyphenols are found in cocoa beans, namely catechins (or also known as flavan-3-ols), anthocyanins and proanthocyanidins, contributing to about 12-18% of the dry weight of the bean (HII et al., 2009). Initially, unfermented cocoa beans have a purple color, but during the fermentation process the polyphenol molecules are oxidized and produce brown colored compounds, indicated a complete fermentation of the cocoa beans (APROTOSOAIE; LUCA; MIRON, 2016). Catechins are oxidized to quinones through enzymatic reactions catalyzed by the polyphenol oxidase (PPO) enzyme after one or two days of fermentation; reactive quinones, in turn, react with nitrogenous compounds, including amino acids, phenolic species, peptides and proteins, to form condensed tannins of high molecular mass and brown pigment (HII et al., 2009; HUE et al., 2016; MUÑOZ et al., 2020). Anthocyanins are hydrolyzed by glycosidases enzymes and generate anthocyanidins (HUE et al., 2016; MUÑOZ et al., 2020). Similarly, proanthocyanidins, also known as condensed tannins, are hydrolyzed under heating and acidic conditions to anthocyanidins, specially into cyanidins and epicatechins (MUÑOZ et al., 2020). In this way, the oxidation and polymerization of polyphenols into insoluble compounds of high molecular weight (tannins) leading to a significant reduction of its concentration and, thus, reducing the bitterness and astringency of the unfermented beans (KONGOR et al., 2016).

3.5 MICROBIOME AND DIVERSITY ANALYSIS IN COCOA FERMENTATION

The fermentation of cocoa beans is a fundamental process to achieve high sensorial quality chocolate. In this process, several microorganisms act metabolically together to enhance cocoa aroma and flavor, resulting beans with unique sensory properties (AFOAKWA et al., 2008; DE VUYST; WECKX, 2016; SCHWAN; WHEALS, 2004). Therefore, the microbial constitution of each producing region is a key to provides a variability of fermented cocoa bean quality. The characterization of microbial diversity among several cocoa regions can reveal which microorganisms are unique to that region and which are present in all of them, making clear the microbial groups involved in the fermentation process, as well as a better explanation of the metabolism fermentation and the sensory characteristics achieved in the final product.

HTS techniques are a powerful tool in the identification of the microbial diversity, as well as its monitoring and dynamics during spontaneous fermentative processes, allowing to discriminate dominant, non-culturable, and low-prevalence microorganisms (QUIGLEY et al., 2012). During the cocoa fermentation, yeasts, LAB, and AAB communities underwent through continuous succession on the dominance that contribute for the death of the embryo and flavor development (DE VUYST; WECKX, 2016; SCHWAN; WHEALS, 2004). However, the total microbiota in the cocoa fermentation is vaster and more complex then only those groups cited. Over the past decade, metagenomic analysis performed in spontaneous cocoa beans fermentation from Brazil, Ivory Coast, Cameroon, Ghana, Nicaragua, and Colombia unveiled the presence of over 222 species of bacteria and fungi (TABLES 1 and 2) (AGYIRIFO et al., 2019; ALMEIDA et al., 2020; BORTOLINI et al., 2016; FIGUEROA-HERNÁNDEZ et al., 2019; ILLEGHEMS et al., 2012; LIMA et al., 2021; PACHECO-MONTEALEGRE et al., 2020; PAPALEXANDRATOU et al., 2019; SERRA et al., 2019; VIESSER et al., 2020). From these, 83 species were reported for the first time when compared to previous works using traditional culture-dependent and PCR-DGGE analyzes (TABLE 3) (CRAFACK et al., 2013; HAMDOUCHE et al., 2019; PAPALEXANDRATOU et al., 2011a; PEREIRA et al., 2013a).

3.5.1 Bacterial world diversity

A qualitative analysis (presence/absence) of the bacterial diversity reported in cocoa fermentations carried out in Brazil, Ghana, Ivory Coast, Cameroon, Nicaragua, and Colombia is shown in Table 1. The results obtained indicate that the bacterial composition can be discriminated by geographical location. The most of NGS studies were conducted with Brazilian cocoa, contributing to a grater bacterial diversity observed in this region.

LAB are largely predominant group in cocoa beans fermentations in all regions, represented mainly by the Firmicutes phylum, which comprises Gram-positive bacteria with a low G+C content (SEONG et al., 2018). A total of 42 LAB species were identified by NGS studies, belonging to the ten taxonomic genera: *Lactobacillus*, *Enterococcus*, *Leuconostoc*, *Weissella*, *Pediococcus*, *Fructobacillus*, *Lactococcus*, *Bifidobacterium*, *Oenococcus*, and *Streptococcus* (TABLE 1).

Lactobacillus was the most frequent genus reported with 22 species identified (TABLE 1). *L. plantarum* and *L. fermentum* have been present in all regions, except in Colombia, indicating their important role during cocoa beans fermentation regardless of the region (FIGURE 3A). In the study carried out in Colombia, the HTS methodology used did not achieve the taxonomic assignment at the specie level, identifying *Lactobacillus* and *Fructobacillus* as the representative LAB genera in this region (PACHECO-MONTEALEGRE et al., 2020). The presence of *L. plantarum* may be related to a high number of sequence reads in its genome that encoding osmotic, oxidative, cold shock, and detoxification stress responses resulting from the fermentation process; therefore, *L. plantarum* is well adapted to the cocoa ecosystem with the capacity to respond to changes in temperature, ethanol concentration and acid stresses encountered in the fermenting cocoa bean-pulp mass (AGYIRIFO et al., 2019; OUATTARA et al., 2017). Generally, at the beginning of the cocoa beans fermentation, *L. plantarum* (facultative heterofermentative metabolism, citrate non-fermenting, and acid-tolerant) predominates, whereas *L. fermentum* (strictly heterofermentative, citrate-fermenting, mannitol-producing, and acid-tolerant) has greater dominance in the final stages of the fermentation (ILLEGHEMS; DE VUYST; WECKX, 2015; LEFEBER et al., 2010). In addition, these two species are able to produce low-molecular weight compounds, such as aldehydes, esters, carboxylic acids, and higher alcohols from catabolism of amino acids present in cocoa pulp (e.g., phenylalanine, isoleucine, and aspartate) (ADEYEYE et al., 2010; ILLEGHEMS; DE VUYST; WECKX, 2015; PEREIRA et al., 2020).

TABLE 1 - BACTERIAL DIVERSITY REPORTED BY NGS STUDIES DURING COCOA BEAN FERMENTATIONS IN DIFFERENT GLOBAL REGIONS.

Microorganisms	**Countries**					
	Brazil	Ghana	Ivory Coast	Cameroon	Nicaragua	Colombia
Acetobacter ascendens	1	0	0	0	0	0
Acetobacter ghanensis	1	0	0	0	0	0
Acetobacter indonesiensis	1	0	0	0	0	0
Acetobacter pasteurianus	1	1	0	1	0	0
Acetobacter senegalensis	1	1	1	1	0	0
Acetobacter sincerae	0	1	0	0	0	0
Acetobacter sp.	1	0	0	0	1	1
Acetobacter syzygii	0	1	1	1	0	0
Acetobacter tropicalis	1	1	0	0	0	0
Achromobacter sp.	1	0	0	0	0	0
Acidiphilium cryptum	0	1	0	0	0	0
Acidisphaera sp.	1	0	0	0	0	0
Acinetobacter baumannii	1	0	0	0	0	0
Acinetobacter guillouiae	0	1	1	1	0	0
Acinetobacter iwoffii	0	0	1	1	0	0
Acinetobacter radioresistens	0	1	1	1	0	0
Acinetobacter rhizosphaerae	0	0	0	1	0	0
Acinetobacter sp.	1	0	0	1	0	0
Actinomycetospora sp.	1	0	0	0	0	0
Agrobacterium sp.	1	0	0	0	0	0
Allobaculum sp.	1	0	0	0	0	0
Anoxybacillus sp.	1	0	0	0	0	0
Aquabacterium sp.	1	0	0	0	0	0
Arthrobacter soli	0	0	1	1	0	0

Asaia sp.	1	0	0	0	0	0
Bacillus circulans	1	1	1	0	0	0
Bacillus clausii	1	1	1	1	0	0
Bacillus coagulans	1	0	0	0	0	0
Bacillus lincheniformis	1	0	0	0	0	0
Bacillus safensis	0	1	1	1	0	0
Bacillus sp.	1	1	1	1	1	0
Bacillus subtilis	1	0	0	0	0	0
Bacteroides sp.	1	0	0	0	0	0
Barnesiella sp.	1	0	0	0	0	0
Bifidobacterium sp.	1	0	0	0	0	0
Blautia sp.	1	0	0	0	0	0
Bradyrhizobium sp.	1	0	0	0	0	0
Brevibacillus sp.	1	0	0	0	0	0
Brevundimonas sp.	1	0	0	0	0	0
Burkholderia sp.	1	1	0	0	0	0
Candidatus Kinetoplastibacterium sp.	1	0	0	0	0	0
Catenibacterium sp.	1	0	0	0	0	0
Caulobacter sp.	1	0	0	0	0	0
Chlorobium sp.	1	0	0	0	0	0
Citrobacter sp.	1	0	0	1	1	0
Collinsella sp.	1	0	0	0	0	0
Coprobacillus sp.	1	0	0	0	0	0
Corynebacterium sp.	1	0	0	0	0	0
Corynebacterium variable	0	0	1	1	0	0
Cronobacter sakazakii	0	1	0	0	0	0
Cronobacter sp.	1	0	0	0	0	0
Cyberlindnera sp.	1	0	0	0	0	0
Dickeya sp.	1	0	0	0	0	0

Dorea sp.	1	0	0	0	0	0
Dyella sp.	0	0	0	1	0	0
Edwarsiella sp.	1	0	0	0	0	0
Enterobacter sp.	1	0	0	0	0	0
Enterococcus camelliae	0	0	1	1	0	0
Enterococcus casseliflavus	0	0	1	1	0	0
Enterococcus cloacae	1	1	0	0	0	0
Enterococcus faecalis	0	1	0	0	0	0
Enterococcus faecium	0	1	0	0	0	0
Enterococcus sp.	1	1	0	1	0	0
Enterococcus tasmaniensis	1	0	0	0	0	0
Entomoplasma sp.	1	0	0	1	0	0
Erwinia amylovora	1	0	0	0	0	0
Erwinia billingiae	1	1	0	0	0	0
Erwinia sp.	1	1	0	1	0	0
Erwinia tasmaniensis	0	1	0	0	0	0
Escherichia coli	1	1	0	0	0	0
Faecalibacterium sp.	1	0	0	0	0	0
Flavobacterium sp.	1	0	0	0	0	0
Fontibacillus pullulanolyticus	1	0	0	0	0	0
Frauteria aurantia	1	0	0	0	0	0
Frauteria sp.	1	0	0	0	0	0
Fructobacillus pseudoficulneus	1	0	0	0	0	0
Fructobacillus sp.	1	0	0	0	0	1
Gluconacetobacter oboediens	1	0	0	0	0	0
Gluconobacter diazotrophicus	0	1	0	0	0	0
Gluconobacter frateurii	1	0	0	0	0	0
Gluconobacter hansenii	1	1	0	0	0	0
Gluconobacter oxydans	1	1	0	0	0	0

Gluconobacter sp.	1	0	0	1	1	0
Granulibacter bethesdensis	0	1	0	0	0	0
Granulibacter sp.	1	0	0	0	0	0
Haemophilus sp.	1	0	0	0	0	0
Halomonas meridiana	1	0	0	0	0	0
Halomonas sp.	1	0	0	0	0	0
Idiomarina sp.	1	0	0	0	0	0
Insolitispirillum sp.	1	0	0	0	0	0
Klebsiella pneumoniae	1	1	0	0	0	0
Klebsiella sp.	1	0	0	1	0	0
Kluyvera sp.	1	0	0	0	0	0
Komagataeibacter hansenii	1	0	0	0	0	0
Komagataeibacter sp.	1	0	0	0	0	0
Kozakia sp.	1	0	0	0	0	0
Lactobacillus antri	0	1	0	0	0	0
Lactobacillus brevis	1	1	0	0	1	0
Lactobacillus bucheri	0	1	0	0	0	0
Lactobacillus cacaonum	1	0	0	0	0	0
Lactobacillus capillatus	0	0	1	1	0	0
Lactobacillus casei	1	1	0	0	0	0
Lactobacillus fermentum	1	1	0	1	1	0
Lactobacillus ghanensis	0	1	0	1	0	0
Lactobacillus murrinus	1	0	0	0	0	0
Lactobacillus nagelii	0	1	0	1	0	0
Lactobacillus paraplantarum	0	1	0	1	0	0
Lactobacillus pentosus	0	0	0	0	1	0
Lactobacillus plantarum	1	1	0	1	1	0
Lactobacillus pontis	1	0	0	0	0	0
Lactobacillus reuteri	1	1	0	0	0	0

Lactobacillus rhamnosus	1	1	0	0	0	0
Lactobacillus ruminis	0	1	0	0	0	0
Lactobacillus sakei	0	1	0	0	0	0
Lactobacillus salivarius	0	1	0	0	0	0
Lactobacillus sp.	1	0	0	0	0	1
Lactobacillus suntoryeus	0	0	1	0	0	0
Lactobacillus vaccinostercus	0	1	0	1	0	0
Lactobacillus vaginalis	0	1	0	1	0	0
Lactococcus lactis	1	1	0	0	0	0
Leptospirillum sp.	1	0	0	0	0	0
Leuconostoc citreum	0	1	0	0	0	0
Leuconostoc gasicomitatum	0	1	0	0	0	0
Leuconostoc kimchii	0	1	0	0	0	0
Leuconostoc mesenteroides	1	1	0	0	0	0
Leuconostoc pseudomesenteroides	1	0	0	0	0	0
Leuconostoc sp.	1	0	0	1	0	0
Lonsdalea sp.	1	0	0	0	0	0
Lysinibacillus halotolerans	1	0	0	0	0	0
Lysinobacillus boronitolerans	0	0	0	1	0	0
Lysinobacillus sp.	0	0	0	1	0	0
Martellela sp.	1	0	0	0	0	0
Methylobacterium sp.	1	0	0	0	0	0
Moraxella sp.	1	0	0	0	0	0
Mycobacterium sp.	1	0	0	0	0	0
Mycoplana sp.	1	0	0	0	0	0
Mycoplasma sp.	1	0	0	0	0	0
Novosphimgobium sp.	1	0	0	0	0	0
Oenococcus oeni	1	1	0	0	0	0
Oscillospora sp.	1	0	0	0	0	0

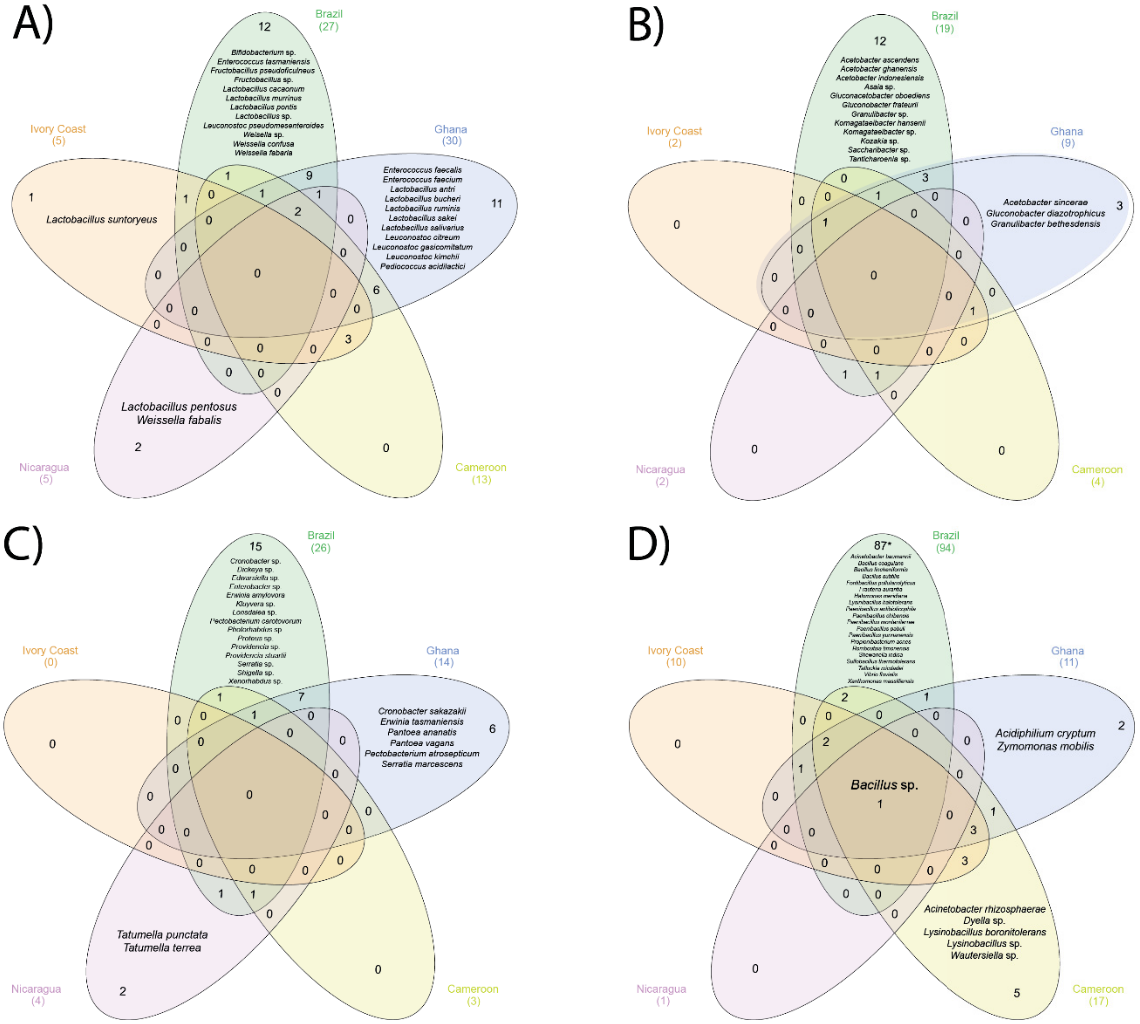
Paenibacillus antibioticophila	1	0	0	0	0	0
Paenibacillus chibensis	1	0	0	0	0	0
Paenibacillus montaniterrae	1	0	0	0	0	0
Paenibacillus pabuli	1	0	0	0	0	0
Paenibacillus sp.	1	0	0	0	0	0
Paenibacillus yunnanensis	1	0	0	0	0	0
Pantoea ananatis	0	1	0	0	0	0
Pantoea sp.	1	1	0	0	0	0
Pantoea vagans	0	1	0	0	0	0
Parabacteroides sp.	1	0	0	0	0	0
Paraburkholderia sp.	1	0	0	0	0	0
Pectobacterium atrosepticum	0	1	0	0	0	0
Pectobacterium carotovorum	1	0	0	0	0	0
Pediococcus acidilactici	0	1	0	0	0	0
Pediococcus pentosaceus	1	1	0	0	0	0
Pediococcus sp.	1	0	1	0	0	0
Peptococcus sp.	1	0	0	0	0	0
Phascolarctobacterium sp.	1	0	0	0	0	0
Photorhabdus sp.	1	0	0	0	0	0
Pleomorphomonas sp.	1	0	0	0	0	0
Prevotella sp.	1	0	0	0	0	0
Propionibacterium acnes	1	0	0	0	0	0
Proteus sp.	1	0	0	0	0	0
Providencia sp.	1	0	0	0	0	0
Providencia stuartii	1	0	0	0	0	0
Pseudomonas sp.	1	0	0	0	0	0
Psychrobacter sp.	1	0	0	0	0	0
Ralstonia sp.	1	0	0	0	0	0
Rhizobium sp.	1	0	0	0	0	0

Rhodanobacter sp.	1	0	0	0	0	0
Rhodococcus sp.	1	0	0	0	0	0
Rhodoplanes sp.	1	0	0	0	0	0
Rhodospirillum sp.	1	0	0	0	0	0
Romboutsia timonensis	1	0	0	0	0	0
Rosenbergiella sp.	1	0	0	0	0	0
Roseomonas sp.	1	0	0	0	0	0
Ruminiclostridium sp.	1	0	0	0	0	0
Ruminococcus sp.	1	0	0	0	0	0
Rummeliibacillus sp.	1	1	1	1	0	0
Saccharibacter sp.	1	0	0	0	0	0
Salinispora sp.	1	0	0	0	0	0
Salmonella enterica	1	1	0	0	0	0
Salmonella sp.	1	1	0	0	0	0
Serratia marcescens	0	1	0	0	0	0
Serratia sp.	1	0	0	0	0	0
Shewanella indica	1	0	0	0	0	0
Shewanella sp.	1	0	0	0	0	0
Shigella sp.	1	0	0	0	0	0
Sphingobium sp.	1	0	0	0	0	0
Sphingomonas sp.	1	0	0	0	0	0
Staphylococcus saprophyticus	0	1	0	1	0	0
Staphylococcus sp.	1	0	0	0	0	0
Stenotrophomonas sp.	1	0	0	0	0	0
Streptococcus sp.	1	1	0	0	0	0
Sulfobacillus sp.	1	0	0	0	0	0
Sulfobacillus thermotolerans	1	0	0	0	0	0
Tanticharoenia sp.	1	0	0	0	0	0
Tatlockia micdadei	1	0	0	0	0	0

Tatumella punctata	0	0	0	0	1	0
Tatumella sp.	1	0	0	0	1	0
Tatumella terrea	0	0	0	0	1	0
Thalassospira sp.	1	0	0	0	0	0
Trabulsiella sp.	0	0	0	1	0	0
Turicibacter sp.	1	0	0	0	0	0
Vibrio fluvialis	1	0	0	0	0	0
Wautersiella sp.	0	0	0	1	0	0
Weisella sp.	1	0	0	0	0	0
Weissella confusa	1	0	0	0	0	0
Weissella fabalis	0	0	0	0	1	0
Weissella fabaria	1	0	0	0	0	0
Weissella paramasenteroides	0	1	0	1	0	0
Xanthomonas massilliensis	1	0	0	0	0	0
Xenorhabdus sp.	1	0	0	0	0	0
Yersinia sp.	1	1	0	0	0	0
Zymomonas mobilis	0	1	0	0	0	0

Source: The author (2020).

FIGURE 3 - VENN DIAGRAMS CONTAINING BACTERIA DIVERSITY FOUND IN COCOA FERMENTATIONS IN BRAZIL, GHANA, IVORY COAST, CAMEROON AND NICARAGUA. A) LACTIC ACID BACTERIA (LAB). B) ACETIC ACID BACTERIA (AAB). C) ENTEROBACTERIA. D) OTHER BACTERIA.



Source: The author (2020).

Weissella is other common genus - with *W. paramesenteroides* being the most common species (TABLE 1). In the Brazilian cocoa beans fermentation were detected *Weissella* sp., *W. confusa*, and *W. fabaria* (FIGURE 3A). The genus *Weissella* has a heterofermentative metabolism and is frequently detected in the beginning of the cocoa fermentation as contaminant from the environment or associated with fruits surfaces, banana/plantain leaves, machetes/knifes and worker's hands (CAMU et al., 2007). *W. fabalis* and *W. paramesenteroides* have also been found, but in less abundance, in the first 48-72 h of fermentation (ORDOÑEZ-ARAQUE et al., 2020; PAPALEXANDRATOU et al., 2019). *W. fabalis* grow well in the presence of fructose, showing a tendency to appear in cocoa beans fermentations where the fructose concentration is higher than the glucose concentration (PAPALEXANDRATOU et al., 2019).

In the literature, *Leuconostoc* is a ubiquitous genus in cocoa beans fermentation; however, its presence has not been reported during cocoa fermentations conducted in Ivory Coast, Nicaragua, and Colombia by NGS studies, probably due to the scarcity of studies in these regions (TABLE 1). *Leu. mesenteroides* and *Leu. pseudomesenteroides* are the most common species in the beginning of the cocoa fermentation (CAMU et al., 2007; OUATTARA et al., 2017), being present in Brazil and Ghana (FIGURE 3A). The genus *Leuconostoc* is characterized by citrate-positive metabolism, which is favored by citric cocoa pulp (CAMU et al., 2007; OUATTARA et al., 2017). Under acidic conditions and in the presence of citrate, *Leuconostoc* species are able to produce metabolites secondaries, including diacetyl, acetoin, and 2,3-butanediol. Diacetyl and acetoin are molecules known to exhibit “nutty” and “buttery” aromatic notes (LAËTITIA; PASCAL; YANN, 2014). Also, *Leu. mesenteroides* is characterized by co-occurrence with *L. plantarum* in cocoa beans fermentation, however, the latter is dominant against *Leu. mesenteroides* in the course of fermentation due to their greater acid and ethanol tolerance (ALMEIDA et al., 2020; OUATTARA et al., 2017).

Other LAB genera reported (i.e., *Enterococcus*, *Pediococcus*, *Fructobacillus*, *Lactococcus*, *Oenococcus*, and *Streptococcus*) are generally detected at low relative abundance in cocoa fermentation and, therefore, they may not play a crucial role in this process. Despite, the rich and complex microbial diversity revealed during cocoa fermentation has been demonstrated the potential of cocoa fermentation process as a source of functional microbial populations with biotechnological purposes (see item 3.6). For instance, fructophilic lactic acid bacteria (FLAB) such as *Fructobacillus* and some *Lactobacillus* strains have a preference for fructose consumption, requiring an electron acceptor when in presence of glucose (ENDO et al., 2018). Previous studies demonstrates a significant residual fructose content at the end of cocoa fermentation (ARDHANA; FLEET, 2003; CAMU et al., 2008a; KRESNOWATI; SURYANI; AFFIFAH, 2013; LAGUNES GÁLVEZ et al., 2007; LIMA et al., 2011; NIELSEN et al., 2007; PAPALEXANDRATOU et al., 2011c; PEREIRA et al., 2012). This residual fructose may be one of the causes of long periods of cocoa beans fermentation, lasting up to 7 days (LIMA et al. 2011). The implementation of fructophilic strains (e.g., *L. plantarum* LPBF35) as starter cultures may assist in the fructose metabolism and aroma formation during cocoa fermentation, contributing to drying of cocoa beans as detailed by Viesser et al. (2020, 2021).

The diversity of AAB species detected in cocoa fermentation is even more restricted than that of the LAB species (TABLE 1). The presence of AAB was detected in all countries analyzed by NGS approaches, belonging to the genera *Acetobacter*, *Gluconobacter*, *Granulibacter*, *Komagataeibacter*, *Asaia*, *Gluconacetobacter*, *Kozakia*, *Saccharibacter*, and *Tanticharoenia*, comprising 15 species (TABLE 1). *Acetobacter* is the most common genus reported in all regions, with *A. senegalensis* found in Brazil, Ghana, Ivory Coast, and Cameroon (FIGURE 3B). Generally, *A. senegalensis* is detected at the start of the cocoa beans fermentation, whereas *A. pasteurianus*, *A. syzygii*, and *A. tropicalis* prevail after 72 h of fermentation, in the AAB phase, when the concentration of ethanol previously produced by yeasts is high (AGYIRIFO et al., 2019; DE VUYST; LEROY, 2020; DE VUYST; WECKX, 2016; HAMDOUCHE et al., 2019; ILLEGHEMS et al., 2012). The prevalence of *A. pasteurianus* during cocoa beans fermentation is due to their ethanol-, lactic acid, and mannitol-oxidizing capacities and their tolerances toward acidity and heat (DE VUYST; LEROY, 2020; ILLEGHEMS; DE VUYST; WECKX, 2013; LEFEBER et al., 2011b; MOENS; LEFEBER; DE VUYST, 2014). Hamdouche et al. (2019) suggested that acetic acid level during cocoa fermentation is mainly related to the *A. pasteurianus* abundance from ethanol-oxidizing, although heterofermentative LAB species also produced minors concentrations of acetic acid from sugars metabolism. In addition, the oxidation of lactic acid by *A. pasteurianus* via tricarboxylic acid cycle (TCA) also favors the formation of acetoin and reduce the acidity of fermented beans (ADLER et al., 2014; ILLEGHEMS; DE VUYST; WECKX, 2013).

Generally, *Gluconobacter* has a higher relative abundance than *Acetobacter* at the beginning of the fermentation process due to its preference for metabolizing the sugars present in cocoa pulp, mainly glucose, as a carbon source via pentose phosphate pathway (PPP) (MAMLOUK; GULLO, 2013) The growth of *Gluconobacter* species during cocoa fermentation is not desirable, indicating a fermentation of poor-quality through the production of gluconic acid and off-flavors (ALMEIDA et al., 2020). As consequence of this metabolism, the prevalence of *Gluconobacter* spp. may cause a late yeast development into cocoa fermentation process and impact on the final quality of cocoa beans (DE VUYST; LEROY, 2020; FIGUEROA-HERNÁNDEZ et al., 2019; PAPALEXANDRATOU et al., 2011c). This partial oxidation of carbohydrates, known as oxidative fermentation, release several secondary metabolites, such as aldehydes, ketones and organic acids, contributing to aroma cocoa formation (MOLINARI et al., 1995).

The *Komagataeibacter* genus was detected only in Brazil (TABLE 1 and FIGURE 3B). The potential functions related to this genus are the cellulose synthesis and production of oxidoreductases (ZHANG et al., 2018); however, its specific role during cocoa beans fermentation remains unclear. Similarly, the genus *Granulibacter* was identified in Brazil and Ghana (TABLE 1 and FIGURE 3B). *Granulibacter bethesdensis* is the only species of this genus described at the moment, which assimilates methanol as a carbon source; in addition, this specie is considered as a pathogen human (SAICHANA et al., 2015; YAMADA; YUKPHAN, 2008).

A significative number of genera included in the group of Enterobacteria (i.e., *Citrobacter*, *Cronobacter*, *Dickeya*, *Edwarsiella*, *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Lonsdalea*, *Pantoea*, *Pectobacterium*, *Photorhabdus*, *Proteus*, *Providencia*, *Salmonella*, *Serratia*, *Shigella*, *Tatumella*, *Trabulsiella*, *Xenorhabdus*, and *Yersinia*) were detected during cocoa beans fermentation by NGS studies (TABLE 1). Nevertheless, no enterobacteria genera was common in all countries studied (FIGURE 3C). The appearance of these bacterial species underlines the importance of monitoring the hygiene of the fermentation process to ensure that these species do not become dominant and spoil the beans (PEREIRA et al., 2012). The genera *Erwinia*, *Tatumella*, and *Pantoea* are derived from soil and considered as phytopathogens of various plants and fruits, which could include cocoa pods (GARCIA-ARMISEN et al., 2010; SCHWAN; PEREIRA; FLEET, 2015). Despite the scarcity of research studies about enterobacteria in cocoa bean fermentation, these three genera may participate in the degradation of pulp pectin and citric acid during the initial phase of cocoa fermentation (GARCIA-ARMISEN et al., 2010; HAMDOUCHE et al., 2019; PAPALEXANDRATOU et al., 2019). Besides, enterobacteria are also associated with methylglyoxal detoxification and mixed-acid fermentation pathways, which involve the production of lactate, acetate, succinate, and ethanol (ILLEGHEMS; WECKX; DE VUYST, 2015; LIMA et al., 2021). According to Hamdouche et al. (2015), *Tatumella* spp. could be involved in pectinolytic degradation of cocoa pulp and citric acid assimilation. Similarly, Mota-Gutierrez et al. (2018) observed a strong correlation between citric acid consumption and *Erwinia* sp. during cocoa beans fermentation in Cameroon. *Pantoea* also related with citrate fermentation, in addition, *Pantoea vagans* has a lactonase activity, which is important metalloprotein involved in bacterial quorum sensing - signals enable bacterial cells to regulate gene expression depending on population density (ALMEIDA et al., 2020).

The group called "other bacteria" comprises genera that are not included in the previous three groups (i.e., LAB, AAB, and Enterobacteria). In this group, more than 70 genera are exclusively found in Brazilian cocoa fermentation, including *Acidisphaera*, *Brevibacillus*, *Chlorobium*, *Entomoplasma*, *Fontibacillus*, *Frauteria*, *Halomonas*, *Methylobacterium*, *Mycobacterium*, *Novosphimgobium*, *Paenibacillus*, *Pseudomonas*, *Rhodoplanes*, *Romboutsia*, *Roseomonas*, *Shewanella*, *Streptomyces*, *Sulfobacillus*, *Tatlockia*, *Vibrio*, and *Xanthomonas*, probably due to a greater number of NGS studies performed in this region. (TABLE 1 and FIGURE 3D).

The genera *Bacillus* and *Paenibacillus*, characterized as spore-forming bacteria, obtained six and five species, respectively, reported during cocoa beans fermentation by NGS studies (TABLE 1). Both genera are frequently associated with soil, reaching counts of approximately 10^5-10^6 spores per g of soil (PEREIRA; SANT'ANA, 2018). However, aerobic spore-forming bacteria (e.g., *B. circulans*, *B. clausii*, *B. coagulans*, *B. lincheniformis*, *B. safensis*, and *B. subtilis*) are the most frequently reported in cocoa fermentation (FIGURE 3D) (BORTOLINI et al., 2016; MOTA-GUTIERREZ et al., 2018; PAPALEXANDRATOU et al., 2019; SERRA et al., 2019). The populations of *Bacillus* rise after 120 h of fermentation due to the increase in temperature, in addition to combination with increase in pH and aeration, of cocoa pulp-bean mass (NIELSEN et al., 2007; SCHWAN; VANETTI; SILVA, 1986). *B. clausii* was detected as the second most abundant specie in cocoa fermentation from Ivory Coast (BORTOLINI et al., 2016). Although the role of *Bacillus* in the cocoa fermentation is not very elucidated, *B. subtilis* strain BS38 shows a high enzymatic activity, mainly pectinases, and production of secondary metabolites such as acetoin, butanediol, methyl butanol, pyrazines, lactic acid and acetic acid, which may contribute to the acidity and as undesirable off-flavors of fermented beans (CASTRO-ALAYO et al., 2019; OUATTARA; ELIAS; DUDLEY, 2020; SCHWAN, 1998).

Paenibacillus has been detected only in Brazil by Serra et al. (2019) and Viesser et al. (2020) (FIGURE 3D). However, the role of this genus during cocoa fermentation has not yet been investigated. In the literature, it is reported that *Paenibacillus* can metabolize hexoses, deoxyhexoses, pentoses, cellulose, and hemicellulose, producing ethanol. For instance, *Paenibacillus macerans* is also able to ferment glycerol in the absence of external electron acceptors (GUPTA et al., 2009).

Other bacteria reported by NGS studies have been associated in the literature with fruit and/or fermented products, such as *Brevibacillus* sp. in Chinese rice wine (XU et al., 2018), *Corynebacterium* spp. in smear-ripened cheeses (SCHRÖDER et al., 2011), *Methylobacterium* sp. in strawberries (KOUTSOMPOGERAS; KYRIACOU; ZABETAKIS, 2007), *Novosphingobium* sp. in Chinese sourdough (ZHANG; HE, 2013), *Rhodoplanes* sp. in grapes (WEI et al., 2018), *Rummeliibacillus* sp. in soya milk (MA et al., 2016), and *Sulfobacillus* sp. in soy sauce (SONG et al., 2017). Although, the role of these bacteria in cocoa fermentation is still unclear.

3.5.2 Fungal world diversity

The fungal diversity reported during cocoa beans fermentations conducted in Brazil, Ghana, Cameroon, Nicaragua, and Colombia by NGS studies is shown in Table 2. The yeast communities have been largely detected in all these studies, whereas filamentous fungi species have been detected only in Brazil (ALMEIDA et al., 2020; ILLEGHEMS et al., 2012; LIMA et al., 2021; SERRA et al., 2019), Ghana (AGYIRIFO et al., 2019), and Cameroon (MOTA-GUTIERREZ et al., 2018). Overall, 73 species of fungi were described in cocoa beans fermentations worldwide. Among them, 43 species are yeasts, represented by the most abundant genera: *Candida*, *Pichia*, *Hanseniaspora*, and *Kluyveromyces*. In contrast, 30 species are filamentous fungi, belonging to the most abundant genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Colletotrichum*. The high incidence of these filamentous fungi species during cocoa fermentation is a worrying factor that deserves to be studied and requires attention to the conditions of beneficial agricultural practices.

A progressive growth pattern of indigenous yeasts is generally observed during spontaneous cocoa beans fermentation due to their differences in acidic, ethanol and heat tolerances. For instance, *Kloeckera*, *Hanseniaspora* and *Candida* predominate in the early stages of fermentation, followed by alcohol-tolerant strains, such as *Pichia* and *Saccharomyces*, in the middle and latter stages of fermentation (DE VUYST; LEROY, 2020; HO; ZHAO; FLEET, 2014; MEERSMAN et al., 2013).

TABLE 2 - FUNGAL DIVERSITY REPORTED BY NGS STUDIES DURING COCOA BEAN FERMENTATIONS IN DIFFERENT GLOBAL REGIONS.

Microorganisms	**Countries**					
	Brazil	Ghana	Ivory Coast	Cameroon	Nicaragua	Colombia
Yeasts						
Candida aaseri	1	0	0	0	0	0
Candida albicans	1	0	0	0	0	0
Candida butyri	0	0	0	1	0	0
Candida diversa	0	0	0	1	0	0
Candida ethanolica	1	0	0	0	0	0
Candida glabrata	1	1	0	0	0	0
Candida inconspicua	0	0	0	1	0	0
Candida jaroonii	1	0	0	1	0	0
Candida orthopsilosis	1	0	0	0	0	0
Candida quercitrusa	0	0	0	1	0	0
Candida sp.	1	0	0	1	1	0
Candida stellimalicola	1	0	0	0	0	0
Candida temnochilae	1	0	0	0	0	0
Candida tropicalis	1	0	0	0	0	0
Debaryomyces hansenii	1	0	0	0	0	0
Hanseniaspora opuntiae	1	0	0	1	1	1
Hanseniaspora sp.	1	0	0	0	0	0
Hanseniaspora uvarum	1	0	0	0	1	0
Hyphopichia burtonii	1	0	0	0	0	0
Issatchenkia sp.	0	0	0	1	0	0
Kazachstania humilis	0	0	0	0	1	0
Kazachstania sp.	1	0	0	0	0	0
Kluyveromyces lactis	1	1	0	0	0	0

Kluyveromyces marxianus	0	0	0	1	0	0
Komagataella sp.	1	0	0	0	0	0
Lachancea thermotolerans	1	1	0	0	0	0
Martiniozyma asiatica	1	0	0	0	0	0
Metschnikowia sp.	1	0	0	0	0	0
Millerozyma sp.	1	0	0	0	0	0
Nakazawaea sp.	1	0	0	0	0	0
Naumovozyma sp.	1	0	0	0	0	0
Pichia angusta	1	0	0	0	0	0
Pichia barkeri	0	0	0	0	1	0
Pichia fermentans	1	0	0	0	0	0
Pichia kluyveri	1	0	0	0	0	0
Pichia kudriavzevii	1	0	0	0	1	1
Pichia manshurica	1	0	0	0	0	0
Pichia pijperi	0	0	0	1	0	0
Pichia rarassimilans	1	0	0	0	0	0
Pichia sp.	1	0	0	1	0	1
Pichia sporocuriosa	1	0	0	0	0	0
Rhodotorula sp.	1	0	0	0	0	0
Saccharomyces cerevisiae	1	1	0	1	1	0
Saccharomyces sp.	1	0	0	0	0	0
Sacchsromycopsis amapae	1	0	0	0	0	0
Sacchsromycopsis crataegensis	1	0	0	0	0	0
Sacchsromycopsis sp.	1	0	0	1	0	0
Sacchsromycopsis synnaedendra	1	0	0	0	0	0
Schizosaccharomyces pombe	1	0	0	0	0	0
Schwanniomyces etchellsii	1	0	0	0	0	0
Starmera sp.	1	0	0	0	0	0
Starnerella bacollaris	1	0	0	0	0	0

Starnerella sp.	1	0	0	0	0	0
Sterigmatomyces sp.	1	0	0	0	0	0
Torulaspora delbrueckii	0	0	0	1	0	0
Vanderwaltozyma polyspora	1	1	0	0	0	0
Wickerhamomyces pijperi	1	0	0	0	0	1
Wickerhamomyces sp.	1	0	0	0	0	0
Zygosaccharomyces bailii	1	0	0	0	0	0
Zygosaccharomyces rouxii	1	0	0	0	0	0
Filamentous fungi						
Amylomyces sp.	1	0	0	0	0	0
Agaricus bisporus	1	0	0	0	0	0
Arthrinium sp.	1	0	0	0	0	0
Aspergillus aculeatus	1	0	0	0	0	0
Aspergillus flavus	1	0	0	0	0	0
Aspergillus sp.	1	0	0	0	0	0
Aureobasidium sp.	1	0	0	0	0	0
Aspergillus tubingensis	1	0	0	0	0	0
Botryosphaeria sp.	0	0	0	1	0	0
Cartera sp.	1	0	0	0	0	0
Ceratocystis sp.	0	0	0	1	0	0
Cladosporium cladodosporioides	1	0	0	0	0	0
Clonostachys rosea	1	0	0	0	0	0
Cokeromyces sp.	1	0	0	0	0	0
Colleotrichum brevisporum	1	0	0	0	0	0
Colleotrichum sp.	1	0	0	0	0	0
Conidiobolus sp.	1	0	0	0	0	0
Corollospora sp.	1	0	0	0	0	0
Cunninghamella sp.	1	0	0	0	0	0
Cophinforma sp.	1	0	0	0	0	0

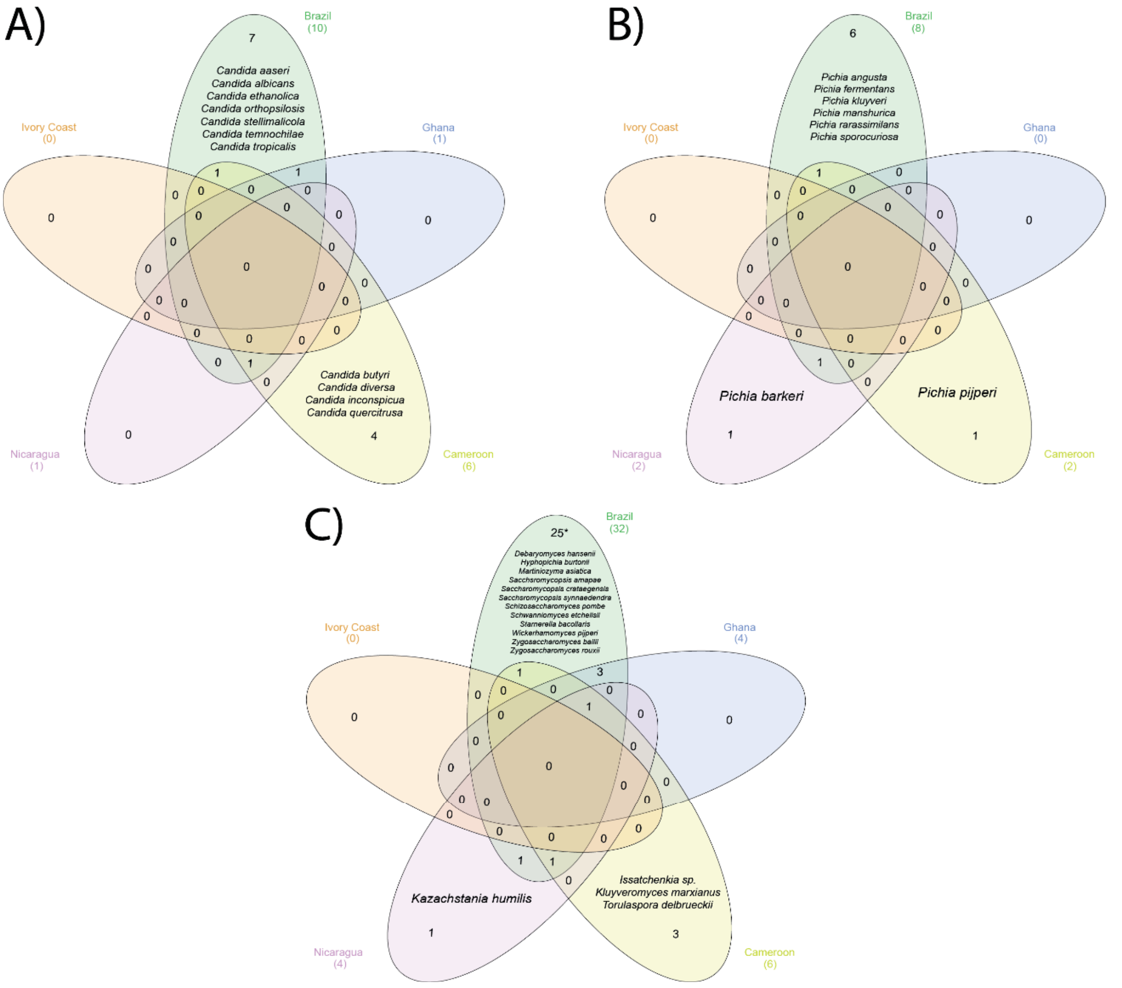
Curvularia lunata	1	0	0	0	0	0
Cylindrocladiella sp.	1	0	0	0	0	0
Cystoagaricus sp.	1	0	0	0	0	0
Eremothecium gossypii	0	1	0	0	0	0
Eremothecium sp.	1	0	0	0	0	0
Fusarium brachygibbosum	1	0	0	0	0	0
Fusarium chlamydosporum	1	0	0	0	0	0
Fusarium solani	1	0	0	0	0	0
Fusarium sp.	1	0	0	0	0	0
Lasiodiplodia theobromae	1	0	0	1	0	0
Lichtheimia sp.	1	0	0	0	0	0
Moniliophthora perniciosa	1	0	0	0	0	0
Nectria pseudotrichia	1	0	0	0	0	0
Nectria haematococca	1	0	0	0	0	0
Neopestalotiopsis formicarum	1	0	0	0	0	0
Nigrospora oryzae	1	0	0	0	0	0
Oidiodendron sp.	1	0	0	0	0	0
Paecilomyces dactylethromorphus	1	0	0	0	0	0
Paraphaeosphaeria sp.	1	0	0	0	0	0
Penicillium citrinum	1	0	0	0	0	0
Penicillium mallochii	1	0	0	0	0	0
Penicillium paxllli	1	0	0	0	0	0
Penicillium simplicissimum	1	0	0	0	0	0
Penicillium sp.	1	0	0	1	0	0
Phycomyces sp.	1	0	0	0	0	0
Porobeltraniella poroso	1	0	0	0	0	0
Purpureocillium lilacinum	1	0	0	0	0	0
Rhizomucor pusillus	1	0	0	0	0	0
Rhizopus sp.	1	0	0	0	0	0

Talaromyces pinophilus	1	0	0	0	0	0
Talaromyces sp.	1	0	0	0	0	0
Thielaviopsis paradoxa	1	0	0	0	0	0
Trichoderma harzianum	1	0	0	0	0	0
Trichoderma sp.	1	0	0	0	0	0
Trichosporon asahii	1	0	0	0	0	0
Wallemia sp.	1	0	0	0	0	0

Source: The author (2020).

Candida was the most frequent yeast genus during cocoa fermentation with 13 species reported (TABLE 2). Among them, seven species were exclusively reported in Brazil (i.e., *C. aaseri*, *C. albicans*, *C. ethanolica*, *C. orthopsilosis*, *C. stellimalicola*, *C. temnochilae*, and *C. tropicalis*), whereas other four species were only cited in Cameroon (i.e., *C. butyri*, *C. diversa*, *C. inconspicua*, and *C. quercitrusa*) (FIGURE 4A). The main role of *Candida* spp. during cocoa beans fermentation is the assimilation of citric acid and causing the pH value to increase in the pulp, which allows growth of bacteria (SCHWAN; WHEALS, 2004). *Candida* spp. also exhibit an antimicrobial activity and can inhibit the growth of pathogenic microorganisms (MAHAZAR et al., 2015; PEREIRA; SOCCOL; SOCCOL, 2016). Although, *C. inconspicua*, is a specie that does not contribute to ethanol production and pectinolytic activity in cocoa beans fermentation; however, it is able to assimilate lactic acid (LAGUNES GÁLVEZ et al., 2007).

FIGURE 4 - VENN DIAGRAMS CONTAINING FUNGI DIVERSITY FOUND IN COCOA FERMENTATIONS IN BRAZIL, GHANA, IVORY COAST, CAMEROON AND NICARAGUA. A) *Candida* SPECIES. B) *Pichia* SPECIES. C) OTHER FUNGI.



Source: The author (2020).

Other common yeast genus revealed was *Pichia* with nine species reported by NGS studies, including *P. angusta*, *P. barkeri*, *P. fermentans*, *P. kudriavzevii*, *P. kluyveri*, *P. manshuricai*, *P. rarassimilans*, *P. pijperi*, and *P. sporocuriosa* (TABLE 2). No *Pichia* species was common in the cocobiota of all six countries, but *P. kudriavzevii* was reported in cocoa fermentations in Brazil, Nicaragua and Colombia (FIGURE 4B). The *Pichia* genus is known for its ability to produce a wide variety of aromatic compounds, such as esters, higher alcohols and aldehydes that have a great impact on the sensory qualities of fermented cocoa beans and chocolate (KONÉ et al., 2016; SCHWAN; WHEALS, 2004). Pereira et al. (2017) reported the importance of *P. kudriavzevii* - an acid- and ethanol-tolerant specie - for flavor modulation in Brazilian cocoa fermentations. Koné et al. (2016) and Papalexandratou et al. (2019) attribute 'fruity', 'floral' and 'sweet' aromas in fermented cocoa beans when it dominates the cocoa fermentation process. In addition, *P. manshurica* and *P. kudriavzevii* have a high polygalacturonase activity and aid in pulp degradation (CRAFACK et al., 2013; JESPERSEN et al., 2005; OUATTARA; ELIAS; DUDLEY, 2020; SANCHEZ; GUIRAUD; GALZY, 1984). Santos et al. (2000) cited toxins produced by *P. kluyveri* strains against other yeast genera, mainly for strains of *Hanseniaspora uvarum*.

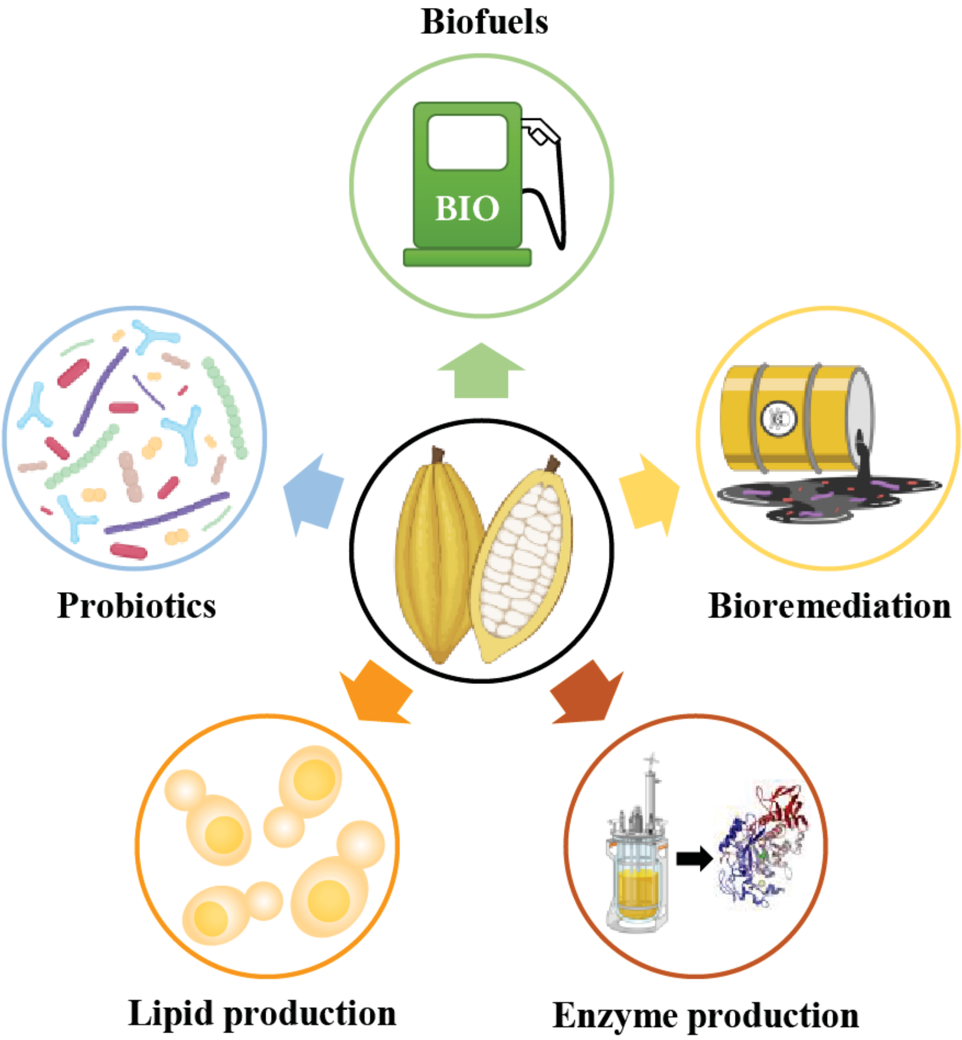
Hanseniaspora (i.e., *H. uvarum* and *H. opuntiae*) and *Saccharomyces* (i.e., *S. cerevisiae*) are yeast genera frequently found in cocoa fermentation and were reported in Brazil, Ghana, Cameroon, Nicaragua, and Colombia (FIGURE 4C). These genera are well adapted to cocoa fermentation process; *H. opuntiae* is capable to tolerate low pH and high citrate concentrations, while *S. cerevisiae* is an ethanol and heat-tolerant yeast (DANIEL et al., 2009; DE VUYST; WECKX, 2016; MEERSMAN et al., 2013). In the literature, *Hanseniaspora* spp. are mainly described as contributing to the development of the cocoa aroma. *H. uvarum* is capable to produce volatile aromatic compounds: aldehydes (e.g., methylbutanal and 2-phenyl-2-butenal) and esters (e.g., ethyl octanoate, ethyl phenylacetate, and phenylethyl acetate) (BATISTA et al., 2016). *H. opuntiae* may produces monoterpenes (e.g., citronellol, geraniol, linalool, and nerolidol) related to the fruity and floral aroma of fermented cocoa beans (SERRA et al., 2019). Similarly, *S. cerevisiae* has been reported as an important producer of desirable sensorial components during cocoa fermentation - higher alcohols (e.g., ethanol, isobutanol, and isoamyl alcohol), esters (e.g., ethyl acetate, isobutyl acetate, and isoamyl acetate) and organic acids (e.g., acetic acid and isovaleric acid) - considered as precursors of chocolate flavor (KONÉ et al., 2016).

Regarding the diversity of filamentous fungi, the most abundant genera were *Penicillium*, *Aspergillus* and *Fusarium* (TABLE 2). Four species of *Penicillium* have been reported during cocoa fermentation in Brazil, including *P. citrinum*, *P. mallochii*, *P. paxllli*, and *P. simplicissimum*. Three species of *Aspergillus* have been mentioned – *A. aculeatus*, *A. flavus*, and *A. tubingensis*. Also, three species of *Fusarium* have been detected – *F. brachygibbosum*, *F. chlamydosporum* and *F. solani* (SERRA et al., 2019). The role of filamentous fungi during cocoa fermentations has not been elucidated; however, these microorganisms may have impact by releasing pectinolytic, amylolytic, and lipolytic enzymes which could be involved in pulp degradation (SARBU; CSUTAK, 2019). Ardhana and Fleet (2003) identified a strain of *P. citrinum* with strong polygalacturonase activity. Angumeenal and Venkappayya (2013) reviewed the production of citric acid by *Penicillium* and *Aspergillus* species; therefore, this production of citric acid may contribute as source to yeast and LAB citrate-metabolism during cocoa fermentation.

3.6 COCOA FERMENTATION AS A SOURCE OF MICROORGANISMS WITH BIOTECHNOLOGICAL APPLICATION

The biotechnological production of products using microbial, plant, or animal cells is an advantageous alternative for industrial purposes, since it has a low cost, occurs under mild conditions, and reduces the generation of toxic wastes and by-products. Food, cosmetic, environmental and pharmaceutical are main fields focused on the search for alternative processes to obtain products in a more sustainable way (PESSÔA et al., 2019). Microorganisms have a wide field of exploration due to their wide range of metabolic pathways and the possibility of modifying it through metabolic engineering and synthetic biology according to the specific industrial application required (ANTRANIKIAN; VORGIAS; BERTOLDO, 2005). Considering the large and complex microbial diversity found during cocoa fermentation by NGS approaches (TABLES 1 and 2), it is expected that these genera and species may be news alternatives for functional microbial populations with biotechnological applications and commercial exploitation (FIGURE 5). In this review, we present the main biotechnological potentials of the microbial strains reported for the first time in cocoa fermentation through NGS approaches (TABLE 3).

FIGURE 5 - BIOTECNOLOGICAL APPLICATIONS OF MICROORGANISMS IDENTIFIED FOR THE FIRST TIME IN COCOA BEANS FERMENTATION BY NGS APPROACHES.



Source: The author (2020). This figure was created using biorender.com.

TABLE 3 - MICROORGANISMS IN COCOA BEANS FERMENTATION IDENTIFIED EXCLUSIVELY BY NGS STUDIES.

Microorganisms	**Biotechnological potential**	**Reference**	**Observance**
Acidiphilium cryptum	Bioremediation of environments contaminated with toxic metals	MAGNUSON et al. (2010)	--
Acidisphaera sp.	--	--	--
Acinetobacter baumanii	--	--	* Human pathogen
Acinetobacter guillouiae	--	--	* Human pathogen
Acinetobacter iwoffii	--	--	* Human pathogen
Acinetobacter radioresistens	Production of bioemulsifiers (e.g., alasan)	ABDEL-EL-HALEEM (2003)	--
Acinetobacter rhizosphaerae	--	--	--
Bacillus circulans	Production of biosurfactant lipopeptides	DAS; MUKHERJEE; SEN (2008)	--
Bacillus clausii	Probiotic activity	LOPETUSO et al. (2016)	--
Bacillus coagulans	Probiotic activity	KONURAY; ERGINKAYA (2018)	--
Brevibacillus sp.	Enzyme production	PANDA et al. (2014)	--
Burkholderia sp.	Bioremediation and production of antimicrobial molecules	DEPOORTER et al. (2016)	--
Candida aaseri	Lipase production	LEE et al. (2017)	--
Candida albicans	--	--	* Human pathogen
Candida butyri	--	--	--
Candida diversa	--	--	--
Candida ethanolitica	--	--	--
Candida glabrata	Production of biosurfactants	LUNA; SARUBBO; CAMPOS-TAKAKI (2009)	--
Candida inconspicua	Bioethanol production	ELOUTASSI et al. (2018)	--
Candida orthopsilosis	--	--	--
Candida quercitrusa	Lipase production	SARKAR; RAO (2016)	--
Candida stellimalicola	--	--	--
Candida temnochilae	--	--	--
Chlorobium sp.	Biohydrogen production	XU (2007)	--
Citrobacter sp.	Production of biosurfactants	GOMAA; EL-MEIHY (2019)	* Human pathogen
Corynebacterium variable	--	--	--

Cronobacter sakazakii	--	--	* Human pathogen
Debaryomyces hansenii	Production and accumulation of lipids	BREUER; HARMS (2006)	--
Dyella sp.	Biocontrol against pathogens in vines	IASUR-KRUH et al. (2018)	--
Enterococcus cloacae	--	--	--
Entomoplasma sp.	--	--	--
Eremothecium gossypii	Riboflavin production	AGUIAR; SILVA; DOMINGUES (2015)	--
Erwinia amylovara	--	--	--
Erwinia tasmaniensis	--	--	--
Fontibacillus pullulanolyticus	--	--	--
Frauteria aurantia	--	--	--
Fructobacillus pseudoficulneus	--	--	--
Gluconobacter diazotrophicus	Nitrogen fixation and phytohormone production	CHAWLA et al. (2014)	--
Granulibacter bethesdensis	--	--	* Human pathogen
Halomonas meridiana	Amylase production	CORONADO et al. (2000)	--
Issatchenkia sp.	Production of bioethanol and organic acids	XIAO et al. (2014)	--
Kazachstania humilis	--	--	--
Kluveromyces lactis	Production of enzymes, heterologous and recombinant proteins	SPOHNER et al. (2016)	--
Komagataeibacter hansenii	Cellulose production	LIMA et al. (2017)	--
Lachancea thermotolerans	Production volatile compounds in wine	MORATA et al. (2018)	--
Lactobacillus antri	--	--	--
Lactobacillus brevis	Probiotic activity	FANG et al. (2018)	--
Lactobacillus murinus	Probiotic activity	PERELMUTER; FRAGA; ZUNINO (2008)	--
Lactobacillus ruminis	--	--	--
Lactobacillus sakei	Probiotic activity	SIM et al. (2016)	--
Lactobacillus salivarius	Probiotic activity	CHEN; ZHU; QIU (2017)	--
Lactobacillus vaginalis	--	--	--
Leptospirillum sp.	--	--	--
Leuconostoc citreum	--	--	--
Leuconostoc gasicomitatum	--	--	--

Leuconostoc kimchii	--	--	--
Lysinibacillus halotolerans	--	--	--
Lysinobacillus boronitolerans	--	--	--
Martiniozyma asiatica	--	--	--
Methylobacterium sp.	Bioremediation and production of bioplastics	DOURADO et al. (2015)	--
Mycobacterium sp.	Bioremediation of PAHs	BOGAN; SULLIVAN (2003)	* Human pathogen
Novosphimgobium sp.	Bioremediation of naphthalene	SUZUKI; HIRAISHI (2007)	--
Oenococcus oeni	Production of β-glucosidases	MICHLMAYR et al. (2010)	--
Paenibacillus antibioticophila	--	--	--
Paenibacillus chibensis	--	--	--
Paenibacillus montaniterrae	--	--	--
Paenibacillus pabuli	Production of chitinase	JUAREZ-JIMENEZ et al. (2008)	--
Paenibacillus yunnanensis	--	--	--
Pantoea ananatis	Production of L-glutamate	HARA et al. (2012)	--
Pectobacterium carotovorum	--	--	* Phytopathogen
Pichia barkeri	--	--	--
Pichia rarassimilans	--	--	--
Pichia sporocuriosa	--	--	--
Providencia stuartii	--	--	* Human pathogen
Rhodoplanes sp.	--	--	--
Romboutsia timonensis	--	--	--
Roseomonas sp.	--	--	--
Rummeliibacillus sp.	--	--	--
Sacchasromycopsis amapa	--	--	--
Sacchasromycopsis crataegensis	--	--	--
Sacchasromycopsis synnaedendra	--	--	--
Schizosaccharomyces pombe	--	--	--
Schwanniomyces etchellsii	--	--	--
Serratia marcescens	Pigment production (prodigiosin)	LIN et al. (2019)	--

Shewanella indica	--	--	--
Sphingomonas sp.	Bioremediation of environmental contaminations	ASAF et al. (2020)(2020)	--
Starmerella bacollaris	Starter culture for the production of low alcohol beverages	LEMOS JUNIOR et al. (2019)	--
Sulfobacillus thermotolerans	--	--	--
Tatlockia micdadei	--	--	--
Trabulsiella sp.	--	--	--
Vagococcus carniphilus	Production of exopolysaccharides	JOSHI; KANEKAR (2011)	--
Vanderwaltozyma polyspora	--	--	--
Vibrio fluvialis	--	--	* Human pathogen
Weisella paramesenteroides	Bacteriocin production	FUSCO et al. (2015)	--
Wickerhamomyces pijperi	Aromatic	IZAWA et al. (2015)	--
Xanthomonas massilliensis	--	--	--
Zygosaccharomyces bailii	Aromatic	XU et al. (2017)	--
Zygosaccharomyces rouxii	D-arabitol production	SAHA; SAKAKIBARA; COTTA (2007)	--
Zymomonas mobilis	Bioethanol production	PENTJUSS et al. (2013)	--

Source: The author (2020).

3.6.1. Probiotics

In the literature, probiotics are described as viable microorganisms that when ingested in appropriated concentrations confers health benefits to the host (PEREIRA et al., 2018). The production of functional foods is an on-growing segment of the food industry with an estimated expansion of the market from $32.06 billion in 2013 to $73.8 billion in 2024 (ZUCKO et al., 2020). Thus, the search for new microbial strains from unusual sources, such as fermented foods and beverages, with probiotic characteristics have become the focus of several researches (CHEN; ZHU; QIU, 2017; FANG et al., 2018; PERELMUTER; FRAGA; ZUNINO, 2008). *Lactobacillus* is the major genus among the LAB group and the earliest with probiotic potential identified (PEREIRA et al. 2018). It is known that *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. acidophilus*, *L. crispatus*, *L. delbrueckii*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. plantarum*, *L. pentosus*, *L. curvatus*, *L. buchneri*, *L. fermentum*, and *L. reuteri* have been used in the industry as probiotic supplements in foods and feeds (ZOTTA; PARENTE; RICCIARDI, 2017). Some of these species have also been detected in the fermentation of cocoa beans by NGS studies (TABLE 1 and FIGURE 3A), therefore, this process can be considered as a source of prospecting for microorganisms with probiotic activity, mainly in Brazil and Ghana (FIGURE 3A). Additionally, strains of *L. plantarum* isolated from Brazilian cocoa fermentation have been considered as suitable candidates for use as probiotics by Ramos et al. (2013) and Teles Santos (2016). Similarly, *L. fermentum* TCUESC01, derived from fermenting cocoa, has been demonstrated a great potential as a safe probiotic food additive (MELO et al., 2017). The NGS studies have also been reveled other LAB species in cocoa fermentation with probiotic potential, such as *L. brevis*, *L. murinus*, *L. sakei*, and *L. salivarus*, which exhibits reported antimicrobial activities and immunoregulatory activities anti-obesity (FANG et al., 2018; HUANG et al., 2016; JI et al., 2012; MESSAOUDI et al., 2013).

Besides LAB group, aerobic spore-forming bacteria, mainly *Bacillus*, are also good candidates for probiotics due their high acid and osmotic tolerance (ELSHAGHABEE et al., 2017). The use of spore-forming bacteria as probiotic includes some advantages compared to the use of lactobacilli, since the products containing these bacteria can be preserved in dry forms at room temperature without any negative effects on the survival of spores (ADIBPOUR et al., 2019). The *Bacillus* is a genus frequently detected as part of the naturally microbial diversity present in cocoa fermentation by NGS studies, as shown in Table 1. Several *Bacillus* spp. has been showed probiotic activity such as *B. coagulans*, *B. subtilis*, *B. indicus*, *B. clausii*, and *B. licheniformis* (ELSHAGHABEE et al., 2017). *B. clausii* and *B. coagulans*, reported for the first time in cocoa fermentation by NGS studies (TABLE 3), are capable to prevent diarrhea and have an inhibitory effect against pathogenic microorganisms tested (e.g., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) (KONURAY; ERGINKAYA, 2018; URDACI; BRESSOLLIER; PINCHUK, 2004). To date, there are no studies on the prospection of *Bacillus* spp. derived from cocoa fermentation with probiotic potential.

3.6.2. Bioremediation

The indiscriminate use of fossil fuels is a major concern not only for its contribution to climate change, but also for representing a direct threat to the human and animals' health, and contamination of environments as resulted from the crude oil and refined fuels spill (ADAMS et al., 2015). For instance, a reliable solution is the use of microorganisms, mainly bacteria and yeasts, for the production of biofuels and for the remediation of contaminated areas (PESSÔA et al., 2019). Table 3 shows the potential of cocoa beans fermentation as a source of the microorganisms with biotechnological application to the use in the remediation of contaminated environments (with PAHs and toxic metals) and in the production of biofuels (bioethanol and biohydrogen).

Polycyclic aromatic hydrocarbons (PAHs) are an extensive group of chemical compounds derived from the incomplete combustion or pyrolysis of numerous organic materials (e.g., coal, oil, petroleum gas, and wood) with a widespread presence in the environment, showing the potential to bio-accumulate, in addition, mutagenic and carcinogenic effects (KADRI et al., 2017). To date, there are no studies that report the isolation and application of microorganisms retrieved from cocoa beans fermentation for use in the bioremediation of contaminated environments. However, some genera of bacteria detected for the first time in cocoa fermentation by NGS studies, such as *Methylobacterium*, *Mycobacterium*, and *Novosphimgobium* (TABLE 3) were described as having a unique metabolism that enables the use of PAH as carbon source under aerobic conditions (ZHANG et al., 2006). Other studies demonstrate that these microorganisms, in single culture or consortium, were able to degrade chemical compounds such as methyl-tert-butyl ether, trichloroethylene, phenanthrene, and pyrene from contaminated soils (DOURADO et al., 2015; LYU et al., 2014; SUN et al., 2019).

Another alternative for the bioremediation of PAH-contaminated environments is the use of microorganisms that produce biosurfactants and bioemulsifiers (KADRI et al., 2017; SATPUTE et al., 2010). These molecules are composed by two parts: a hydrophilic – formed by organic acids, peptides, and polysaccharides – and a hydrophobic – composed by saturated or unsaturated hydrocarbon chains. This singular structure allows the reduction of the surface and interfacial tension and the stabilization of the oil-water mix {Formatting Citation}. Likewise, to date, there are no studies about the isolation and application of microorganisms recovered from the fermentation of cocoa beans with capacity to producing biosurfactants and/or bioemulsifiers. Although, the use of biosurfactants and bioemulsifiers produced by microorganisms detected for the first time in cocoa beans fermentation by NGS techniques, such as *Acinetobacter radioresistens*, *B. circulans*, *C. glabrata*, and *Citrobacter* sp., have been proposed for the oil recovery and removal of hydrocarbon-contaminants (AL-BAHRY et al., 2013; GUSMÃO; RUFINO; SARUBBO, 2010; IBRAHIM, 2018; LUNA; SARUBBO; CAMPOS-TAKAKI, 2009; MUJUMDAR; JOSHI; KARVE, 2019). Therefore, the fermentation process of cocoa beans should be considered a good source capable of discovering new strains with remediation abilities. Moreover, these biomolecules can also be added in detergent, cosmetic formulations, biopesticides, and biodispersants (PESSÔA et al., 2019).

3.6.3 Biofuels

Biofuels are alternative fuels based on the processing of renewable organic biomass, mainly of vegetable origin (e.g., molasses, husks, corn, and wood wastes), including bioethanol, biodiesel, biohydrogen and biogas, produced by bio-chemical pathways such as alcoholic fermentation, anaerobic fermentation and trans-esterification reaction (ARSHAD; ZIA; SHAH, 2018). In recent decades, several studies have investigated the production of biofuels from different organic biomasses through the action of yeasts, bacteria or microalgae (ALFENORE; MOLINA-JOUVE, 2016).

Microorganisms with biotechnological potential for the production of biofuels have also been identified by NGS studies in the cocoa fermentation process (TABLE 3). This potential has already been observed by Pereira et al. (2014a), when verifying the capacity of four strains of *Kluyveromyces marxianus* isolated from cocoa fermentations in Brazil for fermentation of lignocellulosic biomass and bioethanol generation.

In this context, cocoa beans fermentation can be a potential source of discovered of new strains with industrial application in the production of biofuels. Among the microorganisms identified exclusively by NGS during cocoa fermentation (TABLE 3), *C. inconspicua*, *Issatchenkia* sp., and *Zymomonas mobilis* are considered potential starters for the production of bioethanol (ELOUTASSI et al., 2018; LU et al., 2014; PENTJUSS et al., 2013). The facultative anaerobe bacteria *Z. mobilis* has been used as an alternative inoculum for the bioethanol production from mono- and disaccharides: sugarcane broth and saccharified lignocellulosic biomass (PINILLA; TORRES; ORTIZ, 2011; YANASE et al., 2012). On the order hand, *Chlorobium* sp. has been employed for biohydrogen generation from organic wastes (DINESH; CHAUHAN; CHAKMA, 2018).

3.6.4. Oleaginous microorganisms

Several species of yeasts and bacteria are able to synthesize and accumulate lipids in more than 20% of their cell dry weight, regarded as the oleaginous microorganisms (XUE et al., 2018). The oleaginous yeasts and bacteria are the main microorganisms used for "2nd generation biodiesel production (PATEL et al., 2020). As a biofuel, the biodiesel has advantages over other refined fuels (i) it is biodegradable, industrially viable and (ii) causes less environmental contamination and gas emissions (XUE et al., 2018). Yeasts belonging to genera *Candida*, *Rhodosporidium*, *Yarrowia*, *Cryptococcus*, *Rhodotorula*, *Lipomyces*, and *Trichosporon* have been reported as good candidates for biodiesel production, some of which can accumulate lipids up to 80% w/w of their dry cell weight (PATEL et al., 2020). The NGS studies have been revealed the potential of cocoa beans fermentation as a source of oleaginous yeasts (e.g., *Candida* and *Rhodotorula*) (TABLES 2 and 3). In addition, another specie found in cocoa fermentation, i.e. *Debaryomyces hansenii*, has been reported with many biotechnological applications, including its use in the food fermentation (e.g., cheeses and meats), and production of fine chemicals, lytic enzymes, and alditols (BREUER; HARMS, 2006).

3.6.5. Enzyme production

The enzyme-mediated processes have gained attention over usual chemical processes due to the reduction of reaction time, inexistence of toxicity, and low energy input (SINGH; SINGH; PANDEY, 2019). To date, over 500 industrial products uses enzymatic processes and the global market is estimated in over $6.2 billion (ADRIO; DEMAIN, 2014; SINGH et al., 2016). Microorganisms are the main source of enzymes due to its vast range of metabolic pathways and easiness to produce and purify (PESSÔA et al., 2019). Therefore, as the fermentation of cocoa beans has a naturally complex microbial diversity, future research should be focused on the identification of new microorganisms, strains and enzymes-derived that can be used in biotechnological processes.

For instance, lipases are responsible for the hydrolysis of the ester bonds between alcohol and carboxylic acid, being widely used as biocatalysts in the biodiesel production (SHARMA; SHARMA; SHUKLA, 2011). In cocoa beans fermentation, microorganisms previously reported as lipase-producers have been identified by NGS approaches, such as *C. aaseri*, *C. quercitrusa*, and *Brevibacillus* sp. (TABLE 3), which have already been reported in the literature as lipolytic microorganisms (LEE et al., 2017; PANDA et al., 2016; SARKAR; RAO, 2016).

Halomonas meridiana was characterized by its high production of extracellular α-amylases in liquid and solid media (CORONADO et al., 2000), being detected only by NGS studies during Brazilian cocoa fermentation (TABLES 1 and 3). α-Amylases are enzymes that hydrolyze the starch molecules into polymers composed of glucose and maltose units (SUNDARRAM; MURTHY, 2014). Besides, the amylases produced by microorganisms are more stable, with an economical bulk production, and have potential application in a vast number of industrial processes, mainly in the food, textile, paper, detergent and pharmaceutical industries (SOUZA; OLIVEIRA E MAGALHÃES, 2010).

Interestingly, *Paenibacillus pabuli* is other specie identified only by NGS technique in cocoa beans fermentation with biotechnological potential (TABLE 3). It is known by high production of chitinolytic enzymes (chitinases), which hydrolyses chitin, a structural polysaccharide mainly found in the exoskeleton of insects, filamentous fungi, yeast, and algae (JUAREZ-JIMENEZ et al., 2008). The chitinases are used mainly in agriculture fields to control pathogens (biocontrol), pharmaceuticals products, and in the treatment of chitinous waste (HAMID et al., 2013).

3.7 CONCLUSIONS

The popularization of NGS technology has allowed an in-depth analysis of the complex microbial communities in cocoa fermentations across the globe. Among them, 454 Pyrosequencing and Illumina platforms have been the most popular techniques used to monitor the microbial succession, enabled the discovery of dominant, non-culturable, and low-prevalence microorganisms. *Acetobacter*, *Lactobacillus*, *Weissella*, *Candida*, *Pichia*, and *Saccharomyces* were the main genera commonly reported in cocoa beans fermentation in all regions, demonstrating to be well adapted to the cocoa ecosystem regardless of origin and playing important roles in the fermentation process. Besides, a vast diversity of bacteria and fungi has been reported for the first time by NGS studies associated with cocoa beans fermentation (e.g., *Acinetobacter radioresistens*, *Bacillus coagulans*, *Candida inconspicua*, *Debaryomyces hansenii*, and *Paenibacillus pabuli*).

The discovery of new taxa evidences the potential of cocoa beans fermentation as a source for prospecting microorganisms with several biotechnological applications (e.g., bioremediation, enzyme production, lipid accumulation, probiotic activity, and biofuels production). This review opens new perspectives for research on microbial diversity in cocoa fermentation. Furthermore, other omics approaches (proteomics, transcriptomics, and metabolomics) should be further explored in current NGS studies to confirm the functions and metabolic capacity of microbiomes present in cocoa bean fermentations and which may impact the quality of fermented beans, and later, in chocolate.

4 CHAPTER II (RESEARCH RESULTS) - EXPLORING THE CONTRIBUTION OF FRUCTOPHILIC LACTIC ACID BACTERIA TO COCOA BEANS FERMENTATION: ISOLATION, SELECTION AND EVALUATION

Manuscript published in the *Food Research International*, volume 136, October 2020, article 109478 (DOI: 10.1016/j.foodres.2020.109478).

ABSTRACT

Fructophilic lactic acid bacteria (FLAB) are a recently discovered group whose main characteristic is to prefer D-fructose over D-glucose. In this study, laboratory cocoa beans fermentation was analyzed by Illumina-based amplicon sequencing, indicating the presence of potential FLAB of the genera *Fructobacillus* and *Lactobacillus*. Eighty efficient fructose-fermenting isolates, obtained from fermenting cocoa pulp beans mass, were identified by 16S rRNA gene sequencing as *Pediococcus acidilactici* (n = 52), *Lactobacillus plantarum* (n = 10), *Pediococcus pentosaceus* (n = 10), *Bacillus subtilis* (n = 4), and *Leuconostoc pseudomesenteroides* (n = 4). The growth characteristics of all the 10 *L. plantarum* strains classified them as "facultatively" fructophilic bacteria, *i.e.*, they grew on glucose without an external electron acceptor but the growth on fructose was faster. Among them, *L. plantarum* LPBF35 was characterized by producing a range of aroma-impacting compounds (acetaldehyde, ethyl acetate, nonanal, and octanoic acid), being introduced into a cocoa fermentation process. Although the process started with approximately equal amounts of glucose and fructose, a concomitant, but faster utilization of fructose, was observed in cocoa fermentation conducted with *L. plantarum* LPBF35 (with no residual fructose observed) when compared to control fermentation using a glucophilic strain (8.77 mg/g residual fructose) and a spontaneous process (8.38 mg/g residual fructose). *L. plantarum* LPBF 35 also showed an ideal profile of organic acid metabolism (citric acid consumption and lactic acid production) associated with cocoa fermentation. These results proved new insights on cocoa microbial activity and brings new perspectives on the use of lactic acid bacteria as starter culture.

Keywords: Cocoa processing. *L. plantarum*. Fructose. Cocoa fermentation.

4.1 INTRODUCTION

Cocoa is the fruit of the *Theobroma cacao* L., a perennial tree native to the South American tropical region. Cocoa bean, the raw material for chocolate production, is composed of two cotyledons and an embryo enclosed by a seed coat, enveloped in a sweet and white mucilaginous pulp (FIGUEROA-HERNÁNDEZ et al., 2019). When cocoa is ripe, the pulp has a higher proportion of fructose and glucose; in addition to pectin and other polysaccharides, proteins, amino acids, minerals, vitamins and citric acid (CAMU et al., 2008b; DE VUYST; WECKX, 2016; LIMA et al., 2011). Once the cocoa beans are removed from the pod, the pulp is degraded by a natural fermentation process, which is mainly conducted by yeast, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). During fermentation, several metabolites are produced by microbial activity and diffused into seeds (CAMU et al., 2007; SCHWAN; WHEALS, 2004). This leads to a number of biochemical transformation within the seeds, reducing the bitter taste and astringency and, finally, killing the embryo to avoid its germination (KADOW et al., 2013, 2015). Thus, cocoa beans fermentation is considered to be the postharvest treatment stage that most influences the quality of chocolate.

The presence of LAB in the cocoa microbial consortium is associated with the anaerobic fermentation of simple sugars in the early stages of the process (0–48 h) and production of lactic acid, diacetyl, acetoin, 2,3-butanediol, and other minor metabolites (ADLER et al., 2013; CAMU et al., 2007, 2008b; CASTRO-ALAYO et al., 2019; FIGUEROA-HERNÁNDEZ et al., 2019; LEFEBER et al., 2010, 2011a). The LAB species most frequently reported include the genera *Lactobacillus* and *Leuconostoc* (CAMU et al., 2007; FIGUEROA-HERNÁNDEZ et al., 2019; LAGUNES GÁLVEZ et al., 2007; MEERSMAN et al., 2013; OUATTARA et al., 2017; SERRA et al., 2019). Some attempts on designing LAB starter culture have been addressed for cocoa beans fermentation process (KRESNOWATI; SURYANI; AFFIFAH, 2013; LEFEBER et al., 2010, 2011b). Based on knowledge of bacterial metabolism and physiology (lactic acid and volatile organic molecules production, citrate consumption, and heat and ethanol tolerance), two LAB species, *Lactobacillus fermentum* and *Lactobacillus plantarum*, can be considered good candidates for starter culture (ADLER et al., 2013; CAMU et al., 2007; DE VUYST; WECKX, 2016; FIGUEROA-HERNÁNDEZ et al., 2019; KRESNOWATI; SURYANI; AFFIFAH, 2013; LEFEBER et al., 2010, 2011a). These species have been used in conjunction with some yeasts (*Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Pichia kluyveri*, and *Kluyveromyces marxianus*) and AAB (*Acetobacter tropicalis*, *A. pasteurianus* and *A. aceti*) to compose complex microbial starter cultures (BATISTA et al., 2015; CRAFACK et al., 2013; LEFEBER et al., 2010, 2012; PEREIRA et al., 2012; SANDHYA et al., 2016; VISINTIN et al., 2017).

During cocoa beans fermentation, the two main soluble pulp sugars, glucose and fructose, are co-fermented to ethanol (yeast metabolism) and lactic acid (LAB metabolism), as well as other minor but important metabolites (CAMU et al., 2008a; NIELSEN et al., 2007; SCHWAN; WHEALS, 2004). Although fructose is consumed concomitantly with glucose, the latter is depleted first, which gives rise to a discrepancy between the amount of glucose and fructose consumed during cocoa beans fermentation (ARDHANA; FLEET, 2003; CAMU et al., 2008b; KRESNOWATI; SURYANI; AFFIFAH, 2013; LAGUNES GÁLVEZ et al., 2007; LIMA et al., 2011; NIELSEN et al., 2007; PAPALEXANDRATOU et al., 2011c; PEREIRA et al., 2012). Fructose may, thus, be one of the causes of long periods of cocoa beans fermentation, lasting up to 7 days (LIMA et al., 2011). Other factors include the spontaneous nature of the process and the complete metabolism cycle of the three major microbial groups: sugar-to-lactic acid (LAB), sugar-to-ethanol (yeast), and ethanol-to-acetic acid (AAB) (DE VUYST; WECKX, 2016; LIMA et al., 2011; NIELSEN et al., 2007; SCHWAN; WHEALS, 2004). In addition, the residual fructose can be metabolized by undesirable fungi and spoilage bacteria that proliferate when the cocoa bean fermentation actually comes to a finish (MOENS; LEFEBER; DE VUYST, 2014). This overfermentation process favors unwanted production of microbial compounds, especially C3–C5 free fatty acids and extracellular proteases and lipases that might have the potential to access and degrade bean proteins and lipids. The late growth of toxigenic fungi also be- comes a significant public health risk due to the production of myco- toxins, especially ochratoxin A and aflatoxin (SCHWAN; PEREIRA; FLEET, 2015). Whether a causal relation between cocoa fermentation and fructose metabolization exists, it remains unclear.

Although LAB usually have a glucophilic metabolism, recently, a new group called fructophilic lactic acid bacteria (FLAB) has been described by Endo and Okada (2008). It includes all species of the genus *Fructobacillus* and some *Lactobacillus* (*L. kunkeei*, *L. brevis*, *L. apinorum*, *L. florum* and *L. plantarum*) for presenting limited or delayed growth on glucose when compared to fructose (ENDO et al., 2012; ENDO; OKADA, 2008; GUSTAW et al., 2018; MAENO et al., 2017; NEVELING; ENDO; DICKS, 2012). These microorganisms – FLAB – possess an incomplete gene encoding a bifunctional alcohol/acetaldehyde dehydrogenase requiring, thus, additional electrons acceptors (oxygen, fructose or pyruvate) to metabolize glucose (ENDO et al., 2018). FLAB are found in fructose-rich niches, such as fruits, flowers, fermented foods and in the gastrointestinal tracts of animals consuming fructose (ENDO et al., 2018; ENDO; FUTAGAWA-ENDO; DICKS, 2009; ENDO; SALMINEN, 2013). Papalexandratou et al. (2011a) first reported the presence of *Fructobacillus* sp. by culture-independent approach (PCR-DGGE) during the initial phase of cocoa beans fermentation. Then, *Fructobacillus tropaeoli* was found in Ecuadorian cocoa beans fermentation (PAPALEXANDRATOU et al., 2011b) and *F. pseudofilculneus* occurring occasionally in cocoa fermentation in vessels (LEFEBER et al., 2011a). However, the role of FLAB during cocoa fermentation is not understood. Although, *L. plantarum* is a dominant LAB in cocoa beans fermentation, has not been here characterized for its fructophilic metabolism in this environment.

The aims of this work were to: (i) study the presence and diversity of FLAB in laboratory cocoa fermentation, (ii) isolate and characterize FLAB from fermenting material, and (iii) select and evaluate FLAB potential as cocoa starter cultures, with the aim of improving fructose consumption during fermentation process.

4.2 MATERIALS AND METHODS

4.2.1. Laboratory-scale cocoa fermentation and sampling

A 6-day, laboratory-scale, spontaneous cocoa bean fermentation was performed, in triplicate, based on previous works (PEREIRA et al., 2012; ROMANENS et al., 2018). Mature cocoa pods (*Theobroma cacao* L. var. Forastero) were harvested from a cacao farm located in the municipality of Ilhéus (S14°48′17″ W39°08′20″), Bahia State, Brazil, and transported within 1 to 3 days to the Bioprocess Engineering and Bio- technology Laboratory, Federal University of Paraná, Curitiba, Brazil. The pods were manually opened, and 1.8 kg of the cocoa pulp-bean transferred into a 25 cm × 16 cm × 7 cm polypropylene fermentation box containing holes in the bottom to allow drainage of the sweating. The fermentation box was maintained in a laboratory incubator Quimis 0316 M2 (Diadema, São Paulo, Brazil) for 6 days, and the temperature was adjusted every 12 h simulating on-farm process: 28 °C at 0 h, 30 °C at 12 h, 32 °C at 24 h, 35 °C at 36 h, 38 °C at 48 h, 42 °C at 60 h, 46 °C at 72 h, and 48 °C at 84 h, 96 h, 108 h and 120 h

(PEREIRA et al., 2012). In the first 48 h, the fermentation box was kept capped with a lid to create microaerophilic conditions and favor the growth of yeasts and LAB. After this period, the lid was removed, and the fermenting material was revolved every 24 h by manual kneading to promote the development of AAB and allow efficient removal of the pulp.

Ten grams of cocoa beans with adhered pulp were randomly collected every 24 h (0, 24, 48, 72, 96 and 120 h) to perform culture-dependent microbiological analyses immediately after sampling and were posteriorly stored in a freezer at −20 °C for carrying out culture- independent approaches.

4.2.2 Study on the presence of fructophilic lactic acid bacteria (FLAB) by culture-independent approach

Fermenting cocoa pulp bean mass samples from 0, 48 and 72 h were used to assess the presence of FLAB during laboratory-scale cocoa fer mentation using Illumina high-throughput sequencing. The pulp fraction was recovered by decanting after mixing 5 mL of sterile 0.1% (w/v) peptone water with 5 g of the cocoa beans and adhering pulp. One milliliter of cocoa pulp fraction was centrifuged at 12,000 ×g for 1 min (Eppendorf, Hamburg, Germany). Cell pellet was resuspended in 500 μL of Tris-EDTA, and extraction of total DNA was performed according to (CARVALHO NETO et al., 2018). The total DNA obtained previously was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). A total of 20 ng of DNA was used as a template for the amplification of the V4 region of the 16S rRNA gene, using the primers 515F and 806R (CAPORASO et al., 2012) with KlenTaq Master Mix (Sigma-Aldrich, Saint Louis, MO, USA). The PCR products were quantified using the Qubit dsDNA HS kit (Invitrogen, Carlsbad, CA, USA) and sequenced using the v.2 (500 cycles) Sequencing Kit (Illumina, San Diego, CA, USA) on an Illumina MiSeq (Illumina, San Diego, CA, USA) platform. After sequencing, chimeric sequences detection, removal of noises from pre-cluster and taxonomic assignment were done using standard parameters with the QIIME (Quantitative Insights Into Microbial Ecology) software package, version 1.9.0 (http://qiime.org/). Sequences with a similarity above 97% were assigned to the same operational taxonomic units (OTUs) using the SILVA database (https://www.arb-silva.de/) (QUAST et al., 2013).

4.2.3. Isolation of presumptive FLAB

Two approaches, adapted from Endo et al. (2009), were used for the isolation of presumptive FLAB from cocoa beans fermentation. Forty grams of cocoa beans and adhering pulp from samples at 0, 24, 48, 72, 96 and 120 h were deposited into Erlenmeyer flasks containing 100 mL of FYP broth (10 g/L D-fructose, 10 g/L yeast extract, 5 g/L peptone, 2 g/L sodium acetate, 0.5 g/L Tween 80, 0.2 g/L $MgSO4 \cdot 7H2O$, 0.01 g/L $MnSO4 \cdot 4H2O$, 0.01 g/L $FeSO4 \cdot 7H2O$, 0.01 g/L NaCl, 0.05 g/L sodium azide, and 0.2% [v/v] nystatin [pH 6.8]) and incubated at 30 °C and 120 rpm for 24 h. After this incubation, aliquots of 1 mL were homogenized with 9 mL of 0.1% (w/v) peptone water (10^{-1} solution) and diluted serially. Then, 100 µL of each dilution were inoculated on the surface of FYP agar medium supplemented with 0.5% (w/v) $CaCO_3$ to acid production indication. Plates were incubated at 30 °C for 24–48 h, and the number of CFU was recorded, following morphological characterization and counts of each colony type obtained. The colonies that showed a clearance zone surrounding, indicating hydrolysis of $CaCO_3$, were purified on FYP agar, and stored at −80 °C in FYP broth containing 10% (v/v) glycerol.

The second approach consisted of an initial selective pressure, where each fermentation sample (0, 24, 48, 72, 96 and 120 h) was cultivated in FYP broth containing a high concentration of fructose (300 g/L D-fructose). The conditions of plating, isolation and purification of presumptive FLAB were performed as previously described for FYP broth without fructose supplementation.

4.2.4. Screening of carbohydrates consumption

The presumptive FLAB were selected for their efficiency in consuming glucose and fructose. Initially, the isolates were cultured on FYP agar at 30 °C for 24 h. Inoculum were prepared with the bacterial cells suspended in 0.1% (w/v) peptone water and adjusted according to the McFarland 2.0 scale (6.0 × 108 cells/mL). One milliliter of this suspension was inoculated in 9 mL adapted API 50 CHL medium (10 g/L D- glucose, 10 g/L peptone, 5 g/L yeast extract, 5 g/L sodium acetate, 2 g/ L dipotassium phosphate, 2 g/L diammonium citrate, 0.2 g/L magnesium sulfate, 0.17 g/L bromocresol purple, 0.05 g/L manganese sulfate and 1 mL/L Tween 80; pH 6.8), in triplicate, and incubated at 30 °C for 96 h. The glucose-fermenting microorganisms were evaluated by the positive reaction of the color change from medium to yellow and gas production observed with Durham tubes.

Different profiles of isolates were screened (fast or slow glucose fermentation and homofermentative or heterofermentative) by fermentative capacity in medium containing fructose as the sole carbon source. Inoculum was prepared with the bacterial cells suspended in 0.1% (w/v) peptone water and adjusted according to the McFarland 0.5 scale (1.5 × 108 cells/mL). Then, one milliliter of this suspension was inoculated in 9 mL FYP broth, in triplicate, and incubated at 30 °C for 36 h. Differences in growth among the strains were monitored by recording optical density readings at 600 nm. Fructose consumption was evaluated by the colorimetric method for the determination of reducing sugars using 3,5-dinitrosalicylic acid (DNS) described by Miller (1959). After 36 h of fermentation process, the fermented obtained was stored at −20 °C for further analysis of aroma production by gas chromatography (item 4.2.8).

4.2.5 Identification of presumptive FLAB

Eighty efficient fructose-consumers isolates were selected for molecular identification by sequence analysis of the partial 16S rRNA gene. Genomic DNA was extracted from the presumptive FLAB cultured in 10 mL FYP broth at 30 °C for 24 h using phenol/chloroform method adapted by Cheng and Jiang (2006), where cell lysis occurs directly by phenol. The primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGCTACCTTGTTACGACTT-3′) were used to amplify the 16S rRNA gene region (LANE et al., 1985) in a Veriti thermal cycler (Applied Biosystems, Paisley, UK). Amplifications were performed in a final volume of 25 µL containing 5 µL of 5x GoTaq® reaction buffer supplied with $MgCl_2$ (7.5 mM) (Promega, Madison, WI, USA), 0.55 µL of dNTP Mix (10 mM) (Invitrogen, Carlsbad, CA, USA), 0.5 µL of 27F and 1492R primers (10 mM), and 0.2 µL of GoTaq® DNA Polymerase (5U/µL) (Promega). Amplicons were generated by PCR under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles at 93 °C for 60 s, annealing at 50 °C for 60 s, extension at 72 °C for 90 s, and final extension at 72 °C for 5 min. The PCR products were sequenced by automated capillary electrophoresis on ABI 3730xl DNA analyzer (Applied Biosystems, Paisley, UK). The sequences obtained were aligned using the BioEdit 7.7 sequence alignment editor and compared to the GenBank database. The searches were performed to determine the closest known relatives of the partial ribosomal

DNA sequences obtained using the BLAST algorithm - NCBI (National Center for Biotechnology Information, MA, USA).

A phylogenetic tree was constructed based on 16S rRNA gene sequences of previously 28 reference isolates compared to 16S rRNA sequences retrieved from NCBI database. Multiple alignments were performed using the online version of MAFFT program version 7 with the option Auto (FFT-NS-1, FFT-NS-2, FFT-NS-I, or L-INS-i). A neighbor-joining phylogenetic tree was constructed using the MEGA X version 10.1 program (KUMAR et al., 2018) based on the MSA file obtained in MAFFT. The evolutionary distances were computed by Maximum Composite Likelihood method (CAVALLI-SFORZA; EDWARDS, 1967) and Maximum-Parsimony (KLUGE; FARRIS, 1969). The robustness of individual branches was estimated by bootstrapping with 1000 replicates (FELSENTEIN, 1985).

4.2.6 Fructophilic properties of the isolates

The 80 isolates identified by 16S rRNA gene sequencing, encompassing *Pediococcus acidilactici* (n = 52), *Ped. pentosaceus* (n = 10), *L. plantarum* (n = 10), *Bacillus subtilis* (n = 4), and *Leuconostoc pseudomesenteroides* (n = 4), were inoculated in different broths to investigate their fructophilic properties using the method of Gustaw et al. (2018). After 24 h in aerobic incubation at 30 °C, bacterial cultures were centrifuged and removed from the FYP broth. The bacterial cells were resuspended in 0.1% (w/v) peptone water, and the same optical density of 0.5 was set at 600 nm. Growth characteristics of the strain were determined in three different media: FYP, GYP (containing 10 g/L of D-glucose instead of D-fructose) and GYP-P supplemented with 0.5% pyruvate. Two hundred microliters of each medium were transferred to microplates, in triplicate, and the wells were inoculated with 30 µL of the bacterial suspension. The experiment was performed under aerobic and anaerobic conditions by measuring the optical density at 600 nm every 2 h for 24 h. Anaerobic conditions were obtained by cutting off access to oxygen with a few drops of liquid vaseline.

4.2.7 Rep-PCR analysis and computer-assisted analysis of genomic fingerprint

Ten *L. plantarum* fructophilic strains were typed at strain level using repetitive extragenic palindromic (rep)-PCR technique using a $(GTG)_5$ primer (5′-GTGGTGGTG

GTGGTG-3′) (VERSALOVIC et al., 1994). The amplifications were performed following the conditions proposed by Pereira et al. (2017). Amplicons were loaded and separated by electrophoresis in 1.8% (w/v) agarose gels using DNA markers GeneRuler 50 bp DNA Ladder (Invitrogen, Carlsbad, CA, USA) as a reference. Then, the PCR products were stained with ethidium bromide (10 mg/mL) and scanned using a Fluoro Image Analyzer FLA-5000 (Fuji Photo Film Co.).

The genomic fingerprints obtained were converted to a two-dimensional binary matrix (1 = presence of a band; 0 = absence of a band). Dice (Sorensen) coefficient was used to calculate the similarity matrices using the unweighted pair group method with the arithmetic averages clustering algorithm (UPGMA). Computer-assisted analysis was performed with SYSTAT® version 10.0 program for Windows.

4.2.8 Selection of aroma-producing isolates

The ten *L. plantarum* fructophilic strains were selected for their capacity to produce volatile aroma compounds in FYP medium. The extraction of volatile compounds was performed using a headspace (HS) vial coupled to a SPME fiber (CAR/PDMS df75 µm partially crosslinked, Supelco, Saint Louis, MO, USA). For each determination, 2 mL of sample was stored in a 20 mL HS vial, in duplicate. The SPME fiber was exposed for 30 min at 60 °C. The compounds were thermally desorbed into the gas chromatograph injection system gas phase (CGMS TQ Series 8040 and a 2010 Plus GC–MS; Shimadzu, Tokyo, Japan) at 260 °C. The column oven temperature was maintained at 60 °C during 10 min, followed by two heating ramps of 4 and 10 °C/min until reaching the temperatures of 100 and 200 °C, respectively. The compounds were separated on a column 95% PDMS/5% PHENYL (30 m × 0.25 mm × 0.25 mm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). The compounds were identified by comparison to the mass spectra from library databases (Nist'98 and Wiley7n).

4.2.9 Performance of selected FLAB in cocoa beans fermentation

The selected aroma-producing FLAB, *L. plantarum* LPBF35, was introduced into a cocoa fermentation process to evaluate the efficiency of fructose consumption. A glucophilic strain, *P. acidilactici* LPBF66, and a spontaneous cocoa fermentation were used as positive and negative controls, respectively. The frozen strains were initially reactivated from −80 °C to FYP (fructophilic strain) and MRS (glucophilic strain) broths for 48 h at 30 °C. Afterwards, the strains were transferred to 500 mL of FYP and MRS broths, respectively. After incubation, the cells were centrifuged at 7.000 xg for 10 min and washed twice with a buffer phosphate pH 7.0 to remove any culture medium residue.

Laboratory-scale cocoa beans fermentations (as previously described) were conducted with the inoculation of *L. plantarum* LPBF35 and *Ped. acidilactici* LPBF 66. A spontaneous process was carried out as a negative control. The initial bacterial cell concentration was adjusted to 1.5×10^9 cells/mL (O.D. = 0.1). Fermentations were carried out in triplicate at 30 °C for 72 h. The difference in glucose and fructose consumption, and lactic acid production between the three treatments were performed by High-performance Liquid Chromatograph (HPLC). Filtered samples collected from 0, 12, 24, 36, 48, 60 and 72 h were injected into HPLC system equipped with an Aminex HPX 87H column (300 × 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector (HPG1362A; Hewlett-Packard Company, Palo Alto, CA, USA). The column was eluted in an isocratic mode with a mobile phase of 5 mM H_2SO_4 at 60 °C and a flow rate of 0.6 mL/min.

4.2.10 Statistical analysis

The data obtained by the target metabolites analysis were made by post-hoc comparison of means by the Duncan's test. The inoculation efficiency of *L. plantarum* LPBF35 and *Ped. acidilactici* LPBF66 after 72 h of fermentation was analyzed using One-way Analysis of Variance (ANOVA), followed by Dunnett's test. Statistical analyses were done using STATISTICA 7 StatSoft software (STATSOFT, 2007). The level of significance was established using a p-value < 0.05.

4.3 RESULTS AND DISCUSSION

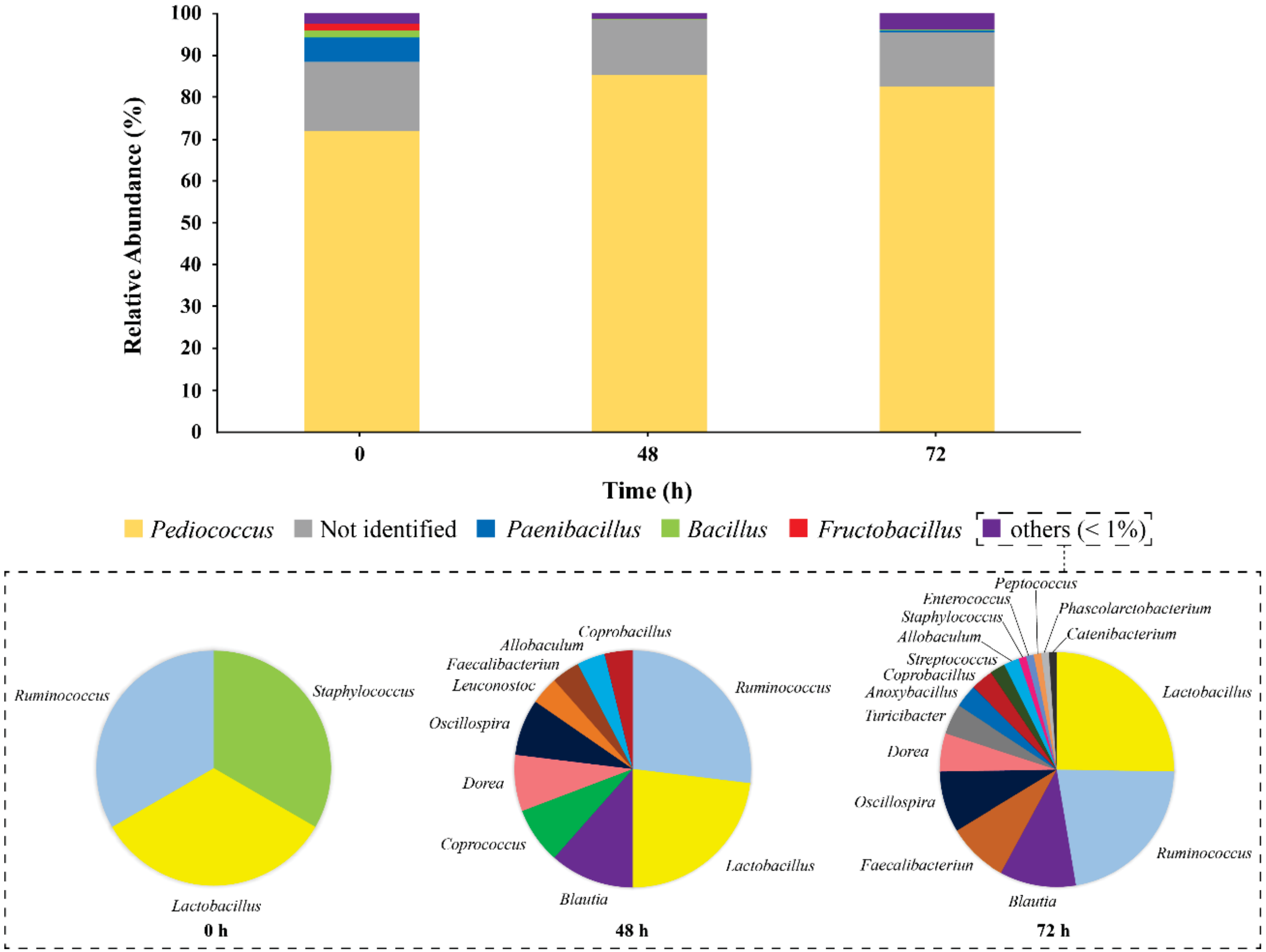
4.3.1 Characteristics of sample sequencing data and LAB community structure

A total of 55,703 sequence reads obtained by Illumina-based sequencing were clustered in 316 OTUs at 97% sequence similarity. The rarefaction analysis indicated a satisfactory coverage of all the samples, suggesting that the majority of bacterial communities was covered (FIGURE A2.1). The identified microbial OTUs, excluding the unclassified, were divided into five phyla, 45 families and 58 genera, after searching in the SILVA database. The complete list of bacteria at the genus level is shown in the supplementary material (TABLE A2.1). Analysis of phyla revealed a high relative abundance of Proteobacteria (82.02%) during the whole cocoa fermentation process, followed by Firmicutes (17.62%), Actinobacteria (0.32%) and Bacteroidetes (0.02%). Proteobacteria was recently reported as the dominant phylum in Brazil and in four regions of cocoa production in Africa (AGYIRIFO et al., 2019; BORTOLINI et al., 2016; ILLEGHEMS et al., 2012; SERRA et al., 2019). The role of Proteobacteria during the cocoa bean fermentation includes the production of degrading enzymes and oxidation of ethanol to acetic acid (ILLEGHEMS; WECKX; DE VUYST, 2015; SERRA et al., 2019).

Fructophilic lactic acid bacteria (FLAB) belong to the phylum Firmicutes, a very diverse group of bacteria with a low G + C content (> 50 mol%) in their genome. Figure 1 shows filtered sequences related to Firmicutes found in this study. *Pediococcus* was the dominant genera reaching 85% relative abundance within 48 h of fermentation. This is the first study to report the dominance of *Pediococcus* in cocoa fermentation, which have been found in lower prevalence in Brazil and Nigeria (AGYIRIFO et al., 2019; ILLEGHEMS et al., 2012; ILLEGHEMS; WECKX; DE VUYST, 2015; KOSTINEK et al., 2008; MIGUEL et al., 2017; PAPALEXANDRATOU et al., 2011a, 2011b, 2011c; PASSOS et al., 1984; SERRA et al., 2019). Other Firmicutes members that were found included *Paenibacillus*, *Lactobacillus*, *Bacillus* and *Leuconostoc*.

Fructobacillus was detected only at 0 h (1.65%) of fermentation (FIGURE 1). Other studies, which used culture-independent approaches, also found low population of *Fructobacillus*, and only at the beginning of cocoa fermentation in Brazil, Ecuador, Malaysia, Ivory Coast and Ghana (AGYIRIFO et al., 2019; MENEZES et al., 2016; PAPALEXANDRATOU et al., 2011a; SERRA et al., 2019). These results indicate that, although *Fructobacillus* is present in cocoa pulp, they are not able to compete with other well-adapted glucophilic bacteria and yeasts. The high ethanol content (6.5–25 mg/g of pulp) produced by yeast may favor the growth of alcohol-tolerant LAB, such as certain species of *Lactobacillus*, *Leuconostoc*, and *Pediococcus* (ENDO et al., 2014). FLAB can compete for fructose with other strict or facultative heterofermentative LAB species, such as *L. fermentum* and *Ped. acidilactici*¸ which use hexose as an alternative external electron acceptor (DE VUYST; WECKX, 2016).

FIGURE 1 - FIRMICUTES DIVERSITY BY ILLUMINA HIGH-THROUGHPUT SEQUENCING DURING LABORATORY-SCALE COCOA BEANS FERMENTATION AT 0, 48 AND 72 H.



Source: The author (2020).

Lactobacillus is another LAB group reported to have members with fructophilic characteristics, including *L. florum*, *L. brevis*, *L. kunkeei*, *L. apinorum* and *L. plantarum* (ENDO et al., 2010, 2012; GUSTAW et al., 2018; MAENO et al., 2017; NEVELING; ENDO; DICKS, 2012). In general, *L. plantarum* and *L. fermentum* have frequently been reported as the dominant species in cocoa beans fermentations around the world (BORTOLINI et al., 2016; CAMU et al., 2007; HAMDOUCHE et al., 2015; ILLEGHEMS; WECKX; DE VUYST, 2015; MEERSMAN et al., 2013; NIELSEN et al., 2007; PAPALEXANDRATOU et al., 2011a; PEREIRA et al., 2012, 2013; SERRA et al., 2019; VISINTIN et al., 2016). The lower *Lactobacillus* abundance found in this study can be associated with the reported high prevalence of *Pediococcus*. It is known that the growth of *Pediococcus* during wine fermentation contribute to subsequent inhibition of other LAB, through production of hydrogen peroxide, bacteriocins and other antibiotic-like substances. However, toxicity has only a transitory effect, i.e., it did not kill and eliminate the bacteria, it only decreases the growth rate and lowered the final population density (LONVAUD-FUNEL; JOYEUX, 1993).

4.3.2. Isolation and identification of fructose-consuming LAB

The initial microbial count on FYP agar plates was 2.14 log CFU/mL and reached a maximum count of 9.0 log CFU/mL after 72 h, followed by a drop to 6.6 log CFU/mL by 120 h (FIGURE A2.2). Overall, 204 isolates were randomly picked up from different fermentation times and evaluated to grow in medium containing glucose as the sole carbon source. A total of 80 isolates, having a low growth efficiency in glucose, were selected since FLAB feature a limited growth when glucose is present as a single carbon source (ENDO et al., 2018; ENDO; FUTAGAWA-ENDO; DICKS, 2009). Subsequently, these 80 isolates were evaluated for fructose consumption efficiency (TABLE A2.2). From an initial fructose concentration of 10 g/L, all the isolates were able to consume > 50% (< 5 g/L) after 36 h of fermentation. In general, the isolates identified as *L. plantarum* showed a more efficient consumption rate with residual fructose reaching values inferior to 4 g/L, which corroborates to the potential fructophilic characteristic attributed to this group (ENDO et al., 2012, 2018).

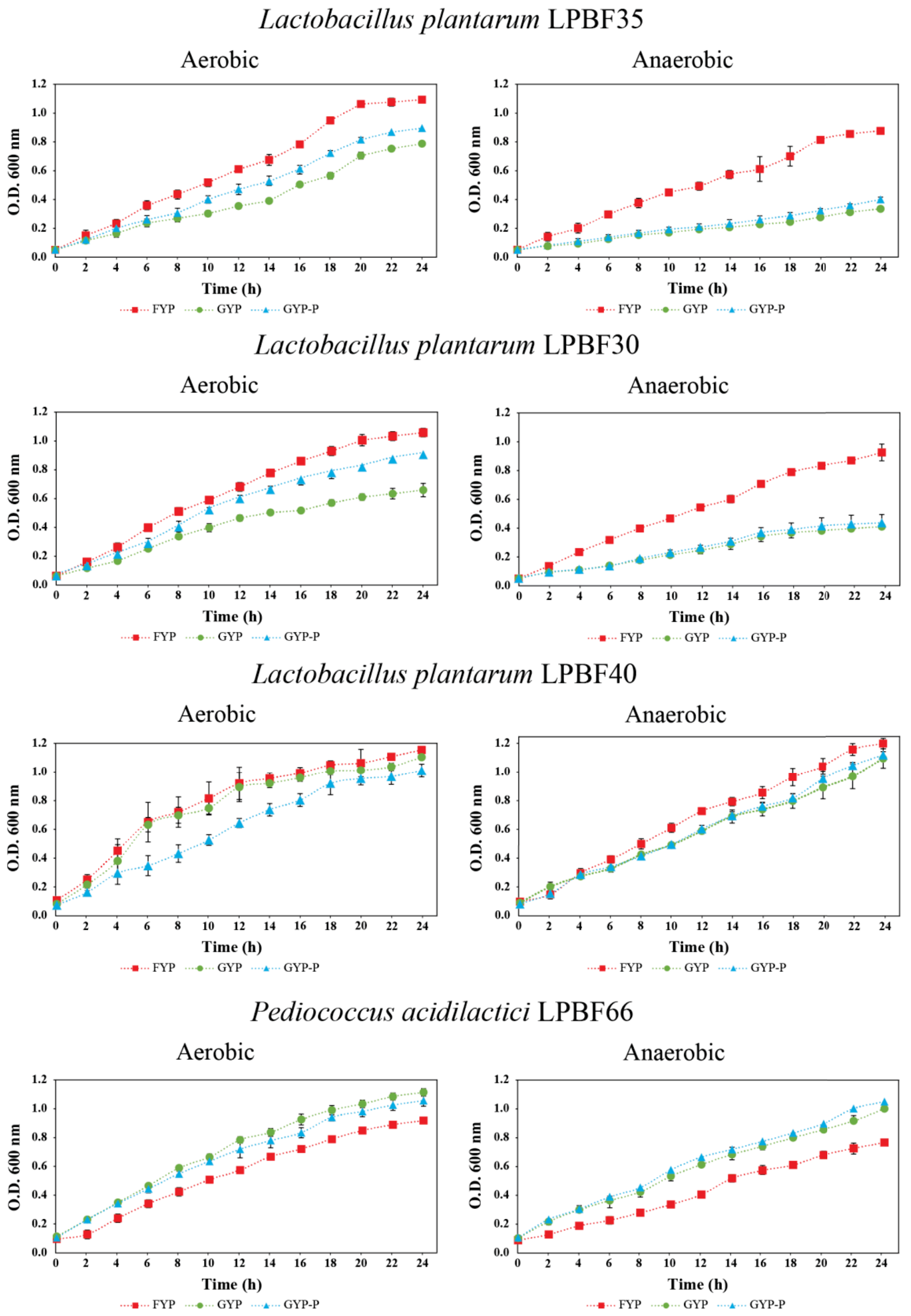
The 80 fructose-fermenting isolates were identified by 16S rRNA gene sequencing as *Ped. acidilactici* (n = 52; 16S rRNA gene 99% sequence similarity), *Ped. pentosaceus* (n = 10; 16S rRNA gene 99% sequence similarity), *L. plantarum* (n =

10; 16S rRNA gene 99% sequence similarity), *B. subtilis* (n = 4; 16S rRNA gene 99% sequence similarity), and *Leu. pseudomesenteroides* (n = 4; 16S rRNA gene 99% sequence similarity) (TABLE A2.2). Although the genus *Fructobacillus* was found by Illumina approach, it was not isolated on FYP medium. The FYP has been successfully applied for isolation of FLAB from flowers and bee guts (ENDO et al., 2010, 2012; ENDO; FUTAGAWA-ENDO; DICKS, 2009; FILANNINO et al., 2016; NEVELING; ENDO; DICKS, 2012). These are sources with significantly lower loads of LAB than cocoa beans fermentation with generally observed LAB populations above 10^8 CFU/g (ARDHANA; FLEET, 2003; CAMU et al., 2007; LEFEBER et al., 2010; PAPALEXANDRATOU et al., 2011a, 2011c; PEREIRA et al., 2012). The nutrient-rich medium of cocoa pulp may have favored the growth of faster growing LAB (i.e., *Pediococcus*) on FYP agar plates at the expense of slow growing of *Fructobacillus*. In any way, with these results, it is possible to speculate that the diversity of bacterial species in cocoa beans fermentation is inevitably underestimated using standard cultivation methods and that organisms of key importance to the community and the entire ecosystem may be overlooked. The use of dilute nutrient media techniques can be tested for recovery of *Fructobacillus* and other slow growing microbial species from cocoa fermentation (CONNON; GIOVANNONI, 2002; RAPPÉ et al., 2002; ZENGLER et al., 2002). These include filtration methods (HAHN et al., 2004), density-gradient centrifugation or elutriation and extinction-dilution, whereby samples are diluted, ideally down to single cells, before their culture in isolation (BEN-DOV; KRAMARSKY-WINTER; KUSHMARO, 2009; CONNON; GIOVANNONI, 2002; SONG; OH; CHO, 2009; WANG et al., 2009).

4.3.3 Fructophilic properties

All ten *L. plantarum* strains showed fructophilic proprieties (preferring D-fructose to D-glucose as a main source of growth), while *Ped. acidilactici*, *B. subtilis* and *Leu. pseudomesenteroides* strains were glucophilic (FIGURE 2). Among the ten strains, two of them (*L. plantarum* LPBF30 and LPBF35) grew faster in FYP than in both GYP and GYP-P, under anaerobic and aerobic conditions. The other eight *L. plantarum* strains showed similar growth in FYP and GYP under aerobic condition, but faster in FYP under anaerobic condition. The characteristics of all the 10 *L. plantarum* strains classified them as "facultatively" fructophilic bacteria, which means they can grow on glucose without an external electron acceptor but the growth on fructose is faster (GUSTAW et al., 2018). Obligate FLAB group, which includes all species of the genus *Fructobacillus* and *L. kunkeei*, require external electron acceptors (i.e., oxygen, fructose, pyruvate, p-coumaric and caffeic acid) in order to maintain the balance of the NAD+/NADH ratio associated with the loss of the alcohol/acetaldehyde dehydrogenase gene (FILANNINO et al., 2019; MAENO et al., 2016). The main end-metabolites produced by obligate FLAB are acetate and CO_2, while ethanol is hardly produced (ENDO et al., 2012, 2018; MAENO et al., 2016). On the other hand, facultative fructophilic properties are not associated with the loss of the alcohol/acetaldehyde dehydrogenase gene, and species of this group, including some strains of *L. florum*, *L. plantarum* and *L. brevis*, can grow on glucose without an external electron acceptor. The end-metabolites of facultative FLAB from glucose includes high rates of lactic acid, ethanol, acetic acid and CO_2 (ENDO et al., 2014, 2018; FILANNINO et al., 2019; NEVELING; ENDO; DICKS, 2012).

FIGURE 2 - GROWTH CURVES MODEL OF FACULTATIVE FRUCTOPHILIC *L. plantarum* STRAINS (LPBF35, LPBF30, AND LPBF40) AND NON-FRUCTOPHILIC *Ped. acidilactici* LPBF66 UNDER AEROBIC AND ANAEROBIC CONDITIONS. FYP = FRUCTOSE YEAST PEPTONE. GYP = GLUCOSE YEAST PEPTONE. GYP-P = GLUCOSE YEAST PEPTONE SUPPLEMENTED WITH PYRUVATE.

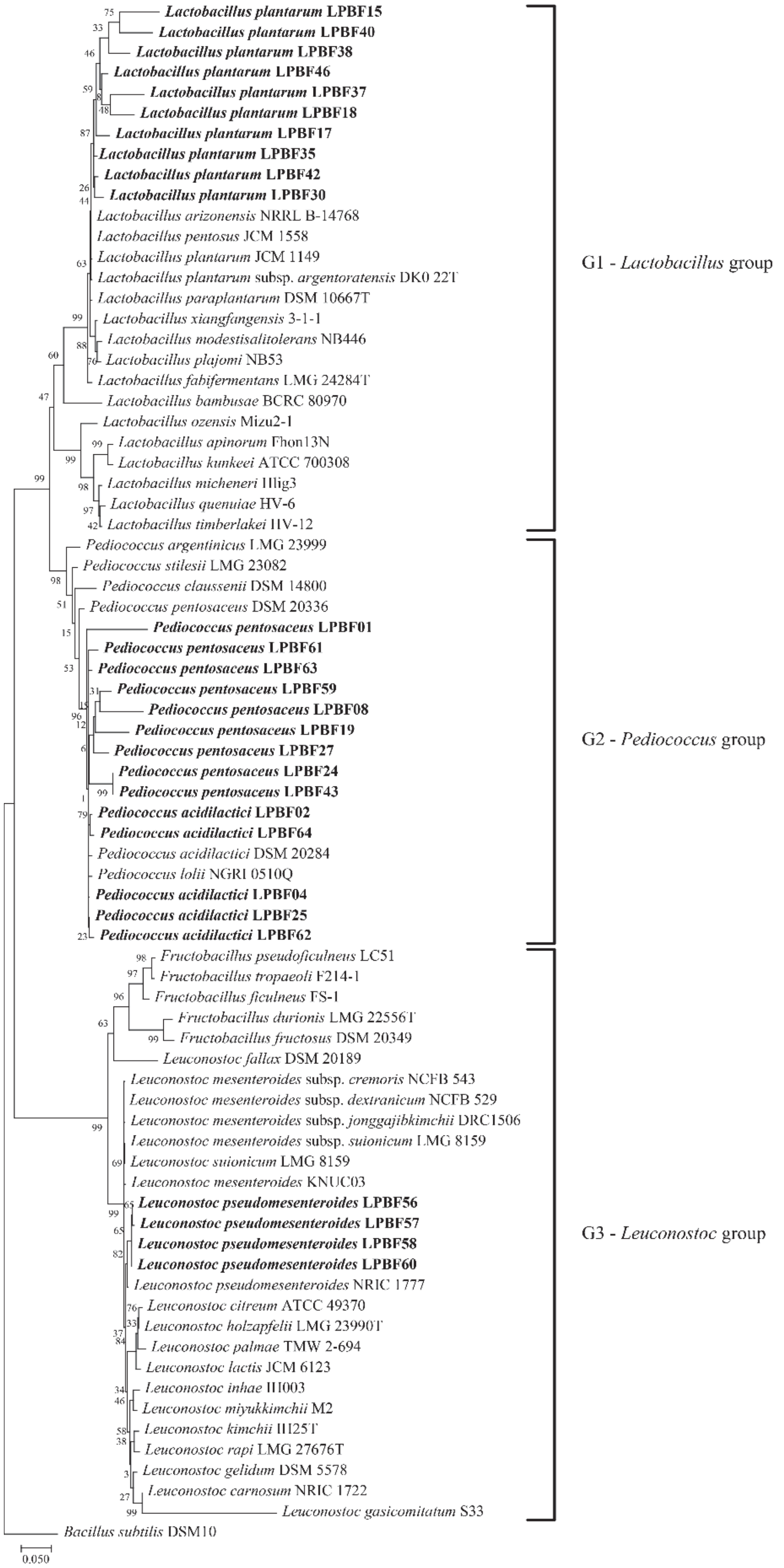


Source: The author (2020).

4.3.4. Genotypic characterization of presumptive FLAB

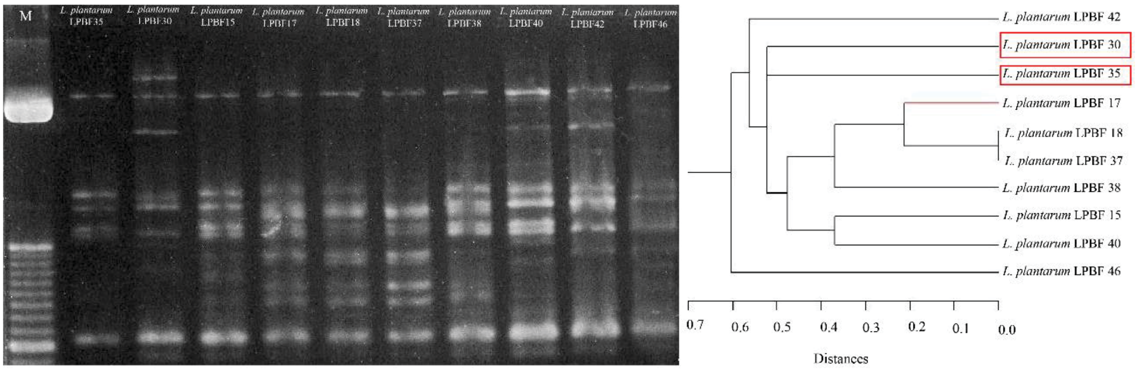
Taxonomic strain characterization was performed by comparing the sequences of each isolate with those reported in the NCBI Reference. Three distinct clusters were formed according to neighbor-joining method, namely G1 - *Lactobacillus* group, G2 - *Pediococcus* group, and G3 - *Leuconostoc* group (FIGURE 3). Interestingly, the two strains that showed fast growth in FYP under anaerobic and aerobic conditions, *L. plantarum* LPBF30 and LPBF35, were grouped into a distinct sub-cluster among the G1 group, along with *L. plantarum* LPBF42. This demonstrates a genetic variation of FLAB within the 16S rRNA gene, which can be used to show the strains differences. $(GTG)_5$-rep-PCR genomic fingerprinting was used to confirm the differentiation of fructophilic strains (FIGURE 4). A computer-assisted analysis clearly differentiated *L. plantarum* LPBF30 and LPBF35 from the other strains. In the same way, *L. plantarum* LPBF42 was also grouped close to both *L. plantarum* LPBF30 and LPBF35, corroborating the observed data from the 16S rRNA gene phylogenetic tree. In addition, both LPBF35 and LPBF30 presented up to seven fewer fragments than the glucophilic strains. FLAB have significantly fewer genes for carbohydrate metabolism than other LAB, especially due to the lack of complete phosphotransferase system transporters (PTS) (ENDO et al., 2015, 2018; FILANNINO et al., 2019; MAENO et al., 2016, 2017). Thus, the ongoing reduction of the genome called “reductive evolution”, together with acquisition or overexpression of genes (GUSTAW et al., 2018; VAN DE GUCHTE et al., 2006), may explain the differentiation of both strains. $(GTG)_5$-rep-PCR genomic fingerprinting revealed itself as a promising genotypic tool for evaluating the rapid and reliable speciation of FLAB.

FIGURE 3 - MAXIMUM-LIKELIHOOD TREE BASED ON 16S rRNA GENE SEQUENCES SHOWING THE PHYLOGENETIC RELATIONSHIPS OF LAB ISOLATED FROM LABORATORY-SCALE COCOA BEANS FERMENTATION. BOOTSTRAP VALUES (%) BASED ON 1000 REPLICATIONS ARE SHOWN AT BRANCH POINTS. THE SUBSTITUTION MODEL USED WAS KIMURA 2-PARAMETER MODEL. BAR = 0.05% SEQUENCE DIVERGENCE.



Source: The author (2020).

FIGURE 4 - (GTG)$_5$-rep-PCR ELECTROPHORESIS BAND PATTERN (A) AND CLUSTERING (B) ACCORDING WITH THE UPGMA ANALYSIS BASED ON DICE COEFFICIENT OF STRAINS OF *L. plantarum* ISOLATED FROM COCOA BEANS FERMENTATION. RED LINES INDICATE FRUCTOPHILIC *L. plantarum* STRAINS LPBF30 AND LPBF35.



Source: The author (2020).

4.3.5. Volatile compounds production and cocoa beans fermentation conducted with selected FLAB

LAB are known for producing a wide range of volatile aroma compounds during cocoa beans fermentation, which contribute for the formation of desirable sensory notes in the composition of chocolate. Aroma production has been used as an important criterion for selecting LAB to be used in cocoa fermentation (AFOAKWA et al., 2008; JANEK et al., 2016; LAGUNES GÁLVEZ et al., 2007; LEFEBER et al., 2011a). The metabolism of aroma formation for all ten fructophilic strains was characterized and reported in Table 1. SPME-GC/MS analysis enabled the identification of a total of 21 compounds, including organic acids (5 compounds), alcohols (5 compounds), aldehydes (4 compounds), furans (1 compound), esters (4 compounds) and ketones (3 compounds). Some flavor-active compounds (i.e., acetaldehyde, ethyl acetate, nonanal, and octanoic acid) are reported in literature for attributing desirable fruity and sweetish sensory notes, thus enriching and modulating the flavor of chocolate (CAMU et al., 2008b; MENEZES et al., 2016; RODRIGUEZ-CAMPOS et al., 2011). Maeno et al. (2016) showed that fructophilic species of *L. kunkeei* possess high number of genes related to amino acids transport and catabolism, which could also explain the elevated production of aldehydes and carboxylic acids. Those low molecular weight compounds are strictly correlated with the catabolism of amino acids in LAB (PEREIRA et al., 2020).

TABLE 1 **-** PERCENTAGE (%) OF THE AREA OF VOLATILE COMPOUNDS PRODUCED BY *L. plantarum* STRAINS, ISOLATED FROM LABORATORY-SCALE COCOA BEANS FERMENTATION, IN FYP BROTH MEDIUM.

	Control	*L. plantarum* LPBF35	*L. plantarum* LPBF30	*L. plantarum* LPBF15	*L. plantarum* LPBF17	*L. plantarum* LPBF18	*L. plantarum* LPBF37	*L. plantarum* LPBF38	*L. plantarum* LPBF40	*L. plantarum* LPBF42	*L. plantarum* LPBF46
Volatile Organic Compound											
Acids											
Octanoic acid	9.5 ± 2.3 a	7.6 ± 4. 1ab	ND	ND	ND	ND	ND	3.1 ± 0.5 a	ND	4.1 ± 0.7 a	ND
Oxalic acid derivative	12.2 ± 5.4 a	3.4 ± 0.5 ab	5.4 ± 1.6 ab	ND	3.7 ± 0.2 ab	ND	ND	2.7 ± 0.2 b	0.9 ± 0.01 c	ND	3.7 ± 0.8 b
2-Methyl butanoic acid	5.0 ± 3.6 a	ND	6.0 ± 1.8 ab	ND	6.0 ± 0.9 b	14.9 ± 5.9 c	12.1 ± 0.7 c	7.4 ± 1.3 b	8.9 ± 1.9 c	8.7 ± 2.6 bc	5.9 ± 1.0 b
3-Methyl butanoic acid	10.8 ± 4.1 a	10.6 ± 3.9 b	5.8 ± 2.2 a	4.4 ± 2.7 a	8.0 ± 0.4 b	13.1 ± 2.9 b	12.1 ± 1.6 b	7.6 ± 0.8 ab	7.3 ± 1.7 ab	10.0 ± 4.2 ab	5.3 ± 0.4 a
2-Decenoic acid	5.9 ± 2.3 a	ND	ND	3.7 ± 1.4 a	ND	ND	ND	4.0 ± 0.3 a	ND	ND	ND
Aldehydes											
Acetaldehyde	7.2 ± 0.9 a	7.3 ± 3.0 b	7.8 ± 3.1 b	6.0 ± 1.6 b	6.9 ± 1.5 b	7.5 ± 1.9 b	6.7 ± 1.2 b	5.6 ± 3.0 ab	4.8 ± 1.1 ab	8.5 ± 1.5 b	6.7 ± 3.5 ab
Benzaldehyde	4.0 ± 1.4 a	9.7 ± 1.9 b	10.5 ± 2.9 bc	7.4 ± 2.8 bc	6.5 ± 0.7 bc	8.3 ± 3.7 bc	8.1 ± 0.5 bc	10.5 ± 2.8 bc	10.0 ± 3.0 b	10.0 ± 2.6 b	9.2 ± 3.1 bc
Nonanal	6.7 ± 4.1 a	8.3 ± 1.2 b	10.3 ± 0.7 b	8.5 ± 0.2 b	9.3 ± 3.2 bc	13.9 ± 5.1 bc	10.5 ± 1.2 b	8.3 ± 3.3 b	8.7 ± 2.0 b	ND	8.8 ± 1.4 b
Decanal	10.8 ± 2.7 ab	7.1 ± 2.7 bc	9.8 ± 0.4 c	8.0 ± 1.8 bc	ND	10.4 ± 2.1 ab	8.4 ± 0.9 ab	ND	8.0 ± 2.8 c	6.3 ± 0.4 ab	4.5 ± 1.6 ab
Alcohols											
1-Hexanol	5.4 ± 2.3 a	5.1 ± 2.3 ab	5.6 ± 3.4 ac	8.0 ± 0.4 c	6.0 ± 1.7 bc	ND	ND	3.3 ± 0.2 a	ND	0.4 ± 0.01 a	7.8 ± 0.4 bc
1-Heptanol	3.6 ± 0.9 a	5.1 ± 1.4 bc	8.7 ± 3.8 bc	11.0 ± 0.7 c	10.8 ± 2.2 c	ND	15.6 ± 5.1 c	5.8 ± 0.5 c	3.7 ± 0.4 b	3.0 ± 0.2 b	5.5 ± 1.0 b
1-Octanol	4.5 ± 1.8 a	6.0 ± 3.4 b	ND	10.6 ± 1.4 c	11.4 ± 0.9 c	9.6 ± 1.3 b	ND	6.9 ± 1.0 b	2.0 ± 0.1 a	5.4 ± 1.3 b	9.6 ± 2.7 bc
1-Decanol	6.3 ± 2.7 a	5.3 ± 2.8 b	ND	7.4 ± 2.3 b	ND	ND	ND	1.5 ± 0.1 a	5.9 ± 1.3 b	2.6 ± 0.9 a	ND
2-Propyl-1-pentanol	2.3 ± 0.2 a	ND	8.3 ± 2.9 b	ND	ND	ND	ND	2.9 ± 0.1 a	3.5 ± 0.7 b	ND	ND

Esters											
Ethyl acetate	ND	6.4 ± 1.2 b	ND	5.0 ± 1.1 ab	3.2 ± 1.1 a	6.7 ± 1.1 ab	8.6 ± 1.4 ab	6.5 ± 1.3 ab	ND	6.3 ± 0.7 b	3.1 ± 1.4 a
Methyl acetate	ND	3.0 ± 0.1 a	4.0 ± 1.1 a	4.2 ± 1.9 a	4.7 ± 1.9 a	7.2 ± 0.5 a	5.3 ± 2.6 a	7.6 ± 1.5 b	4.1 ± 0.9 a	7.9 ± 0.2 b	ND
3-Methyl-1-butanol acetate	ND	ND	ND	ND	6.3 ± 0.9 b	ND	7.2 ± 1.6 b	ND	5.9 ± 2.4 ab	5.2 ± 2.2 ab	2.2 ± 1.0 a
Furans											
2-Pentylfuran	5.9 ± 2.7 a	4.4 ± 2.5 ab	ND	ND	ND	ND	ND	5.4 ± 1.8 ab	5.0 ± 1.5 ab	5.9 ± 1.1 b	ND
Ketones											
2-Heptanone	ND	ND	8.3 ± 0.7 b	6.7 ± 1.6 bc	7.6 ± 2.6 bc	5.6 ± 1.3 a	ND	7.6 ± 1.5 bc	8.3 ± 3.5 bc	5.0 ± 0.9 a	9.6 ± 4.3 c
2-Octanone	ND	6.5 ± 2.3 bc	ND	ND	ND	ND	1.9 ± 0.1 a	3.1 ± 0.1 a	6.3 ± 1.1 b	6.3 ± 1.3 b	10.8 ± 3.7 c
2-Nonanone	ND	4.1 ± 0.5 b	9.5 ± 1.1 b	9.1 ± 3.2 bc	9.5 ± 3.9 bc	2.9 ± 0.8 a	3.5 ± 0.1 a	ND	6.7 ± 1.5 b	4.6 ± 0.2 b	7.3 ± 2.2 b

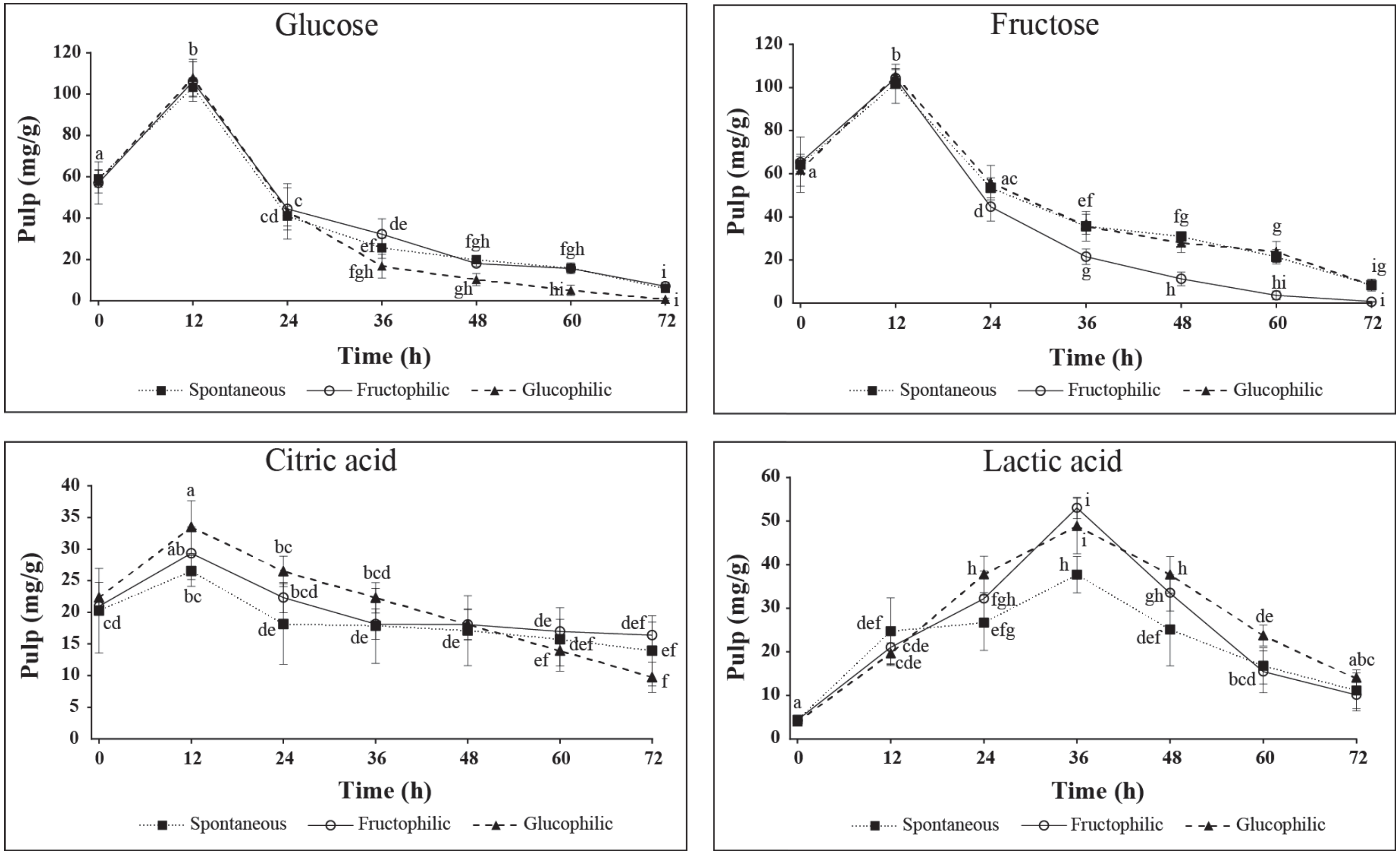
Source: The author (2020).

* ND = not detected. Means of duplicate in each row bearing the same letters are not significantly different ($p > 0.05$) from one another using Duncan's Test (mean ± standard variation).

Among the two-faster fructose-consuming strains (LPBF30 and LPBF35), *L. plantarum* LPBF35 was introduced as a starter culture in laboratory cocoa beans fermentation due to its higher production ($p > 0.05$) and diversity of volatile aroma compounds. The metabolism of sugar consumption and lactic acid production was compared to fermentations conducted with a glucophilic bacterium (*Ped. acidilactici* LPBF66) and a spontaneous process (FIGURE 5). The observed increase in the concentration of glucose (from approx. 103.19 mg/g up to 107.96 mg/g) and fructose (from approx.101.64 mg/g up to 105.31 mg/g) at 12 h of fermentation can be attributed to the hydro lysis of sucrose, pectin and other complex polysaccharides present in cocoa pulp (PEREIRA et al., 2012, 2013a; RODRIGUEZ-CAMPOS et al., 2011). After 24 h, fructose was more rapidly metabolized ($p > 0.05$) in fermentation containing the fructophilic strain in comparison with the glucophilic strain and the spontaneous process; the opposite was observed for glucose metabolism. Therefore, residual fructose was not observed in the FLAB process. Previous studies have reported a residual fructose content (5 mg/g up to 17 mg/g) at 120 h of spontaneous cocoa fermentation (ARDHANA; FLEET, 2003; CAMU et al., 2008b; KRESNOWATI; SURYANI; AFFIFAH, 2013; LAGUNES GÁLVEZ et al., 2007; LIMA et al., 2011; NIELSEN et al., 2007; PAPALEXANDRATOU et al., 2011a; PEREIRA et al., 2012). Thus, the use of FLAB can assist in the fructose metabolism, contributing to the drying of beans.

Metabolism of organic acids is another mechanism by which LAB are considered to impact on cocoa bean composition and quality. The utilization of citric acid and production of lactic acid are considered the two main functions of LAB, decreasing the acidity of the pulp, and equilibration of the bean pH to values around 5.0–5.5, which are considered optimal for endogenous proteolytic and other enzymatic activities (DE VUYST et al., 2010; SCHWAN; PEREIRA; FLEET, 2015). Although the different fermentative processes showed similar profile of organic acid metabolism, citric acid was most efficiently metabolized in the process by *P. acidilactici* LPBF66 (residual concentration of 16.40 mg/g, 9.73 mg/g and 13.91 mg/g in *L. plantarum* LPBF35, *P. acidilactici* LPBF66 and spontaneous process, respectively). On the other hand, both fructophilic and glucophilic inoculated processes achieved similar lactic acid concentration (approx. 50 mg/g at 36 h), which was significantly higher than the spontaneous process (37.71 mg/g). These results demonstrate that FLAB has an ideal profile of organic acid metabolism for cocoa fermentation.

FIGURE 5 - PROFILE OF CONSUMPTION OF GLUCOSE AND FRUCTOSE, AND METABOLISM OF CITRIC ACID AND LACTIC ACID, DURING COCOA BEANS FERMENTATION CONDUCTED WITH *L. plantarum* LPBF35 (FRUCTOPHILIC BACTERIUM), *Ped. acidilactici* LPBF66 (NON-FRUCTOPHILIC BACTERIUM) AND SPONTANEOUS PROCESS.



Source: The author (2020).

Means of triplicate in each row bearing the same letters are not significantly different ($p > 0.05$) from one another using Duncan's test.

4.4. CONCLUSIONS

FLAB of the genera *Fructobacillus* and *Lactobacillus* inhabit cocoa pulp; however, *Fructobacillus* remains as a yet-to-be-cultivated genus from this environment. The bioprospecting of fructophilic *L. plantarum* promoted a faster utilization of fructose and ideal profile of organic acid metabolism associated with cocoa fermentation. The exploration of these new taxa will promote the best opportunities to isolate novel microorganisms with functional proprieties and, ultimately, their use as improved starters.

5 CHAPTER III (RESEARCH RESULTS) - CO-CULTURING FRUCTOPHILIC LACTIC ACID BACTERIA AND YEAST ENHANCED SUGAR METABOLISM AND AROMA FORMATION DURING COCOA BEANS FERMENTATION

Manuscript published in the *International Journal of Food Microbiology*, volume 339, February 2021, article 109015 (DOI:10.1016/j.ijfoodmicro.2020.109015).
Patented process under publication number: **BR 102020011064-0**.

ABSTRACT

Glucose and fructose are the main fermentable sugars in cocoa pulp. During fermentation, glucose is consumed within 48–72 h and fructose only after 120 h, mainly associated with the preferential use of glucose by microorganisms. In the first stage of this study, the complete genome sequence of a lactic acid bacterium with high fructose consumption capacity (*Lactobacillus plantarum* LPBF35) was reported. The notable genomic features of *L. plantarum* LPBF35 were the presence of alcohol/acetaldehyde dehydrogenase gene and improved PTS system, confirming its classification as a “facultatively” fructophilic bacterium. Subsequently, this bacterium was introduced into cocoa fermentation process in single and mixed cultures with *Pediococcus acidilactici* LPBF66 or *Pichia fermentans* YC5.2. Community composition by Illumina-based amplicon sequencing and viable counts indicated suppression of wild microflora in all treatments. At the beginning of the fermentation processes, cocoa pulp consisted of approximately 73.09 mg/g glucose and 73.64 mg/g fructose. The *L. plantarum* LPBF35 + *P. fermentans* YC5.2 process showed the lowest levels of residual sugars after 72 h of fermentation (7.89 and 4.23 mg/g, for fructose and glucose, respectively), followed by *L. plantarum* LPBF35 + *Ped. acidilactici* LPBF66 (8.85 and 6.42 mg/g, for fructose and glucose, respectively), single *L. plantarum* LPBF35 treatment (4.15 and 10.15 mg/g, for fructose and glucose, respectively), and spontaneous process (22.25 and 14.60 mg/g, for fructose and glucose, respectively). The positive interaction between *L. plantarum* LPBF35 and *P. fermentans* YC5.2 resulted in an improved formation of primary (ethanol, lactic acid, and acetic acid) and secondary (2-methyl-1-butanol, isoamyl acetate, and ethyl acetate) metabolites during fermentation. The primary metabolites accumulated significantly in cocoa beans fermented by *P. fermentans* YC5.2 + *L. plantarum* LPBF35, causing important reactions of color development and key flavor molecules formation. The results of this study suggest that fructophilic lactic acid bacteria and yeast is a microbial consortium that could improve sugar metabolism and aroma formation during cocoa beans fermentation.

Keywords: Cocoa processing. Fructose. *Lactobacillus plantarum*. *Pichia fermentans*. *Pediococcus acidilactici.*

5.1. INTRODUCTION

During cocoa fermentation, yeasts and lactic acid bacteria (LAB) convert, within 48–72 h, glucose, fructose, and other sugars into mainly ethanol and lactic acid, respectively; the ethanol content is later oxidized to acetic acid by acetic acid bacteria (AAB), increasing the temperature of the fermenting mass to 50 °C (DE VUYST; LEROY, 2020; OUATTARA et al., 2008). The degradation of cocoa pulp enables the diffusion of these microbial metabolites into the seeds, which, in conjecture with high fermentation temperature, are responsible for the color development and flavor formation of the cocoa beans. Various species of *Bacillus* are also present during cocoa fermentation process, producing pectinolytic enzymes that assists in the degradation of cocoa pulp (OUATTARA et al., 2008, 2011). Due to the glucophilic character of cocoa microbiota, glucose is consumed at a higher rate than fructose, and the presence of residual fructose is generally observed during the acetic acid fermentation phase (LIMA et al., 2011; PAPALEXANDRATOU et al., 2011a; PEREIRA et al., 2012). The residual fructose content allied with the reduction of the water activity can stimulate the proliferation of spoilage bacteria and filamentous fungi during the last days of fermentation (COPETTI et al., 2014; MOENS; LEFEBER; DE VUYST, 2014). This overfermentation process favors unwanted production of microbial compounds, mainly C3–C5 free fatty acids and extracellular proteases and lipases. The late growth of toxigenic fungi also becomes a significant public health risk due to the production of mycotoxins, especially ochratoxin A and aflatoxin (NGANG et al., 2015).

The world demand for fine cocoa and chocolate has been increasing. As fermentation is considered as the “core stage” for cocoa flavor formation, starter culture consortia, including some yeasts (*Kluyveromyces marxianus*, *Pichia kluyveri*, and *Saccharomyces cerevisiae*), LAB (*Lactobacillus fermentum* and *L. plantarum*), and AAB (*Acetobacter pasteurianus*, *A. aceti*, and *A. tropicalis*), have been proposed for process control (CRAFACK et al., 2013; LEFEBER et al., 2012; PEREIRA et al., 2012;

SANDHYA et al., 2016). Each microbial member has defined functions, including: (i) lactic acid production and citric acid metabolization by LAB; (ii) ethanol and volatile compounds production by yeast; and (iii) ethanol and lactic acid oxidation into acetic acid by AAB (PEREIRA et al., 2012). In addition, the acceleration of carbohydrate consumption, pectin degradation, inhibition of growth of undesirable microorganisms, and cocoa beans brown coloring are other functions attributed to the mutualistic growth of these three microbial groups (CRAFACK et al., 2013; LEFEBER et al., 2012; SANDHYA et al., 2016).

Among LAB, a new group called fructophilic lactic acid bacteria (FLAB) has been described by Endo and Okada (2008). It includes all species belonging to the genus *Fructobacillus* and *Apilactobacillus apinorum* (formerly *Lactobacillus apinorum*), *Apilactobacillus kunkeei* (formerly *Lactobacillus kunkeei*), *Fructilactobacillus florum* (formerly *Lactobacillus florum*), *Levilactobacillus brevis* (formerly *Lactobacillus brevis*), and *L. plantarum*, which preferentially metabolize fructose as carbon source and exhibit limited or delayed growth on glucose (ENDO et al., 2018; GUSTAW et al., 2018; VIESSER et al., 2020; ZHENG et al., 2020). FLAB metabolism has been reported during cocoa fermentation (PAPALEXANDRATOU et al., 2011a) and, recently, Viesser et al. (2020) showed that the inoculation of *L. plantarum* LPBF35 (metabolically classified as a "facultatively" fructophilic bacterium) reduced the residual content of cocoa pulp fructose during fermentation compared to a glucophilic strain (*Pediococcus acidilactici* LPBF66) and spontaneous process. In this study, the effect of co-culture with *L. plantarum* LPBF35 and *Pichia fermentans* YC5.2 or *Ped. acidilactici* LPBF66 was investigated by Illumina-based amplicon sequencing, plate counts, and chromatographic techniques. The results may contribute to further elucidate the interaction between FLAB and yeast during food fermentations and improve the quality of cocoa beans.

5.2. MATERIALS AND METHODS

5.2.1. Genome assemble of *L. plantarum* LPBF35

The draft genome of *L. plantarum* LPBF35 was sequenced and analyzed. Genomic DNA was extracted using the phenol/chloroform method adapted by Cheng and Jiang (2006) and quantified by Qubit Fluorometer (Life Technologies, Carlsbad, CA, USA). The total DNA was shredded into 350 bp-sized fragments for library construction and its quality was verified using the 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA). The libraries were sequenced on an Illumina HiSeq platform (Illumina Inc., San Diego, CA, USA) using the PE150 (paired-end, 150 bp reads) strategy. Overall, 8,267,763 reads were obtained, giving a 33-fold coverage for the strain sequenced. The Illumina reads were subjected to adapter trimming and quality filtering by Trimmomatic 0.38.0 (BOLGER; LOHSE; USADEL, 2014) with the following parameters: SLIDINGWINDOW (4 bases) with an average of PHRED score 10 for read quality required. The quality of trimmed reads was verified in FastQC 0.72 (http://www.bioinformatics.babraham.ac.uk/ projects/fastqc/). A de novo assembly was performed by SPAdes 3.14.1 (BANKEVICH et al., 2012) with the careful mode enabled.

The *L. plantarum* LPBF35 whole genome sequence (WGS) available data has been deposited in GenBank under the accession number JACADL000000000. The data information sequence was also deposited under the BioSample accession number SAMN15369627 and Bioproject accession number PRJNA641895 at National Center for Biotechnology Information (NCBI).

5.2.2. Starter culture and inoculum preparation

L. plantarum LPBF35 and *Ped. acidilactici* LPBF66 were previously isolated from cocoa fermentation, as detailed in Viesser et al. (2020). The yeast *P. fermentans* YC5.2 was selected for this study due to its high production of flavor-active compounds and tolerance to high temperatures (≥ 40 °C), osmotic pressure (≥ 50% hexose), and metabolite accumulation (12–15% of ethanol, 2% of lactic acid, and 2% of acetic acid), as described in de Pereira (2014b). For biomass production, *L. plantarum* LPBF35 was cultivated in Erlenmeyer flasks containing 4 L of synthetic medium composed by fructose 2% (w/v), yeast extract 2% (w/v), ammonium citrate 2% (w/v), ammonium phosphate 2% (w/v), sodium acetate 0.8% (w/v), and manganese sulfate 0.02% (w/v). *Ped. acidilactici* LPBF66 was grown in Erlenmeyer flasks containing 4 L of similar medium with glucose 2% (w/v) instead fructose as carbon source. *P. fermentans* YC5.2 was prepared in Erlenmeyer flasks containing 4 L of medium composed of glucose 2% (w/v) and yeast extract 2% (w/v). All microbial cultures were incubated at 30 °C for 24 h. After incubation, the yeast and LAB cells were separated from the medium by centrifugation at 5000 ×g during 15 min, washed twice, and resuspended in 250 mL of sterile saline solution (0.9% NaCl [w/v]). The inoculum was stored at 4 °C until use. This process was repeated four times for each inoculum. After the concentration step, the microbial load of the inoculum was quantified by plate count at 30 °C during 24 h using FYP (*L. plantarum* LPBF35; 10 g/L D-fructose, 10 g/L yeast extract, 5 g/L peptone, 2 g/L sodium acetate, 0.5 g/L Tween 80, 0.2 g/L MgSO4·7H2O, 0.01 g/L MnSO4·4H2O, 0.01 g/L FeSO4·7H2O, 0.01 g/L NaCl, 0.05 g/L sodium azide), MRS (*Ped. acidilactici* LPBF66; Himedia, Marg, India), and YEPG agar (*P. fermentans* YC5.2; Himedia), revealing an approximate concentration of 9 log CFU/mL for each starter culture.

5.2.3. Fermentation experiment and sampling

Cocoa seeds were retrieved from mature cocoa fruits (*Theobroma cacao* L. var. Forastero) and deposited in 20-L plastic buckets. Four sets of inoculation protocols were performed in triplicate: (i) single culture fermentation with *L. plantarum* LPBF35, (ii) combined fermentation with L. *plantarum* LPBF35 and *Ped. acidilactici* LPBF66, (iii) combined fermentation with *L. plantarum* LPBF35 and *P. fermentans* YC5.2, and (iv) spontaneous, non-inoculated control. In each set of inoculation, 100 mL of the concentrated starter culture was added to 10 kg of fresh cocoa beans to achieve an approximate initial cell population of 7 log CFU/g of cocoa beans. The fermentations were conducted in triplicate. Samples of approximately 50 g were collected at intervals of 24 h (0, 24, 48, 72, 96, 120, 144 and 168 h) for microbiological and physicochemical analyses.

5.2.4. Plate counts

Fifteen grams of cocoa beans and adhering pulp from samples at 0, 24, 48, 72, 96, 120, 144 and 168 h were added to 30 mL of sterile saline solution and homogenized using vortex (Kasvi, Curitiba, Brazil). Serial dilutions in sterile 0.1% (w/v) peptone water were prepared in triplicate for enumeration of total LAB on MRS agar (Himedia) containing 0.2% (v/v) nystatin, presumptive FLAB on FYP agar supplemented with 0.5% (w/v) $CaCO_3$ and 0.2% (v/v) nystatin, and yeasts on YEPG agar containing 0.1% (w/v) chloramphenicol. Plates were incubated at 30 °C for 24–48 h, and the number of CFU was recorded.

5.2.5. Total DNA extraction and high-throughput sequencing

Samples of fermenting cocoa-bean mass at 0, 24 and 48 h (first phase of LAB and yeast cocoa beans fermentation) were used for total DNA extraction. The pulp fraction was recovered by decanting, after mixing 5 mL of sterile 0.1% (w/v) peptone water with 5 g of the cocoa beans and adhering pulp. One milliliter of each sample was centrifuged at 12,000 ×g for 1 min (Eppendorf, Hamburg, Germany) and the supernatant removed. The pellets were resuspended in 500 µL of Tris-EDTA, homogenized with 10 µL of lysozyme solution (Sigma-Aldrich, Saint Louis, MO, USA), and incubated at 30 °C for 60 min. Then, 50 µL of SDS 10% (w/v) and 10 µL of proteinase K (Sigma-Aldrich) were added, followed by homogenization and incubation at 60 °C for 60 min. A volume of 150 µL of phenol/chloroform solution (25:24; Sigma-Aldrich) was added, homogenized by inversion, and centrifuged (12,000 ×g for 5 min). Posteriorly, supernatant was removed, and the DNA precipitated with absolute ethanol (Sigma-Aldrich). Pellets were washed with 80% ethanol, dried and suspended in Milli-Q® ultrapure water (Merck, Darmstadt, Germany). Extracted DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A total of 20 ng of DNA were used as template for the amplification of the V4 region of the 16S rRNA gene, using the primers 515F and 806R (CAPORASO et al., 2012) with KlenTaq Master Mix (Sigma- Aldrich). The PCR products were quantified using the Qubit dsDNA HS kit (Invitrogen, Carlsbad, CA, USA) and sequenced using the v.2 (500 cycles) Sequencing Kit on an Illumina MiSeq (Illumina Inc.) platform. After sequencing, chimeric sequences detection, removal of noises from pre-cluster and taxonomic assignment were done using standard parameters with the QIIME (Quantitative Insights into Microbial Ecology) software package, version 1.9.0 (http://qiime.org/). Sequences with a similarity above 97% were assigned to the same operational taxonomic units (OTUs) using the SILVA database (https://www.arb-silva.de/) (Quast et al., 2013).

5.2.6. High performance liquid chromatography (HPLC) analysis

Concentrations of sugars (glucose and fructose), organic acids (citric, lactic and acetic acids), and ethanol were monitored by high-performance liquid chromatography (HPLC) at 0, 24, 48, and 72 h. After homogenization, about 200 mg of the surrounding pulp was diluted in 5 mL of sterile saline solution (0.9% NaCl [w/v]) and centrifuged at

6000 ×g for 15 min. The supernatant was recovered and filtered through 0.22 µm pore size filter (Millipore Corp., Billerica, MA, USA). A volume of 0.02 mL of each filtered sample was injected into HPLC system (Agilent Technologies) equipped with an Aminex HPX-87H column (7.8 mm i.d. × 300 mm, 5 µm; Bio-Rad, Hercules, CA, USA) and a refractive index (RI) detector (HPG1362A; Hewlett–Packard Company, Palo Alto, CA, USA). All samples were analyzed in triplicate, and the average values and standard deviations are presented. The column was eluted in an isocratic mode with a mobile phase of 5 mM H_2SO_4 at 60 °C and a flow rate of 0.6 mL/min.

5.2.7. Gas chromatography coupled to mass spectrophotometry (GC–MS) analysis

The volatile organic compositions were monitored by gas chromatography coupled to mass spectrophotometry (GC–MS) at 0, 24, 48, and 72 h. The extraction of volatile compounds was performed using a headspace-solid phase microextraction (HS-SPME) coupled to a DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA). Aliquots (5 mL) from fermenting cocoa pulp were placed in 20 mL headspace vials, in triplicate. The vials were heated at 70 °C for 10 min without agitation, followed by 15 min of exposition of the fiber in a COMBI-PAL system. The compounds were desorbed into the gas chromatograph injection system gas phase (CGMS TQ Series 8040 and a 2010 Plus GC–MS; Shi- madzu, Tokyo, Japan) at 260 ◦C. The column oven temperature was maintained at 60 °C during 10 min, followed by heating ramps of 4 and 10 °C/min until reaching the temperatures of 100 and 200 °C, respectively. The compounds were separated on a column 95% PDMS/5% PHENYL (30 m × 0.25 mm × 0.25 mm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett-Packard). Helium was used as carrier gas at a rate of 1.0 mL/min. Mass spectra were obtained by electron impact at 70 eV and a start and end mass-to-charge ratio (m/z) of 30 and 200, respectively. Identification of volatile aroma compounds were obtained by comparing the mass spectra of individual compounds with the Nist'98 and Wiley7N. databases. The volatile aroma compounds in each sample were extracted and analyzed in triplicate, and the average values and standard deviations are presented. A maximum coefficient of variation of 30% was considered acceptable for a given compound (CEVALLOS-CEVALLOS et al., 2018).

5.2.8. Cocoa bean quality determination

5.2.8.1. Instrumental color parameters analysis

Samples of fermented and unfermented cocoa bean were ground and sieved through a standard mesh size number 20 (850 µm). The powder obtained was used to carry out color measurements using a spectrophotometer (HunterLab MiniScan XE Plus, Reston, VA, USA). 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°. The results were expressed in the L^* (luminosity), a* (red-green component) and b^* (yellow-blue component). Other parameters were analyzed according to Misnawi et al. (2003):

$$\mathrm{C}^*(\text{chroma or saturation}) = (\mathrm{a}^{*2} + \mathrm{b}^{*2})^{\frac{1}{2}} \quad (1)$$

$$\mathrm{h}^{\circ *}(\text{hue angle, dominant colour}) = \tan^{-1} \mathrm{b}^*/\mathrm{a}^* \quad (2)$$

$$\mathrm{TCD}^*(\text{Total colour differences}) = \left((\mathrm{L_f} - \mathrm{L_0})^2 + (\mathrm{a_f} - \mathrm{a_0})^2 + (\mathrm{b_f} - \mathrm{b_0})^2\right)^{\frac{1}{2}} \quad (3)$$

5.2.8.2. Sugars and non-volatile organic acids content

Sugars (glucose and fructose), organic acids (citric, lactic, and acetic acids), and ethanol content of fermented sun-dried and unfermented cocoa beans were evaluated by HPLC. Cocoa bean samples were ground and prepared as described by Ho et al. (2014). Five grams of each sample were mixed with 60 mL of in Milli-Q® ultrapure water (Merck) and homogenized with a vortex (Kasvi) for 10 min. The homogenate was centrifuged at 12,000 ×g for 20 min and the supernatant was recovered. The sediment was washed twice with 20 mL of Milli-Q® water and all the supernatants were pooled and clarified by filtering through 0.22 µm pore size filter (Millipore Corp.). The HPLC analysis was performed as previously described (item 5.2.6). All samples were analyzed in triplicate, and the average values and standard deviations were reported.

5.2.8.3. Volatile organic compounds determination

The volatile compounds from sun-dried fermented and unfermented cocoa samples were extracted using the technique of HS-SPME, followed GC–MS analysis. For each determination, 1 g of ground cocoa sample was stored in a 20 mL HS vial. The conditions of analysis and identification were performed as previously described in item 5.2.7. Analyses of individual samples were done in triplicate, and the average values and standard deviations were reported.

5.2.9. Statistical analysis

The data obtained (means and standard deviations) using target metabolite analysis were calculated and subjected to ANOVA followed by post-hoc comparison of means by Duncan's test. Statistical analyses were performed using the SAS program (Statistical Analysis System, Cary, NC, USA). Level of significance was established in a two-sided p-value < 0.05.

5.3. RESULTS AND DISCUSSION

5.3.1. Genome properties and classification of *L. plantarum* LPBF35

The circular genome of *L. plantarum* LPBF35 is comprised of 3,154,517 bp and a G + C content of 44.61 mol%, which are similar to reported values of *L. plantarum* type strains (KLEEREBEZEM et al., 2003). The genome was predicted to contain a total of 3060 coding sequences (CDS), of which 2972 code for functional proteins and 78 code for RNAs (rRNA: 8, tRNA: 70). Among the 2972 functional protein coding genes, 1173 were assigned hypothetical proteins. A total of 486 genes were categorized into SEED subsystems, with the majority being assigned to protein metabolism (104), miscellaneous (64), carbohydrates (58), co-factors, vitamins, prosthetic groups, pigments (34) and amino acids and derivatives (28). A N50 value of 345,348 bp was achieved for the correctly assembled contigs.

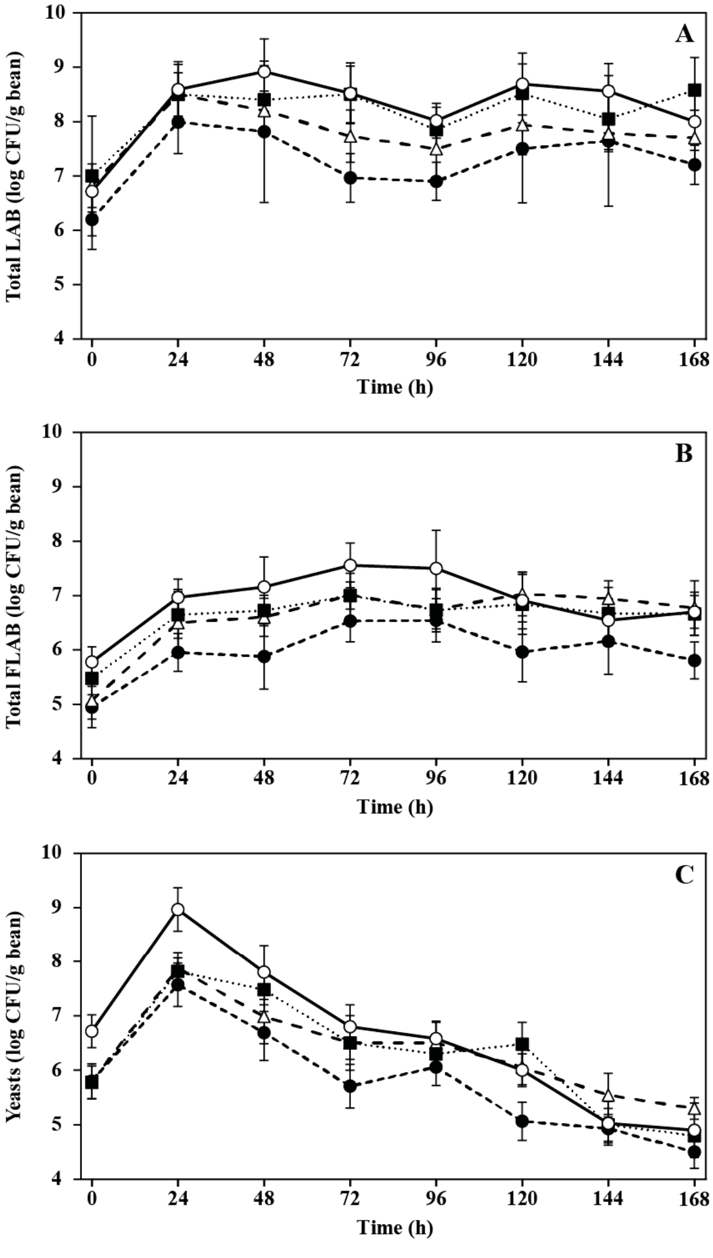
The notable genomic features of *L. plantarum* LPBF35 are the presence of both aldehyde and alcohol dehydrogenase (*adhE1* and *adhE2*) genes, a NADPH dependent R-specific alcohol dehydrogenase (*AdhR*), and a putative helix-to-helix (HTH)-type transcriptional regulator. Previous studies reported that the loss of the *adhE* gene in *Fructobacillus* results in the production of acetate instead of ethanol and causes an imbalance of NAD+/NADH in the heterolactic phosphoketolase pathway (ENDO et al., 2018). Thus, *Fructobacillus* spp. require an electron acceptor (fructose, pyruvate or oxygen) to metabolize glucose. On the other hand, the fructophilic behavior of *L. plantarum* and *L. brevis* is not associated with the loss of the *adhE* gene, but with the enhanced systems of sugar translocation, such as the overexpression of the fructose-PTS and H+ symporters (GUSTAW et al., 2018). Therefore, “facultatively” fructophilic species are able to grow on glucose without the external electron acceptor but show a faster growth on fructose. The presence of the *adhE* gene in *L. plantarum* LPBF35 supports its classification as a “facultatively” fructophilic bacterium, as previously determined by growth characteristics on fructose- and glucose-containing culture media (VIESSER et al., 2020). Finally, both genome length and total number of CDS of the *L. plantarum* LPBF35 are superior to those of *Fructobacillus* species and similar to *L. plantarum* type strains (ENDO et al., 2015). *Fructobacillus* spp. has significantly less protein CDS in their small genomes and lacks several metabolic systems, including respiratory chains, phosphotransferase systems, pyruvate dehydrogenase complex subunits, and ABC transporters (ENDO et al., 2018). The more complex genome showed by the *L. plantarum* LPBF35 supports its facultatively fructophilic behavior. Further proteomic and transcriptomic studies are needed to characterize the overexpression of the PTS system of this strain.

5.3.2. Microbial dynamics by counts and species diversity

The microbial growth dynamics and diversity in single and mixed culture fermentations were estimated using plate count and Illumina-based metagenomic sequencing (FIGURES 1 e 2). For all treatments, LAB, presumptive FLAB and yeasts raised until 24–48 h, followed by a plateau or a decline until the end of the fermentation process. The inoculated treatments showed higher counts in comparison to the spontaneous process, showing starters’ dominance over the indigenous microflora. The maximum populations of total LAB (8.9 log CFU/g), FLAB (7.6 log CFU/g), and yeasts

(9.0 log CFU/g) were reached during co-fermentation of *L. plantarum* LPBF35 + *P. fermentans* YC5.2, when compared to *L. plantarum* LPBF35 + *Ped. acidilactici* LPBF66 and single *L. plantarum* LPBF35 treatment. The highest microbial activity is relevant to warrant the desirable cocoa and chocolate quality. These counts are similar to literature reports (HO; FLEET; ZHAO, 2018; SANDHYA et al., 2016).

FIGURE 1 - COUNTS OF TOTAL LACTIC ACID BACTERIA - LAB (A), PRESUMPTIVE FRUCTOPHILIC LACTIC ACID BACTERIA - FLAB (B), AND YEASTS (C) DURING SPONTANEOUS (●), SINGLE *L. plantarum* LPBF35 (Δ), COMBINED *L. plantarum* LPBF35 + *Ped. acidilactici* LPBF66 (■), AND COMBINED *L. plantarum* LPBF35 + *P. fermentans* YC5.2 (○) COCOA BEANS FERMENTATION.

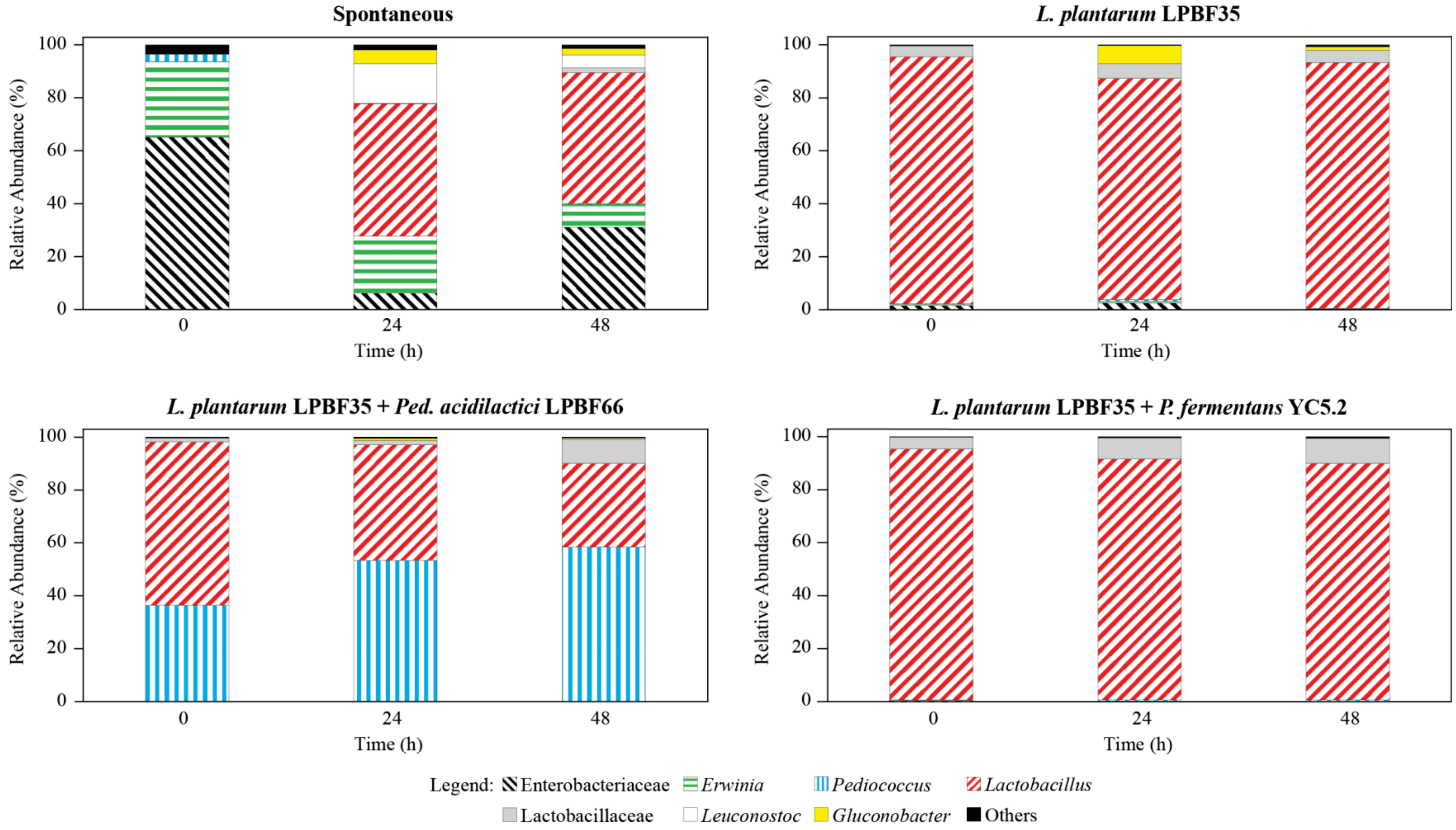


Source: The author (2020).
Each sample was analyzed in triplicate (n = 3) and the standard deviation is represented as error bar.

Illumina Miseq sequencing obtained 155,609 effective 16S rRNA gene sequences, which were clustered into 340 OTUs with the 97% identity level. The observed community richness and community diversity indexes are shown in Figure 2. The 99% Good's coverage of sample meant that most of microbial phylotypes were observed (FIGURE A3.1). It was observed that the bacterial diversity was most abundant in the early stages of the spontaneous process, mainly associated with the high incidence of enterobacteria members (65.13%). Some Enterobacteriaceae genus, such as *Erwinia*, *Serratia*, and *Pantoea*, produce hydrolytic enzymes enabling their growth on cocoa leaves and fruits (GARCIA-ARMISEN et al., 2010)), which have been reported accessing cocoa fermentations in Brazil, Ecuador, Ghana, and Ivory Coast (LEFEBER et al., 2011a; PAPALEXANDRATOU et al., 2011a, 2011b). These bacteria are frequently associated with the production of *off-flavor* compounds (GARCIA-ARMISEN et al., 2010). Other minor bacterial groups reported included *Bacillus*, *Staphylococcus*, *Sphingomonas*, *Pseudomonas*, *Fusobacterium*, *Acinetobacter*, and *Vibrio* (TABLE A3.1). However, this high bacterial diversity was partially reduced after 48 h due to the dominance of *Lactobacillus* sp. (48.53%). It might be that part of exogenous bacteria was inhibited because of the decrease in pH and anaerobic environment created by *Lactobacillus* group (MOREIRA et al., 2013).

On the other hand, the single inoculation of *L. plantarum* resulted in high OTU reads for *Lactobacillus* since the beginning of the fermentation process, reaching 92.81% at 72 h. These results show the adaptation of *L. plantarum* LPBF35 to the cocoa bean fermentation process, which, in turn, enabled the suppression of the wild microbiota. *L. plantarum* is one of the most frequent species in cocoa fermentation, due to its ability to tolerate high temperatures and ethanol concentrations (PEREIRA et al., 2012). These physiological proprieties make this species more often used as a cocoa starter specie (SANDHYA et al., 2016).

FIGURE 2 - MICROBIAL DYNAMICS AND DIVERSITY BY HIGH THROUGHPUT 16S rDNA GENE SEQUENCE AT 0, 24, AND 48 H OF SPONTANEOUS AND INOCULATED COCOA BEANS FERMENTATIONS.



Source: The author (2020).
The genera grouped as “others” have a low relative abundance (<1%) and are reported in Table A3.1.

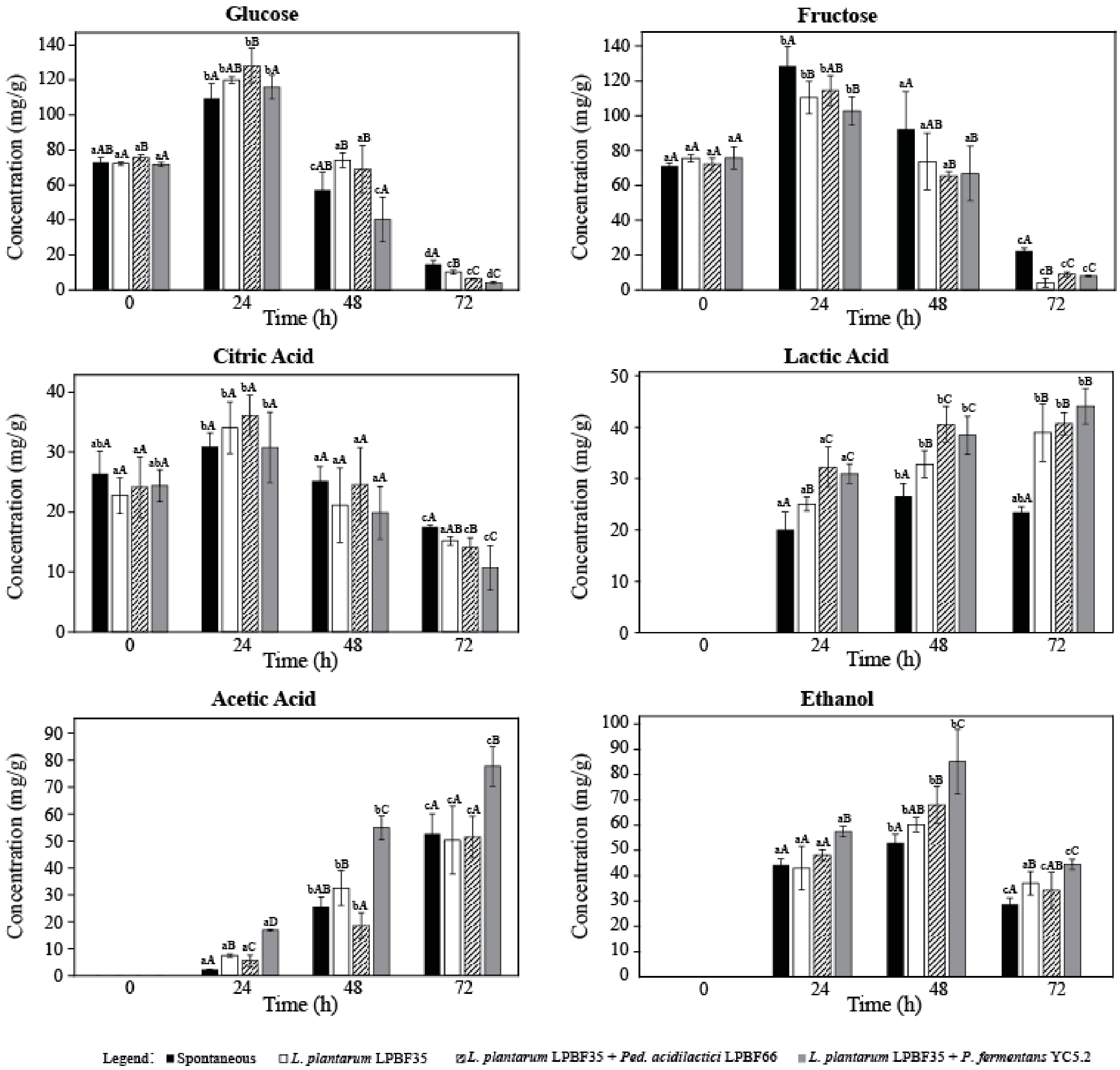
Interestingly, *Lactobacillus* and *Pediococcus* genera shared dominance until 24 h of *L. plantarum* LPBF35 + *Ped. acidilactici* LPBF66 fermentation (FIGURE 2); and, at 48 h, *Pediococcus* showed a slight overlap on the total OTU reads compared to *Lactobacillus* (53.22 and 43.60%, respectively). This competitive interaction between the two starters can be associated with the production of hydrogen peroxide, bacteriocins, and other antibiotic-like substances produced by *Pediococcus*, which contribute to the subsequent inhibition of other LAB species. Nonetheless, this toxicity has only a transitory effect, i.e., it does not kill or eliminate the bacteria, it only decreases the growth rate and lowers the final population density (LONVAUD-FUNEL; JOYEUX, 1993). Finally, the addition of *P. fermentans* YC5.2 resulted in the highest relative prevalence values of *Lactobacillus* (96.52% at 24 h) in comparison to all other treatments - this was also observed in the viable cell count showed in Figure 1. Yeasts and LAB are known to co-exist and share a stable mutualistic relationship in fermented beverages and food products, such as wine, kefir, cheeses, and sourdough (SIEUWERTS; BRON; SMID, 2018). During the initial phase of cocoa fermentation, the production of carbon dioxide by yeasts enables the creation of a microaerophilic environment and, thus, stimulates the growth of LAB; in conjecture, the acidification of the cocoa pulp-bean mass by LAB creates a favorable environment for yeast development (DE VUYST; WECKX, 2016). This is the first study to report the dominance of a *L. plantarum* LPBF35 strain with fructophilic character and its behavior during co-inoculation with an aromatic yeast strain.

5.3.3. Target-metabolites analysis during fermentation

5.3.3.1. Primary metabolites

Cocoa pulp consisted of approximately 73.09 mg/g glucose, 73.64 mg/g fructose, and 24.42 mg/g citric acid (FIGURE 3). The sugar concentration increased during the initial 24 h in all fermentation processes, associated with the hydrolysis of sucrose, pectin, and other complex polysaccharides present in cocoa pulp (PEREIRA et al., 2012). Subsequently, co-culture treatments were more efficient to utilize the reducing sugars, reaching the lower residual contents after 72 h of *L. plantarum* LPBF35 + *P. fermentans* YC5.2 fermentation (4.23 and 7.89 mg/g, respectively), followed by *L. plantarum* LPBF35 + *Ped. acidilactici* LPBF66 (6.42 and 8.85 mg/g, respectively), single *L. plantarum* LPBF35 treatment (10.15 and 4.15 mg/g, respectively), and spontaneous process (14.60 and 22.25 mg/g, respectively). Previous studies reported total glucose consumption within 48–72 h, while 17 mg/g residual fructose is still generally observed after 120 h of fermentation (LIMA et al., 2011; PEREIRA et al., 2012). Interestingly, the single culture fermentation had the lowest residual fructose content, showing that *L. plantarum* was the microorganism responsible for the use of this sugar in the LAB-yeast co-fermentation process.

FIGURE 3 - COURSE OF SUGARS (GLUCOSE AND FRUCTOSE), NON-VOLATILE ORGANIC ACIDS (CITRIC, LACTIC, AND ACETIC ACIDS), AND ETHANOL DURING SPONTANEOUS AND INOCULATED COCOA BEANS FERMENTATION.



Source: The author (2020).
The significance of the results was assessed using an ANOVA with Duncan's post-hoc test at $p < 0.05$. Lower-case letters (a, b, c, and d) show significant differences during fermentation time (0, 24, 48, and 72 h) and upper-case letters (A, B, and C) show significant differences between different treatments.

The formation of primary metabolites (ethanol, acid lactic and acid acetic) was significantly higher in the inoculated treatments compared to the spontaneous process (FIGURE 3). Ethanol showed a continuous and steady production over 48 h, followed by a decrease until 72 h in parallel with acetic acid increase. Fermentation with *L. plantarum* LPBF 35 + *P. fermentans* YC5.2 increased the ethanol (85.11 mg/g at 48 h) and acid acetic (77.73 mg/g at 72 h) content much more significantly than the other inoculated treatments. Ethanol plays crucial role for cocoa fermentation process since it becomes a carbon source for AAB to pro- duce acetic acid. The oxidation of ethanol into acetic acid elevates the temperatures above 50 °C, killing the embryo, and triggering complex biochemical reactions that contributes for the chocolate flavor development (DE VUYST; WECKX, 2016; HO; FLEET; ZHAO, 2018; LIMA et al., 2011). In this sense, the higher availability of ethanol during *L. plantarum* LPBF35 + *P. fermentans* YC5.2 treatment created a favorable environment for the development of AAB. Despite the low prevalence of *Gluconobacter* during the spontaneous and inoculated processes (≤ 6.88%), this AAB can produce high concentrations of acetic acid with a low cell yield (0.09 $g_{cdw}/g_{glucose}$) due to its oxidative metabolism (KRAJEWSKI et al., 2010). This particular metabolism enables the production of acetic acid under aerobic conditions via Pentose Phosphate Pathway (PPP) or through the incomplete oxidation of acetaldehyde in the periplasm via a membrane-bound, pyrroloquinoline quinone (PQQ)-dependent acetaldehyde dehydrogenase (BRINGER; BOTT, 2016).

Lactic acid was progressively produced in all fermentation processes. The highest concentrations were observed after 72 h of *L. plantarum* LPBF35 + *P. fermentans* YC5.2 (44.02 mg/g), *L. plantarum* LPBF35 + *Ped. acidilactici* LPBF66 (40.62 mg/g), and single *L. plantarum* LPBF35 treatment (38.92 mg/g) (with no statistical differences between treatments), followed by the spontaneous process (23.45 mg/g). Lactic acid is responsible for the acidification of the cocoa bean-pulp mass and also acts as a C-source for the late-growing of AAB (DE VUYST; LEROY, 2020). The diffusion of this organic acid into the cocoa beans reduces the pH to 5.5, value considered optimum for the activity of endogenous enzymes responsible for the generation of cocoa flavor precursors (HO; ZHAO; FLEET, 2015). In addition, this compound is a potent inhibitor of spoilage filamentous fungi and bacteria associated with production of mycotoxin and *off-flavors*, respectively (NGANG et al., 2015).

Finally, citric acid had a decrease in its content over 72 h of all fermentation processes (FIGURE 3). LAB and yeast have the ability to assimilate citric acid resulting in a slight increase in pH, around 5.0–5.5, considered ideal for proteolytic and other microbial enzymatic activities (MOENS; LEFEBER; DE VUYST, 2014). Nevertheless, as citric acid is not a preferred carbon source for LAB and yeast growth, there was no significant difference between inoculated treatments and spontaneous process (FIGURE 3).

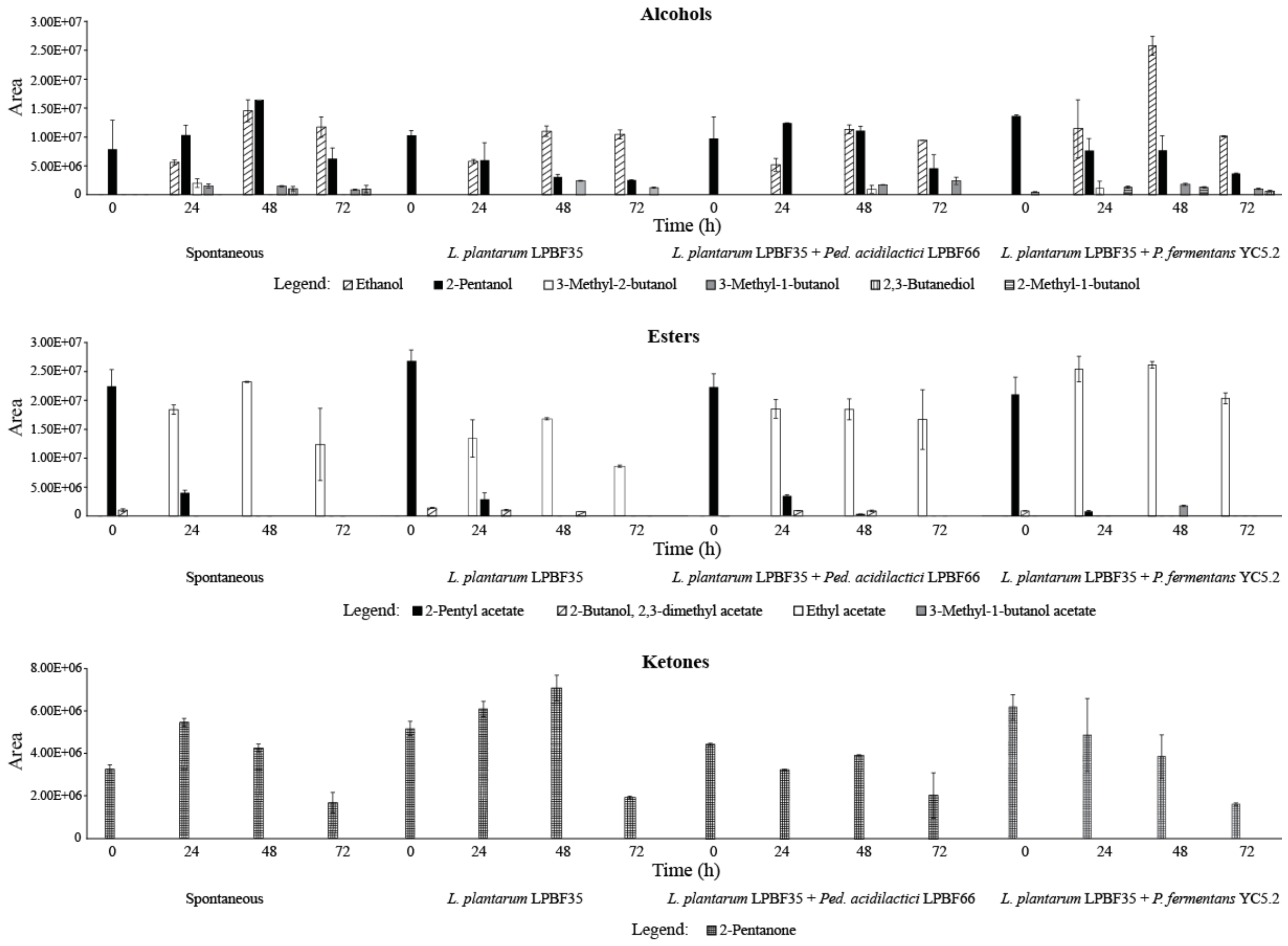
5.3.3.2. Volatile compounds

There were 11 volatile compounds identified by SPME-GC/MS during fermentation processes, classified as alcohols (6), esters (4), and ketone (1) (FIGURE 4). 2-Pentyl acetate was found in higher proportions in cocoa pulp and reduced drastically after 24 h in all treatments. The reduction of this compound can be associated to the evaporation process or diffusion into the cocoa seeds (CAMU et al., 2008b). Other minor volatiles, including 2-pentanol and 2,3-dimethyl-2-butanol acetate, were also associated to cocoa pulp. The presence of these compounds have been reported in fresh cocoa beans of *Forastero*, *Criollo*, and *Trinitario* cultivars (CEVALLOS-CEVALLOS et al., 2018; ROTTIERS et al., 2019; UTRILLA-VÁZQUEZ et al., 2020). The production of acetate esters by plants plays an important role in the defense mechanism in response to mechanical damages or to surrounding parasite plants (GOULET et al., 2012).

The co-inoculation of *L. plantarum* LPBF35 and *P. fermentans* YC5.2 showed the highest peaks of ethanol and ethyl acetate (2.64E+07 and 2.67E+07 at 48 h, respectively). Ouattara et al. (2020) and Pereira et al. (2017) also reported the accumulation of these metabolites during cocoa fermentations using *Pichia* strains as starter cultures. The production of ethyl acetate is assigned to the secondary metabolism of *Pichia* and occurs via esterification of ethanol and acetic acid by esterase under aerobic conditions (LÖSER; URIT; BLEY, 2014). The accumulation and diffusion of this fruity-flavoring molecule into the cocoa seeds during the fermentation has a direct relationship with the production of a high-quality chocolate (MOTA-GUTIERREZ et al., 2019). Interestingly, the spontaneous process showed a higher accumulation of ethanol (1.45E+07 at 48 h), ethyl acetate (2.38E+07 at 48 h), and 2-pentanol (1.64 + 07 at 48 h) when compared to the treatments with the addition of only LAB (*L. plantarum* LPBF35 and co-inoculation of *L. plantarum* LPBF35 and *Ped. acidilactici* LPBF66.). This result suggest that LAB does not have a significant impact on the formation of alcohols or esters during cocoa fermentation (HO; FLEET; ZHAO, 2018; HO; ZHAO; FLEET, 2015) and that these metabolites were produced by indigenous yeasts growing during the spontaneous process (HO; ZHAO; FLEET, 2014).

2-Pentanone showed a continuous production over 48 h, followed by a decrease until 72 h. The single inoculation of *L. plantarum* LPBF35 resulted in higher 2-pentanone production, achieving a maximum peak of 7.08E+06 at 48 h. This compound is derived from the incomplete β-oxidation of free fatty acids and has been already attributed to the lipolytic metabolism of *Lactobacillus* species (GALLEGOS et al., 2017). The persistence of this volatile compound in cocoa beans is associated with desirable herbal and fruity aroma (CEVALLOS-CEVALLOS et al., 2018). 2-Methyl-1-butanol and 3-methyl-1-butanol acetate were only reported in the *L. plantarum* LPBF35 and *P. fermentans* YC5.2 treatment (FIGURE 4). 2-Methyl-1-butanol is produced by yeast through the decarboxylation and further NAD+-dependent reduction of isoleucine found in cocoa pulp via Ehrlich pathway (HAZELWOOD et al., 2008), while 3-methyl-1-butanol acetate is formed by condensation of 2-methyl-1-butanol and acetic acid reported in the cocoa fermentation at 48 h. These secondary metabolites were also reported during cocoa fermentations using *Pichia* strains and are associated with the development of floral, green, and fruity notes in cocoa beans (BATISTA et al., 2016; CRAFACK et al., 2013; OUATTARA; ELIAS; DUDLEY, 2020).

FIGURE 4 - PREVALENCE OF VOLATILE ORGANIC COMPOUNDS (AREA) IN COCOA PULP DURING SPONTANEOUS AND INOCULATED COCOA BEANS FERMENTATIONS.



Source: The author (2020).

5.3.4. Quality assessment

5.3.4.1. Chemical composition

The contents of primary and secondary compounds of fermented cocoa beans are presented in Table 1. Ethanol was not detected probably because of its evaporation during the drying process (PAPALEXANDRATOU et al., 2011b). Cocoa beans from *L. plantarum* LPBF35 + *Ped. acidilactici* LPBF66 and *L. plantarum* LPBF35 + *P. fermentans* YC5.2 treatment had significantly superior concentrations of lactic acid (4.17 and 0.95 mg/g, respectively) and acetic acid (3.23 and 5.24 mg/g, respectively), when compared to spontaneously fermented cocoa beans. These values are within the range considered ideal (≤ 5.0 mg/g and ≤ 10.0 mg/g, respectively) to achieve an internal pH value of 5.4–5.5 for the optimum activity of endogenous enzymes (HO; ZHAO; FLEET, 2014, 2015).

Interestingly, cocoa beans generated from inoculated treatments had reduced, on average, 2.4- to 3.6-fold the contents of fructose and glucose in comparison to unfermented and spontaneously fermented cocoa beans (TABLE 1). It has been demonstrated that, under internal acidic pH and high temperatures, microbial-derived compounds produced during the fermentation can interact with free sugars and free amino acids inside the beans, leading to the non-enzymatic formation of volatiles precursors (e.g., Amadori compounds) (AFOAKWA et al., 2008). The Amadori products are considered important intermediates for the generation of several short chain, flavor-active molecules during roasting process (MUÑOZ et al., 2020).

TABLE 1 - PREVALENCE OF VOLATILE COMPOUNDS (AREA*10^5) AND CONCENTRATION OF ORGANIC ACIDS AND REDUCING SUGARS (mg/g OF COCOA BEANS) IN SUN-DRIED COCOA BEANS BEFORE (UNFERMENTED) AND AFTER FERMENTATION.

		Cocoa Beans				
Compounds	**Aroma Perception**	**Unfermented**	**Spontaneous**	***L. plantarum* LPBF35**	***L. plantarum* LPBF35 + *Ped. acidilactici* LPBF66**	***L. plantarum* LPBF35 + *P. fermentans* YC5.2**
Alcohols (5)						
2-Pentanol	Mild and green	4.36 ± 0.32 a	ND	0.79 ± 0.05 b	1.04 ± 0.30 b	1.12 ± 0.02 b
2,3-Butanediol	--	ND	ND	0.71 ± 0.05 a	0.46 ± 0.02 a	3.52 ± 0.17 b
3-Methyl-1-butanol	Malty, bitter and chocolate	ND	ND	0.48 ± 0.06 a	0.35 ± 0.02 b	0.39 ± 0.07 b
5-Methyl-2-hexanol	--	ND	ND	0.47 ± 0.06 a	ND	0.35 ± 0.04 b
Phenylethyl alcohol	Floral, sweet and bready	ND	1.27 ± 0.21 a	2.41 ± 0.44 b	1.59 ± 0.17 a	2.51 ± 0.03 b
Aldehydes (6)						
2-Methylpropanal	Fresh, floral and green	1.14 ± 0.39 a	0.78 ± 0.04 b	ND	0.68 ± 0.01 c	ND
2-Methylbutanal	Musty, cocoa, coffee and nutty	1.11 ± 0.15 a	ND	ND	0.70 ± 0.05 b	0.40 ± 0.06 c
3-Methylbutanal	Fruity and peach-like in dilution	ND	0.37 ± 0.02	ND	ND	ND
Benzaldehyde	Fruity and cherry	ND	1.14 ± 0.20 a	2.24 ± 0.45 b	2.30 ± 0.34 b	3.35 ± 0.27 c
Pentanal	Almond, malty and pungent	1.8 ± 0.15 a	ND	0.14 ± 0.03 b	ND	0.25 ± 0.02 c
Phenylacetaldehyde	Honey, floral and sweet	ND	ND	0.26 ± 0.00	ND	ND
Esters (10)						
2-Heptanol acetate	Fruity	ND	ND	ND	ND	0.32 ± 0.02
2-Methyl-1-butanol acetate	--	ND	0.43 ± 0.05 a	1.57 ± 0.17 b	1.01 ± 0.13 c	1.46 ± 0.13 b
2-Pentanol acetate	Tropical and herbal	ND	ND	0.91 ± 0.10 a	1.23 ± 0.05 a	0.97 ± 0.09 a
2-Phenylethyl acetate	--	ND	0.40 ± 0.02 a	1.06 ± 0.05 b	0.59 ± 0.05 c	0.70 ± 0.02 d
3-Methyl-1-butanol acetate	--	ND	0.73 ± 0.03 a	3.64 ± 0.39 b	2.30 ± 0.30 c	3.17 ± 0.27 d
Ethyl acetate	Pineapple	ND	ND	0.58 ± 0.03 a	1.11 ± 0.37 b	2.34 ± 0.12 c
Hexanoic acid, ethyl ester	Fruity and pineapple	ND	ND	0.20 ± 0.01	ND	ND
Isobutyl acetate	Fruity, apple and banana	ND	ND	0.42 ± 0.03 a	ND	0.23 ± 0.01 b

Methyl acetate	--	0.89 ± 0.5 ab	1.38 ± 0.04 b	0.66 ± 0.07 a	1.44 ± 0.25 b	0.46 ± 0.15 a
Octanoic acid, ethyl ester	--	ND	ND	0.16 ± 0.00	ND	ND
Ketones (5)						
2-Pentanone	Fruity	0.67 ± 0.02 a	ND	0.32 ± 0.03 b	0.41 ± 0.07 c	0.41 ± 0.05 c
2-Heptanone	Fruity, spice and herbal	ND	0.35 ± 0.18 ab	0.83 ± 0.51 b	0.70 ± 0.12 a	0.45 ± 0.07 a
2-Nonanone	Green fruity, dairy, cheese, buttery	ND	0.4 ± 0.03	ND	ND	ND
2,3-Butanedione	Buttery	ND	ND	ND	ND	0.36 ± 0.05
Acetoin	Buttery, creamy	ND	0.47 ± 0.21 a	1.43 ± 0.56 b	1.58 ± 0.25 b	0.84 ± 0.33 a
Organic acids (3)						
Acetic acid	Sour, astringent, vinegar	1.71 ± 0.16 a	7.23 ± 1.18 b	6.33 ± 1.84 b	6.62 ± 0.85 b	6.73 ± 0.98 b
Isobutyric acid	Rancid, buttery, cheesy and hammy	ND	3.73 ± 0.47 a	1.98 ± 0.10 b	2.09 ± 0.10 b	1.24 ± 0.79 b
Isovaleric acid	Sweat, acid and rancid	ND	4.37 ± 0.13 a	2.51 ± 0.29 b	3.37 ± 0.15 c	2.50 ± 0.28 b
Pyrazines (1)						
Tetramethylpyrazine	Roasted and chocolate	ND	1.09 ± 0.24 a	0.69 ± 0.31 a	2.10 ± 0.15 b	1.88 ± 0.31 b
Terpenes (2)						
β-myrcene	Balsamic, peppery and spice	ND	ND	ND	ND	0.33 ± 0.02
Linalool	Citrus, orange and lemon	ND	0.43 ± 0.13 a	0.25 ± 0.04 b	ND	0.30 ± 0.07 ab
HPLC						
Glucose	--	11.97 ± 1.07 a	1.34 ± 0.16 b	0.13 ± 0.04 c	0.55 ± 0.27 bc	0.18 ± 0.16 c
Fructose	--	13.91 ± 1.16 a	8.79 ± 0.20 b	1.51 ± 0.10 c	8.51 ± 1.72 b	5.93 ± 0.70 d
Citric acid	--	2.35 ± 0.20 a	1.29 ± 0.10 b	0.26 ± 0.06 d	0.92 ± 0.38 bc	0.58 ± 0.14 cd
Lactic acid	--	ND	0.27 ± 0.14 a	0.36 ± 0.03 a	4.17 ± 0.33 b	0.95 ± 0.03 c
Acetic acid	--	ND	2.21 ± 0.16 a	1.39 ± 0.26 b	3.23 ± 0.23 c	5.24 ± 0.06 d

Source: The author (2020).

ND = not detected; HPLC = high-performance liquid chromatography; 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).

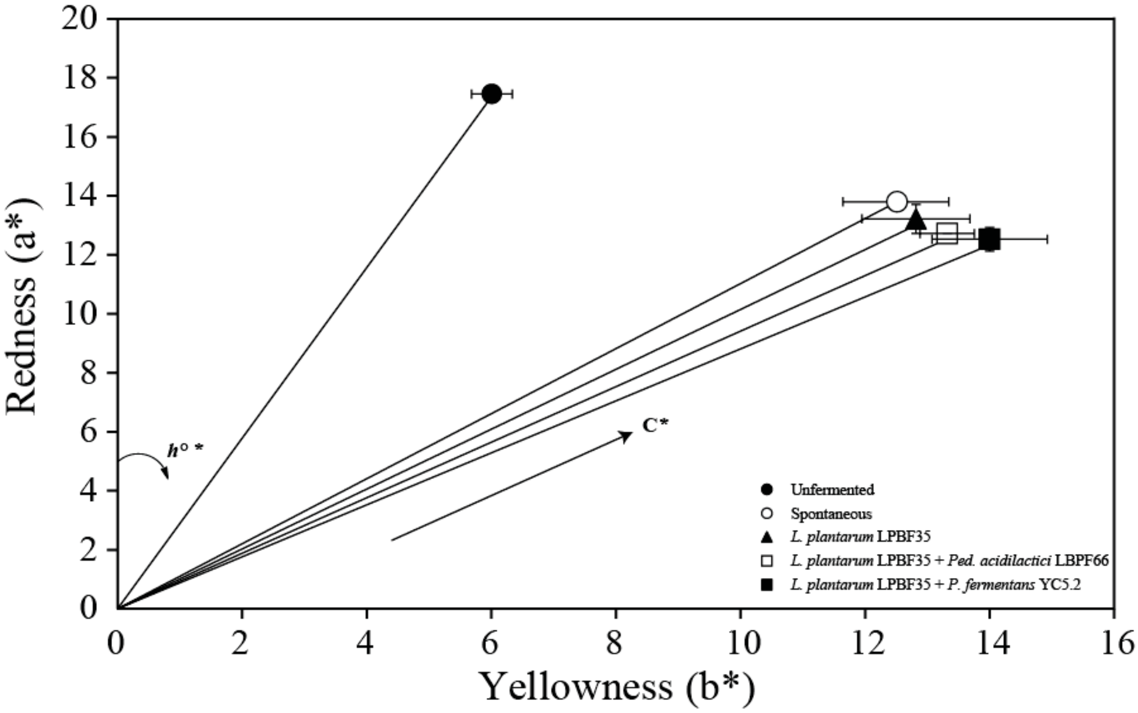
A total of 32 volatile organic compounds were identified in fermented cocoa beans, including esters (10), aldehydes (6), alcohols (5), ketones (5), organic acids (3), terpenes (2), and pyrazine (1) (TABLE 1). Cocoa beans generated from inoculated treatments had superior prevalence of several volatile compounds in comparison to spontaneous treatment, including 3-methyl-1-butanol acetate, acetoin, 2-phenylethyl acetate, 2-methyl-1-butanol acetate, benzaldehyde, and phenylethyl alcohol. The higher production and diffusion of primary metabolites (ethanol, lactic acid, and acetic acid) into cocoa beans during inoculated treatments may have caused an improved destruction of cocoa beans' cellular structure and released key enzymes (DE VUYST; WECKX, 2016). The biochemical and structural modifications trigger several biochemical reactions, such as proteolysis, hydrolysis, and oxidation, which generates both non-volatile precursors and volatile compounds (MUÑOZ et al., 2020). In addition, these compounds may have been accumulated due to diffusion from the secondary metabolism of the starter cultures (CRAFACK et al., 2013; LEFEBER et al., 2012; SANDHYA et al., 2016).

Interestingly, cocoa beans from *L. plantarum* LPBF35 + *P. fermentans* YC5.2 treatment showed a higher prevalence of specific compounds, such as ethyl acetate, 2,3-butanediol, benzaldehyde, and 2,3-butanedione, among the inoculated treatments. Both ethyl acetate and benzaldehyde are strictly associated with the metabolism of *P. fermentans* yeast (PEREIRA et al., 2014b), while 2,3-butanediol and 2,3-butanedione are associated with the citrate catabolism in *L. plantarum* (CUI et al., 2019). This result corroborates the existence of a non-competitive nature for primary carbon source between these microorganisms.

5.3.4.2. Color development

Unfermented cocoa bean powders presented a predominance of the red color ($h°* < 45°$), as revealed by colorimetric analysis reported in Figure 5. After fermentation, yellowness (b*) became dominant, with no statistical differences between treatments. Red color reduction and yellow increase is attributed to anthocyanin hydrolysis and polyphenol oxidation, respectively, that occurs in cocoa beans during fermentation (ROMERO-CORTES et al., 2013). The reduction of anthocyanins imparts on the brown color development, and the polyphenol oxidation process reduces the bitterness and astringency of the final chocolate (MISNAWI et al., 2003). From the color data obtained, total color differences (TCD) were estimated for each fermentation treatment using non-fermented beans as reference (control). Despite the use of starter cultures increased the production and diffusion of ethanol and acetic acid into the cotyledons, the inoculation of *L. plantarum* LPBF35, whether in pure (9.41 ± 1.33) or mixed inoculation with *Ped. acidilactici* LPBF66 (9.91 ± 1.28) or *P. fermentans* YC5.2 (12.52 ± 1.48), did not show statistical difference of TCD in comparison to the spontaneous fermentation (11.30 ± 0.55). However, the co-inoculation of *L. plantarum* LPBF35 + *P. fermentans* YC5.2 was the only treatment achieving a value above 12.0, implying absolutely different color spaces in comparison to the non-fermented beans (FRANCIS, 1983). This result is similar to those re- ported by Pereira et al. (2017), which demonstrated that the volatile and non-volatile composition changes in cocoa beans promoted by the inoculation of *P. kudriavzevii* did not affected the color development in comparison to spontaneous fermentations.

FIGURE 5 – CHANGES IN HUE ANGLE (*h*°*) AND CHROMA (C*).



Source: The author (2020).
(Mean ± standard deviation) of unfermented and fermented cocoa beans.

5.4 CONCLUSIONS

Recently, an increasing attention has been given to the co-culture of yeast and LAB, which is widely used in fermentation of wine, cheese, and other food products. Nevertheless, there is still a lack of knowledge on the interactions between LAB and yeast during cocoa beans fermentation. In this study, co-culturing FLAB and yeast enhanced sugar metabolism and aroma formation during cocoa beans fermentation. The faster consumption of fructose promoted by *L. plantarum* LPBF35 could significantly reduce the fermentation time required for cocoa beans fermentation and drying. In addition, the non-competitive nature for primary carbon sources between *L. plantarum* LPBF35 and *P. fermentans* YC5.2 allowed higher productions of ethanol, lactic acid, and ethyl acetate in comparison to the other treatments. The resulting cocoa beans from the co-starters also had a high content of primary and secondary metabolites in relation to the single fermentation treatment. The results of this study suggest that FLAB and yeast is a microbial consortium that can improve sugar metabolism and aroma formation during cocoa beans fermentation. Further research will be needed to fully evaluate the technological suitability and aromatic contribution of the distinct selected strains with AAB.

6 CONCLUSIONS

The action of microorganisms during cocoa beans fermentation is crucial for obtaining quality cocoa beans. In this process are formed precursor molecules of aroma, flavor and color, reducing the bitterness and astringency of the unfermented cocoa beans. The composition microbiota associated with cocoa fermentation has been studied through culture -dependent and -independent techniques. The culture-independent approaches, mainly NGS technologies, have allowed an in-depth analysis of the complex microbial communities in cocoa fermentations across the globe (e.g., Brazil, Ghana, Ivory Coast, Cameroon, Nicaragua, and Colombia), monitoring the dominance of lactic acid bacteria, acetic acid bacteria, and yeasts during the process, in addition, revealed a more complex microbial diversity, comprising uncultivable microorganisms, sub-dominant populations, and late-growing species with 83 species reported for the first time in cocoa fermentation.

Fructophilic lactic acid bacteria (FLAB) belonging to the genera *Fructobacillus* and *Lactobacillus* were identified during cocoa beans fermentation by NGS technology (Illumina MiSeq). *Lactobacillus plantarum* LPBF35 shows a “facultatively” fructophilic metabolism, preferring fructose over glucose in both anaerobic and aerobic conditions. Its implementation as a pure culture starter promoted a faster utilization of fructose and an ideal profile of organic acids metabolism (i.e., lactic and citric acids) associated with cocoa fermentation in laboratory-scale fermentations.

The *L. plantarum* LPBF35’ facultative fructophilic metabolism was confirmed by the presence of the *adhE1* and *adhE2* genes, which are associated with its growth without an external electron acceptor and a faster growth on fructose. Similarly, the inoculation of *L. plantarum* LPBF35 in mixed culture with *P. fermentans* YC5.2 during cocoa on-farm fermentation resulted in lowest levels of residual sugars after 72 h of fermentation (7.89 and 4.23 mg/g, for fructose and glucose, respectively). Besides, this mixed culture improved formation of primary (ethanol, lactic acid, and acetic acid) and secondary (2-methyl-1-butanol, isoamyl acetate, and ethyl acetate) metabolites during fermentation, which are fundamental characteristics for obtaining quality cocoa beans. Therefore, the results of this study suggest that fructophilic lactic acid bacteria and yeast is a microbial consortium that could improve sugar metabolism and aroma formation during cocoa beans fermentation.

REFERENCES

ABDEL-EL-HALEEM, D. *Acinetobacter*: environmental and biotechnological applications. **African Journal of Biotechnology**, v. 2, n. 4, p. 71–74, 2003.

ADAMS, G. O. et al. Bioremediation, biostimulation and bioaugmention: A review. **International Journal of Environmental Bioremediation & Biodegradation**, v. 3, n. 1, p. 28–39, 2015.

ADEYEYE, E. I. et al. Effect of farm and industrial processing on the amino acid profile of cocoa beans. **Food Chemistry**, v. 118, n. 2, p. 357–363, 2010.

ADIBPOUR, N. et al. A review on *Bacillus coagulans* as a spore-forming probiotic. **Applied Food Biotechnology**, v. 6, n. 2, p. 91–100, 2019.

ADLER, P. et al. Core fluxome and metafluxome of lactic acid bacteria under simulated cocoa pulp fermentation conditions. **Applied and Environmental Microbiology**, v. 79, n. 18, p. 5670–5681, 2013.

ADLER, P. et al. The key to acetate: Metabolic fluxes of acetic acid bacteria under cocoa pulp fermentation-simulating conditions. **Applied and Environmental Microbiology**, v. 80, n. 15, p. 4702–4716, 2014.

ADRIO, J. L.; DEMAIN, A. L. Microbial enzymes: tools for biotechnological processes. **Biomolecules**, v. 4, n. 1, p. 117–139, 2014.

AFOAKWA, E. O. **Chocolate Science and Technology**. Hoboken: Wiley-Blackwell, 2010.

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

AFOAKWA, E. O. et al. Changes in acidification, sugars and mineral composition of cocoa pulp during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans. **International Food Research Journal**, v. 20, n. 3, p. 1215–1222, 2013.

AGUIAR, T. Q.; SILVA, R.; DOMINGUES, L. *Ashbya gossypii* beyond industrial riboflavin production: A historical perspective and emerging biotechnological applications. **Biotechnology Advances**, v. 33, n. 8, p. 1774–1786, 2015.

AGYIRIFO, D. S. et al. Metagenomics analysis of cocoa bean fermentation microbiome identifying species diversity and putative functional capabilities. **Heliyon**, v. 5, n. 7, p. e02170, 2019.

AL-BAHRY, S. N. et al. Biosurfactant production by *Bacillus subtilis* B20 using date molasses and its possible application in enhanced oil recovery. **International Biodeterioration and Biodegradation**, v. 81, p. 141–146, 2013.

ALFENORE, S.; MOLINA-JOUVE, C. Current status and future prospects of conversion of lignocellulosic resources to biofuels using yeasts and bacteria. **Process Biochemistry**, v. 51, n. 11, p. 1747–1756, 2016.

ALMEIDA, O. G. G. et al. Does Quorum Sensing play a role in microbial shifts along spontaneous fermentation of cocoa beans? An in silico perspective. **Food Research International**, v. 131, p. 109034, 2020.

ANGUMEENAL, A. R.; VENKAPPAYYA, D. An overview of citric acid production. **LWT - Food Science and Technology**, v. 50, n. 2, p. 367–370, 2013.

AMOA-AWUA, W. K. Methods of cocoa fermentation and drying. In: **Cocoa and coffee fermentations**. SCHWAN, R.F.; FLEET, G.H. (Eds). Boca Raton: CRC Press, p. 71-128, 2015.

ANTRANIKIAN, G.; VORGIAS, C. E.; BERTOLDO, C. Extreme environments as a resource for microorganisms and novel biocatalysts. **Advances in Biochemical Engineering/Biotechnology**, v. 96, n. August, p. 219–262, 2005.

APROTOSOAIE, A. C.; LUCA, S. V.; MIRON, A. Flavor chemistry of cocoa and cocoa products - An overview. **Comprehensive Reviews in Food Science and Food Safety**, v. 15, n. 1, p. 73–91, 2016.

ARANA-SÁNCHEZ, A. et al. Identification of predominant yeasts associated with artisan Mexican cocoa fermentations using culture-dependent and culture-independent approaches. **World Journal of Microbiology and Biotechnology**, v. 31, n. 2, p. 359–369, 2015.

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

ARSHAD, M.; ZIA, M. A.; SHAH, F. A. **An overview of biofuel**. Springer International Publishing, 2018.

ASAF, S. et al. *Sphingomonas*: from diversity and genomics to functional role in environmental remediation and plant growth. **Critical Reviews in Biotechnology**, v. 40, n. 2, p. 138–152, 2020.

BADRIE, N. et al. Cocoa agronomy, quality, nutritional, and health aspects. **Critical Reviews in Food Science and Nutrition**, v. 55, n. 5, p. 620–659, 2015.

BANAT, I. M. et al. Microbial biosurfactants production, applications and future potential. **Applied Microbiology and Biotechnology**, v. 87, n. 2, p. 427–444, 2010.

BANKEVICH, A. et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. **Journal of Computational Biology**, v. 19, n. 5, p. 455–477, 2012.

BATISTA, N. N. et al. Dynamic behavior of *Saccharomyces cerevisiae*, *Pichia kluyveri* and *Hanseniaspora uvarum* during spontaneous and inoculated cocoa fermentations and

their effect on sensory characteristics of chocolate. **LWT - Food Science and Technology**, v. 63, n. 1, p. 221–227, 2015.
BATISTA, N. N. et al. The impact of yeast starter cultures on the microbial communities and volatile compounds in cocoa fermentation and the resulting sensory attributes of chocolate. **Journal of Food Science and Technology**, v. 53, n. 2, p. 1101–1110, 2016.

BEG, M. S. et al. Status, supply chain and processing of cocoa - A review. **Trends in Food Science and Technology**, v. 66, p. 108–116, 2017.

BEN-DOV, E.; KRAMARSKY-WINTER, E.; KUSHMARO, A. An in situ method for cultivating microorganisms using a double encapsulation technique. **FEMS Microbiology Ecology**, v. 68, n. 3, p. 363–371, 2009.

BOGAN, B. W.; SULLIVAN, W. R. Physicochemical soil parameters affecting sequestration and mycobacterial biodegradation of polycyclic aromatic hydrocarbons in soil. **Chemosphere**, v. 52, n. 10, p. 1717–1726, 2003.

BOLGER, A. M.; LOHSE, M.; USADEL, B. Trimmomatic: A flexible trimmer for Illumina sequence data. **Bioinformatics**, v. 30, n. 15, p. 2114–2120, 2014.

BORTOLINI, C. et al. Detailed analyses of the bacterial populations in processed cocoa beans of different geographic origin, subject to varied fermentation conditions. **International Journal of Food Microbiology**, v. 236, p. 98–106, 2016.

BREUER, U.; HARMS, H. Debaryomyces hansenii - an extremophilic yeast with biotechnological potential. **Yeast**, v. 23, n. 6, p. 415–437, 2006.

BRINGER, S.; BOTT, M. Central carbon metabolism and respiration in *Gluconobacter oxydans*. In: MATSUSHITA, K. et al. (Eds.). **Acetic acid bacteria: Ecology and physiology**. Japan: Springer, 2016. p. 235–254.

CALIGIANI, A. et al. Influence of fermentation level and geographical origin on cocoa bean oligopeptide pattern. **Food Chemistry**, v. 211, p. 431–439, 2016.

CAMU, N. et al. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. **Applied and Environmental Microbiology**, v. 73, n. 6, p. 1809–1824, 2007.

CAMU, N. et al. Influence of turning and environmental contamination on the dynamics of populations of lactic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation in Ghana. **Applied and Environmental Microbiology**, v. 74, n. 1, p. 86–98, 2008a.

CAMU, N. et al. Fermentation of cocoa beans: influence of microbial activities and polyphenol concentrations on the flavour of chocolate. **Journal of the Science of Food and Agriculture**, v. 88, p. 2288–2297, 2008b.

CAPORASO, J. G. et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. **ISME Journal**, v. 6, n. 8, p. 1621–1624, 2012.

CARVALHO NETO, D. P. et al. High-throughput rRNA gene sequencing reveals high and complex bacterial diversity associated with brazilian coffee bean fermentation. **Food Technology and Biotechnology**, v. 56, n. 1, p. 90–95, 2018.

CASTRO-ALAYO, E. M. et al. Formation of aromatic compounds precursors during fermentation of Criollo and Forastero cocoa. **Heliyon**, v. 5, n. 1, p. e01157, 2019.

CAVALLI-SFORZA, L. L.; EDWARDS, A. W. Phylogenetic analysis. Models and estimation procedures. **American Journal of Human Genetics**, v. 19, n. 3, p. 233–257, 1967.

CEVALLOS-CEVALLOS, J. M. et al. Time-related changes in volatile compounds during fermentation of bulk and fine-flavor cocoa (*Theobroma cacao*) beans. **Journal of Food Quality**, v. 2018, p. 1–14, 2018.

CHAWLA, N. et al. *Gluconacetobacter diazotrophicus*: An overview. **Research in Environment and Life Sciences**, v. 7, n. 1, p. 1–10, 2014.

CHEN, F.; ZHU, L.; QIU, H. Isolation and probiotic potential of *Lactobacillus salivarius* and *Pediococcus pentosaceus* in specific pathogen free chickens. **Revista Brasileira de Ciencia Avicola**, v. 19, n. 2, p. 325–332, 2017.

CHENG, H. R.; JIANG, N. Extremely rapid extraction of DNA from bacteria and yeasts. **Biotechnology Letters**, v. 28, n. 1, p. 55–59, 2006.

CONNON, S. A.; GIOVANNONI, S. J. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. **Applied and Environmental Microbiology**, v. 68, n. 8, p. 3878–3885, 2002.

COPETTI, M. V. et al. Fungi and mycotoxins in cocoa: From farm to chocolate. **International Journal of Food Microbiology**, v. 178, p. 13–20, 2014.

CORONADO, M.-J. et al. Production and biochemical characterization of an α-amylase from the moderate halophile *Halomonas meridiana*. **FEMS Microbiology Letters**, v. 183, n. 1, p. 67–71, 2000.

CRAFACK, M. et al. Influencing cocoa flavour using *Pichia kluyveri* and *Kluyveromyces marxianus* in a defined mixed starter culture for cocoa fermentation. **International Journal of Food Microbiology**, v. 167, n. 1, p. 103–116, 2013.

CUI, S. et al. Effect of different *Lactobacillus* species on volatile and nonvolatile flavor compounds in juices fermentation. **Food Science and Nutrition**, v. 7, n. 7, p. 2214–2223, 2019.

DANIEL, H.-M. et al. Yeast diversity of Ghanaian cocoa bean heap fermentations. **FEMS Yeast Research**, v. 9, n. 5, p. 774–783, 2009.

DAS, P.; MUKHERJEE, S.; SEN, R. Antimicrobial potential of a lipopeptide biosurfactant derived from a marine *Bacillus circulans*. **Journal of Applied Microbiology**, v. 104, n. 6, p. 1675–1684, 2008.

DE VUYST, L.; LEROY, F. Functional role of yeasts, lactic acid bacteria and acetic acid bacteria in cocoa fermentation processes. **FEMS Microbiology Reviews**, v. 44, n. 4, p. 432–453, 2020.

DE VUYST, L.; WECKX, S. The cocoa bean fermentation process: from ecosystem analysis to starter culture development. **Journal of Applied Microbiology**, v. 121, n. 1, p. 5–17, 2016.

DEPOORTER, E. et al. *Burkholderia*: an update on taxonomy and biotechnological potential as antibiotic producers. **Applied Microbiology and Biotechnology**, v. 100, n. 12, p. 5215–5229, 2016.

DINESH, G. K.; CHAUHAN, R.; CHAKMA, S. Influence and strategies for enhanced biohydrogen production from food waste. **Renewable and Sustainable Energy Reviews**, v. 92, p. 807–822, 2018.

DOURADO, M. N. et al. Biotechnological and agronomic potential of endophytic pink-pigmented methylotrophic Methylobacterium spp. **BioMed Research International**, v. 2015, 2015.

ELOUTASSI, N. et al. Biotechnological recovery of industrial waste: Production of bioethanol from free yeast and immobilized from whey. **Journal of Applied Science and Environmental Studies**, v. 1, n. 1, p. 28–35, 2018.

ELSHAGHABEE, F. M. F. et al. *Bacillus* as potential probiotics: Status, concerns, and future perspectives. **Frontiers in Microbiology**, v. 8, p. 1–15, 2017.

ENDO, A. et al. *Lactobacillus florum* sp. nov., a fructophilic species isolated from flowers. **International Journal of Systematic and Evolutionary Microbiology**, v. 60, n. 10, p. 2478–2482, 2010.

ENDO, A. et al. Characterization and emended description of *Lactobacillus kunkeei* as a fructophilic lactic acid bacterium. **International Journal of Systematic and Evolutionary Microbiology**, v. 62, n. PART 3, p. 500–504, 2012.

ENDO, A. et al. Fructophilic characteristics of *Fructobacillus* spp. may be due to the absence of an alcohol/acetaldehyde dehydrogenase gene (adhE). **Current Microbiology**, v. 68, n. 4, p. 531–535, 2014.

ENDO, A. et al. Comparative genomics of *Fructobacillus* spp. and *Leuconostoc* spp. reveals niche-specific evolution of *Fructobacillus* spp. **BMC Genomics**, v. 16, n. 1, p. 1–13, 2015.

ENDO, A. et al. Fructophilic lactic acid bacteria, a unique group of fructose-fermenting microbes. **Applied and Environmental Microbiology**, v. 84, n. 19, p. 1–14, 2018.

ENDO, A.; FUTAGAWA-ENDO, Y.; DICKS, L. M. T. Isolation and characterization of fructophilic lactic acid bacteria from fructose-rich niches. **Systematic and Applied Microbiology**, v. 32, n. 8, p. 593–600, 2009.

ENDO, A.; OKADA, S. Reclassification of the genus *Leuconostoc* and proposals of *Fructobacillus fructosus* gen. nov., comb. nov., *Fructobacillus durionis* comb. nov., *Fructobacillus ficulneus* comb. nov. and *Fructobacillus pseudoficulneus* comb. nov. **International Journal of Systematic and Evolutionary Microbiology**, v. 58, n. 9, p. 2195–2205, 2008.

ENDO, A.; SALMINEN, S. Honeybees and beehives are rich sources for fructophilic lactic acid bacteria. **Systematic and Applied Microbiology**, v. 36, n. 6, p. 444–448, 2013.

ENGESETH, N. J.; AC PANGAN, M. F. Current context on chocolate flavor development — a review. **Current Opinion in Food Science**, v. 21, p. 84–91, 2018.

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

FANG, F. et al. Characterization of a *Lactobacillus brevis* strain with potential oral probiotic properties. **BMC Microbiology**, v. 18, n. 221, p. 1–9, 2018.

FELSENTEIN, J. Confidence limits on phylogenies: An approach using the bootstrap. **Evolution**, v. 39, n. 4, p. 783–791, 1985.

FIGUEROA-HERNÁNDEZ, C. et al. The challenges and perspectives of the selection of starter cultures for fermented cocoa beans. **International Journal of Food Microbiology**, v. 301, p. 41–50, 2019.

FILANNINO, P. et al. Metabolism of fructophilic lactic acid bacteria isolated from the *Apis mellifera* L. bee gut: Phenolic acids as external electron acceptors. **Applied and Environmental Microbiology**, v. 82, n. 23, p. 6899–6911, 2016.

FILANNINO, P. et al. Fructose-rich niches traced the evolution of lactic acid bacteria toward fructophilic species. **Critical Reviews in Microbiology**, v. 45, n. 1, p. 65–81, 2019.

FLEET, G. H. Yeasts in foods and beverages: impact on product quality and safety. **Current Opinion in Biotechnology**, v. 18, n. 2, p. 170–175, 2007.

FOWLER, M. S. **Cocoa Beans: From Tree to Factory**. Fourth ed. Blackwell Publishing Ltd., 2009.

FOOD AND AGRICULTURE ORGANIZATION OF UNITED NATIONS (FAO). **FAOSTAT**. Disponível em: <http://www.fao.org/faostat/en/#data/QC/visualize>. Acesso em: 16 jul 2020.

FRANCIS, F. J. Colorimetry of foods. In: PELEG, M.; BAGLEY, E.B. (Eds.). **Physical Properties of Foods**. Westport: AVI Publishing, 1983.pp. 105–124.

FUSCO, V. et al. The genus *Weissella*: Taxonomy, ecology and biotechnological potential. **Frontiers in Microbiology**, v. 6, p. 1–22, 2015.

GALLEGOS, J. et al. Target identification of volatile metabolites to allow the differentiation of lactic acid bacteria by gas chromatography-ion mobility spectrometry. **Food Chemistry**, v. 220, p. 362–370, 2017.

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

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

GOMAA, E. Z.; EL-MEIHY, R. M. Bacterial biosurfactant from *Citrobacter freundii* MG812314.1 as a bioremoval tool of heavy metals from wastewater. **Bulletin of the National Research Centre**, v. 43, n. 69, p. 1–14, 2019.

GOULET, C. et al. Role of an esterase in flavor volatile variation within the tomato clade. **Proceedings of the National Academy of Sciences of the United States of America**, v. 109, n. 46, p. 19009–19014, 2012.

GUPTA, A. et al. Anaerobic fermentation of glycerol in *Paenibacillus macerans*: Metabolic pathways and environmental determinants. **Applied and Environmental Microbiology**, v. 75, n. 18, p. 5871–5883, 2009.

GUSMÃO, C. A. B.; RUFINO, R. D.; SARUBBO, L. A. Laboratory production and characterization of a new biosurfactant from *Candida glabrata* UCP1002 cultivated in vegetable fat waste applied to the removal of hydrophobic contaminant. **World Journal of Microbiology and Biotechnology**, v. 26, n. 9, p. 1683–1692, 2010.

GUSTAW, K. et al. Isolation and characterization of a new fructophilic *Lactobacillus plantarum* FPL strain from honeydew. **Annals of Microbiology**, v. 68, n. 7, p. 459–470, 2018.

HAHN, M. W. et al. The filtration-acclimatization method for isolation of an important fraction of the not readily cultivable bacteria. **Journal of Microbiological Methods**, v. 57, n. 3, p. 379–390, 2004.

HAMDOUCHE, Y. et al. Dynamics of microbial ecology during cocoa fermentation and drying: Towards the identification of molecular markers. **Food Control**, v. 48, p. 117–122, 2015.

HAMDOUCHE, Y. et al. Impact of turning, pod storage and fermentation time on microbial ecology and volatile composition of cocoa beans. **Food Research International**, v. 119, p. 477–491, 2019.

HAMID, R. et al. Chitinases: An update. **Journal of Pharmacy and Bioallied Sciences**, v. 5, n. 1, p. 21–29, 2013.

HANSEN, C. E.; OLMO, M. DEL; BURRI, C. Enzyme activities in cocoa beans during fermentation. **Journal of Food Science and Agriculture**, v. 77, p. 273–281, 1998.
HARA, Y. et al. The complete genome sequence of *Pantoea ananatis* AJ13355, an organism with great biotechnological potential. **Applied Microbiology and Biotechnology**, v. 93, n. 1, p. 331–341, 2012.

HATMI, R. U.; KOBARSIH, M.; CAHYANINGRUM, N. Fungi level analysis of cocoa beans based on fermentation box type and duration. **Procedia Food Science**, v. 3, p. 371–382, 2015.

HAZELWOOD, L. A. et al. The Ehrlich pathway for fusel alcohol production: A century of research on *Saccharomyces cerevisiae* metabolism. **Applied and Environmental Microbiology**, v. 74, n. 8, p. 2259–2266, 2008.

HEBERLE, H. et al. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. **BMC Bioinformatics**, v. 16, n. 169, p. 1–7, 2015.

HII, C. L. et al. Polyphenols in cocoa (*Theobroma cacao* L.). **Asian Journal of Food & Agro-Industry**, v. 2, n. 204, p. 702–722, 2009.

HO, V. T. T.; FLEET, G. H.; ZHAO, J. Unravelling the contribution of lactic acid bacteria and acetic acid bacteria to cocoa fermentation using inoculated organisms. **International Journal of Food Microbiology**, v. 279, n. September 2017, p. 43–56, 2018.

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

HO, V. T. T.; ZHAO, J.; FLEET, G. The effect of lactic acid bacteria on cocoa bean fermentation. **International Journal of Food Microbiology**, v. 205, p. 54–67, 2015.

HUANG, C.-H. et al. The probiotic activity of *Lactobacillus murinus* against food allergy. **Journal of Functional Foods**, v. 25, n. 1, p. 231–241, 2016.

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

HUGENHOLTZ, P.; GOEBEL, B. M.; PACE, N. R. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. **Journal of Bacteriology**, v. 180, n. 18, p. 4765–4774, 1998.

IASUR-KRUH, L. et al. *Dyella*-Like bacterium isolated from an insect as a potential biocontrol agent against grapevine yellows. **Phytopathology**, v. 108, n. 3, p. 336–341, 2018.

IBRAHIM, H. M. M. Characterization of biosurfactants produced by novel strains of *Ochrobactrum anthropi* HM-1 and *Citrobacter freundii* HM-2 from used engine oil-contaminated soil. **Egyptian Journal of Petroleum**, v. 27, n. 1, p. 21–29, 2018.

ILLEGHEMS, K. et al. Phylogenetic analysis of a spontaneous cocoa bean fermentation metagenome reveals new insights into its bacterial and fungal community diversity. **PLoS**

ONE, v. 7, n. 5, p. e38040, 2012.

ILLEGHEMS, K.; DE VUYST, L.; WECKX, S. Complete genome sequence and comparative analysis of *Acetobacter pasteurianus* 386B, a strain well-adapted to the cocoa bean fermentation ecosystem. **BMC Genomics**, v. 14, n. 1, p. 1–14, 2013.

ILLEGHEMS, K.; DE VUYST, L.; WECKX, S. Comparative genome analysis of the candidate functional starter culture strains *Lactobacillus fermentum* 222 and *Lactobacillus plantarum* 80 for controlled cocoa bean fermentation processes. **BMC Genomics**, v. 16, n. 1, p. 1–13, 2015.

ILLEGHEMS, K.; WECKX, S.; DE VUYST, L. Applying meta-pathway analyses through metagenomics to identify the functional properties of the major bacterial communities of a single spontaneous cocoa bean fermentation process sample. **Food Microbiology**, v. 50, p. 54–63, 2015.

The International Cocoa Organization (ICCO). **ICCO Quarterly Bulletin of Cocoa Statistics**, v. XLIV, n. 3, cocoa year 2017/18.

IZAWA, N. et al. Production of aroma compounds from whey using *Wickerhamomyces pijperi*. **AMB Express**, v. 5, n. 23, p. 1–9, 2015.

JANEK, K. et al. The cleavage specificity of the aspartic protease of cocoa beans involved in the generation of the cocoa-specific aroma precursors. **Food Chemistry**, v. 211, p. 320–328, 2016.

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

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

JI, Y. S. et al. Modulation of the murine microbiome with a concomitant anti-obesity effect by *Lactobacillus rhamnosus* GG and *Lactobacillus sakei* NR28. **Beneficial Microbes**, v. 3, n. 1, p. 13–22, 2012.

JOSHI, A. A.; KANEKAR, P. P. Production of exopolysaccharide by *Vagococcus carniphilus* MCM B-1018 isolated from alkaline Lonar Lake, India. **Annals of Microbiology**, v. 61, n. 4, p. 733–740, 2011.

JUAREZ-JIMENEZ, B. et al. Production of chitinolytic enzymes by a strain (BM17) of Paenibacillus pabuli isolated from crab shells samples collected in the east sector of central Tyrrhenian Sea. **International Journal of Biological Macromolecules**, v. 43, n. 1, p. 27–31, 2008.

KADOW, D. et al. Identification of main fine or flavour components in two genotypes of the cocoa tree (*Theobroma cacao* L.). **Journal of Applied Botany and Food Quality**, v. 86, n. 1, p. 90–98, 2013.

KADOW, D. et al. Fermentation-like incubation of cocoa seeds (*Theobroma cacao* L.) -

Reconstruction and guidance of the fermentation process. **LWT - Food Science and Technology**, v. 62, n. 1, p. 357–361, 2015.
KADRI, T. et al. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by fungal enzymes: A review. **Journal of Environmental Sciences**, v. 51, p. 52–74, 2017.

KLEEREBEZEM, M. et al. Complete genome sequence of *Lactobacillus plantarum* WCFS1. **Proceedings of the National Academy of Sciences of the United States of America**, v. 100, n. 4, p. 1990–1995, 2003.

KLUGE, A. G.; FARRIS, J. S. Quantitative phyletics and the evolution biologists. **Systematic Zoology**, v. 18, n. 1, p. 1–32, 1969.

KONÉ, M. K. et al. Contribution of predominant yeasts to the occurrence of aroma compounds during cocoa bean fermentation. **Food Research International**, v. 89, p. 910–917, 2016.

KONGOR, J. E. et al. Factors influencing quality variation in cocoa (*Theobroma cacao*) bean flavour profile - A review. **Food Research International**, v. 82, p. 44–52, 2016.

KONURAY, G.; ERGINKAYA, Z. Potential use of *Bacillus coagulans* in the food industry. **Foods**, v. 7, n. 92, p. 1–10, 2018.

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

KOUTSOMPOGERAS, P.; KYRIACOU, A.; ZABETAKIS, I. The formation of 2,5-dimethyl-4-hydroxy-2H-furan-3-one by cell-free extracts of *Methylobacterium extorquens* and strawberry (Fragaria x ananassa cv. Elsanta). **Food Chemistry**, v. 104, p. 1654–1661, 2007.

KRAJEWSKI, V. et al. Metabolic engineering of *Gluconobacter oxydans* for improved growth rate and growth yield on glucose by elimination of gluconate formation . **Applied and Environmental Microbiology**, v. 76, n. 13, p. 4369–4376, 2010.

KRESNOWATI, M. T. A. P.; SURYANI, L.; AFFIFAH, M. Improvement of cocoa beans fermentation by LAB starter addition. **Journal of Medical and Bioengineering**, v. 2, n. 4, p. 274–278, 2013.

KUMAR, S. et al. MEGA X: Molecular evolutionary genetics analysis across computing platforms. **Molecular Biology and Evolution**, v. 35, n. 6, p. 1547–1549, 2018.

LAËTITIA, G.; PASCAL, D.; YANN, D. The citrate metabolism in homo- and heterofermentative LAB: A selective means of becoming dominant over other microorganisms in complex ecosystems. **Food and Nutrition Sciences**, v. 05, n. 10, p. 953–969, 2014.

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

LALIBERTÉ, B. **A Global strategy for the conservation and use of cacao genetic resources as the foundation for a sustainable cocoa economy**. Montpellier: CacaoNet, 2012.

LANE, D. J. et al. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses (reverse transcriptase/dideoxynudeotide). **Evolution**, v. 82, n. October, p. 6955–6959, 1985.

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

LEE, S. H. et al. Draft genome sequence of a lipolytic yeast, *Candida aaseri* SH-14. **Genome Annoucements**, v. 5, n. 20, p. e00373-17, 2017.

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

LEFEBER, T. et al. Dynamics and species diversity of communities of lactic acid bacteria and acetic acid bacteria during spontaneous cocoa bean fermentation in vessels. **Food Microbiology**, v. 28, n. 3, p. 457–464, 2011a.

LEFEBER, T. et al. Interesting starter culture strains for controlled cocoa bean fermentation revealed by simulated cocoa pulp fermentations of cocoa-specific lactic acid bacteria. **Applied and Environmental Microbiology**, v. 77, n. 18, p. 6694–6698, 2011b.

LEFEBER, T. et al. On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. **Food Microbiology**, v. 30, n. 2, p. 379–392, 2012.

LEMOS JUNIOR, W. J. F. et al. Potential use of *Starmerella bacillaris* as fermentation starter for the production of low-alcohol beverages obtained from unripe grapes. **International Journal of Food Microbiology**, v. 303, p. 1–8, 2019.

LIMA, C. O. DE C. et al. Integrating microbial metagenomics and physicochemical parameters and a new perspective on starter culture for fine cocoa fermentation. **Food Microbiology**, v. 93, p. 103608, 2021.

LIMA, H. L. S. et al. Bacterial cellulose production by *Komagataeibacter hansenii* ATCC 23769 using sisal juice - An agroindustry waste. **Brazilian Journal of Chemical Engineering**, v. 34, n. 3, p. 671–680, 2017.

LIMA, LÍ. J. R. et al. *Theobroma cacao* L., “The food of the Gods”: Quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. **Critical Reviews in Food Science and Nutrition**, v. 51, n. 8, p. 731–761, 2011.

LIN, C. et al. Enhanced production of prodigiosin by *Serratia marcescens* FZSF02 in the form of pigment pellets. **Electronic Journal of Biotechnology**, v. 40, p. 58–64, 2019.

LONVAUD-FUNEL, A.; JOYEUX, A. Antagonism between lactic acid bacteria of wines: inhibition of *Leuconostoc oenos* by *Lactobacillus plantarum* and *Pediococcus pentosaceus*. **Food Microbiology**, v. 10, p. 411–419, 1993.

LOPETUSO, L. R. et al. *Bacillus clausii* and gut homeostasis: state of the art and future perspectives. **Expert Review of Gastroenterology and Hepatology**, v. 10, n. 8, p. 943–948, 2016.

LÖSER, C.; URIT, T.; BLEY, T. Perspectives for the biotechnological production of ethyl acetate by yeasts. **Applied Microbiology and Biotechnology**, v. 98, n. 12, p. 5397–5415, 2014.

LU, M. et al. Fine structure of Tibetan kefir grains and their yeast distribution, diversity, and shift. **PLoS ONE**, v. 9, n. 6, p. e101387, 2014.

LUNA, J. M.; SARUBBO, L.; CAMPOS-TAKAKI, G. M. A new biosurfactant produced by *Candida glabrata* UCP 1002: Characteristics of stability and application in oil recovery. **Brazilian Archives of Biology and Technology**, v. 52, n. 4, p. 785–793, 2009.

LYU, Y. et al. Biodegradation of polycyclic aromatic hydrocarbons by *Novosphingobium pentaromativorans* US6-1. **PLoS ONE**, v. 9, n. 7, p. e1014438, 2014.

MA, X. et al. The quality changes and microflora analysis of commercial instant soya milk. **Food Science and Nutrition**, v. 5, n. 1, p. 123–130, 2016.

MACORI, G.; COTTER, P. D. Novel insights into the microbiology of fermented dairy foods. **Current Opinion in Biotechnology**, v. 49, p. 172–178, 2018.

MAENO, S. et al. Genomic characterization of a fructophilic bee symbiont *Lactobacillus kunkeei* reveals its niche-specific adaptation. **Systematic and Applied Microbiology**, v. 39, n. 8, p. 516–526, 2016.

MAENO, S. et al. *Lactobacillus apinorum* belongs to the fructophilic lactic acid bacteria. **Bioscience of Microbiota, Food and Health**, v. 36, n. 4, p. 147–149, 2017.

MAGNUSON, T. S. et al. Proteogenomic and functional analysis of chromate reduction in *Acidiphilium cryptum* JF-5, an Fe(III)-respiring acidophile. **BioMetals**, v. 23, n. 6, p. 1129–1138, 2010.

MAHAZAR, N. H. et al. *Candida* sp. as a starter culture for cocoa (*Theobroma cacao* L.) beans fermentation *Candida* sp. as a starter culture for cocoa (Theobroma cacao L.) beans fermentation. **International Food Research Journal**, v. 22, n. 5, p. 1783–1787, 2015.

MAMLOUK, D.; GULLO, M. Acetic acid bacteria: Physiology and carbon sources oxidation. **Indian Journal of Microbiology**, v. 53, n. 4, p. 377–384, 2013.

MAYO, B. et al. Impact of Next Generation Sequencing techniques in food microbiology. **Current Genomics**, v. 15, n. 4, p. 293–309, 2014.

MEERSMAN, E. et al. Detailed analysis of the microbial population in Malaysian spontaneous cocoa pulp fermentations reveals a core and variable microbiota. **PLoS ONE**, v. 8, n. 12, p. e81559, 2013.

MELO, T. A. et al. Functional profile evaluation of *Lactobacillus fermentum* TCUESC01: A New potential probiotic strain isolated during cocoa fermentation. **BioMed Research International**, v. 2017, p. 1–7, 2017.

MENEZES, A. G. T. et al. Investigation of chocolate produced from four different Brazilian varieties of cocoa (*Theobroma cacao* L.) inoculated with *Saccharomyces cerevisiae*. **Food Research International**, v. 81, p. 83–90, 2016.

MESSAOUDI, S. et al. *Lactobacillus salivarius*: Bacteriocin and probiotic activity. **Food Microbiology**, v. 36, n. 2, p. 296–304, 2013.

MICHLMAYR, H. et al. A β-glucosidase from *Oenococcus oeni* ATCC BAA-1163 with potential for aroma release in wine: Cloning and expression in *E. coli*. **World Journal of Microbiology and Biotechnology**, v. 26, n. 7, p. 1281–1289, 2010.

MIGUEL, M. G. DA C. P. et al. Cocoa fermentation: Microbial identification by MALDI-TOF MS, and sensory evaluation of produced chocolate. **LWT - Food Science and Technology**, v. 77, p. 362–369, 2017.

MILLER, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Chemistry**, v. 31, n. 3, p. 426–428, 1959.

MISNAWI et al. Effects of incubation and polyphenol oxidase enrichment on colour, fermentation index, procyanidins and astringency of unfermented and partly fermented cocoa beans. **International Journal of Food Science and Technology**, v. 38, n. 3, p. 285–295, 2003.

MOENS, F.; LEFEBER, T.; DE VUYST, L. Oxidation of metabolites highlights the microbial interactions and role of *Acetobacter pasteurianus* during cocoa bean fermentation. **Applied and Environmental Microbiology**, v. 80, n. 6, p. 1848–1857, 2014.

MOLINARI, F. et al. Aldehyde production by alcohol oxidation with *Gluconobacter oxydan*. **Applied Microbiology and Biotechnology**, v. 43, p. 989–994, 1995.

MORATA, A. et al. *Lachancea thermotolerans* applications in wine technology. **Fermentation**, v. 4, n. 3, p. 1–12, 2018.

MOREIRA, I. M. DA V. et al. Microbial succession and the dynamics of metabolites and sugars during the fermentation of three different cocoa (*Theobroma cacao* L.) hybrids. **Food Research International**, v. 54, n. 1, p. 9–17, 2013.

MOTA-GUTIERREZ, J. et al. Dynamics and biodiversity of bacterial and yeast communities during fermentation of cocoa beans. **Applied and Environmental Microbiology**, v. 84, n. 19, p. 1–17, 2018.

MOTA-GUTIERREZ, J. et al. Traceability of functional volatile compounds generated on inoculated cocoa fermentation and its potential health benefits. **Nutrients**, v. 11, n. 4, p. 884, 2019.

MOTAMAYOR, J. C. et al. Geographic and genetic population differentiation of the Amazonian chocolate tree (*Theobroma cacao* L). **PLoS ONE**, v. 3, n. 10, p. e3111, 2008.

MOUNJOUENPOU, P. et al. Filamentous fungi producing ochratoxin a during cocoa processing in Cameroon. **International Journal of Food Microbiology**, v. 121, p. 234-241.

MUJUMDAR, S.; JOSHI, P.; KARVE, N. Production, characterization, and applications of bioemulsifiers (BE) and biosurfactants (BS) produced by *Acinetobacter* spp.: A review. **Journal of Basic Microbiology**, v. 59, n. 3, p. 1–11, 2019.

MUÑOZ, M. S. et al. An overview of the physical and biochemical transformation of cocoa seeds to beans and to chocolate: Flavor formation. **Critical Reviews in Food Science and Nutrition**, v. 60, n. 10, p. 1593–1613, 2020.

NEVELING, D. P.; ENDO, A.; DICKS, L. M. T. Fructophilic *Lactobacillus kunkeei* and *Lactobacillus brevis* isolated from fresh flowers, bees and bee-hives. **Current Microbiology**, v. 65, n. 5, p. 507–515, 2012.

NGANG, J.-J. E. et al. Antifungal properties of selected lactic acid bacteria and application in the biological control of ochratoxin A producing fungi during cocoa fermentation. **Biocontrol Science and Technology**, v. 25, n. 3, p. 245–259, 2015.

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

NIELSEN, D. S.; SNITKJAER, P.; VAN DEN BERG, F. Investigating the fermentation of cocoa by correlating Denaturing Gradient Gel Electrophoresis profiles and Near Infrared spectra. **International Journal of Food Microbiology**, v. 125, n. 2, p. 133–140, 2008.

ORDOÑEZ-ARAQUE, R. H. et al. Microorganisms during cocoa fermentation: systematic review. **Foods and Raw Materials**, v. 8, n. 1, p. 155–162, 2020.

OSTOVAR, K.; KEENEY, P. G. Isolation and characterization of microrganisms involved in the fermentation of Trinidad’s cacao beans. **Journal of Food Science**, v. 38, n. 7973, p. 611–6117, 1973.

OUATTARA, H. D. et al. Lactic acid bacteria involved in cocoa beans fermentation from Ivory Coast: Species diversity and citrate lyase production. **International Journal of Food Microbiology**, v. 256, p. 11–19, 2017.

OUATTARA, H. G. et al. Implication of *Bacillus* sp. in the production of pectinolytic enzymes during cocoa fermentation. **World Journal of Microbiology and Biotechnology**, v. 24, n. 9, p. 1753–1760, 2008.

OUATTARA, H. G. et al. Molecular identification and pectate lyase production by Bacillus strains involved in cocoa fermentation. **Food Microbiology**, v. 28, n. 1, p. 1–8, 2011.

OUATTARA, H. G.; ELIAS, R. J.; DUDLEY, E. G. Microbial synergy between *Pichia kudriazevii* YS201 and *Bacillus subtilis* BS38 improves pulp degradation and aroma production in cocoa pulp simulation medium. **Heliyon**, v. 6, n. 1, p. e03269, 2020.

OZTURK, G.; YOUNG, G. M. Food evolution: The impact of society and science on the fermentation of cocoa beans. **Comprehensive Reviews in Food Science and Food Safety**, v. 16, n. 3, p. 431–455, 2017.

PACHECO-MONTEALEGRE, M. E. et al. Fine resolution analysis of microbial communities provides insights into the variability of cocoa bean fermentation. **Frontiers in Microbiology**, v. 11, p. 1–15, 2020.

PANDA, A. K. et al. Brevibacillus as a biological tool: A short review. **Antonie van Leeuwenhoek**, v. 105, n. 4, p. 623–639, 2014.

PANDA, A. K. et al. Cloning and in silico analysis of a high-temperature inducible lipase from *Brevibacillus*. **Arabian Journal for Science and Engineering**, v. 41, n. 6, p. 2159–2170, 2016.

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

PAPALEXANDRATOU, Z. et al. Species diversity, community dynamics, and metabolite kinetics of the microbiota associated with traditional Ecuadorian spontaneous cocoa bean fermentations. **Applied and Environmental Microbiology**, v. 77, n. 21, p. 7698–7714, 2011b.

PAPALEXANDRATOU, Z. et al. Spontaneous organic cocoa bean box fermentations in Brazil are characterized by a restricted species diversity of lactic acid bacteria and acetic acid bacteria. **Food Microbiology**, v. 28, n. 7, p. 1326–1338, 2011c.

PAPALEXANDRATOU, Z. et al. *Hanseniaspora opuntiae*, *Saccharomyces cerevisiae*, *Lactobacillus fermentum*, and *Acetobacter pasteurianus* predominate during well-performed Malaysian cocoa bean box fermentations, underlining the importance of these microbial species for a successful cocoa. **Food Microbiology**, v. 35, n. 2, p. 73–85, 2013.

PAPALEXANDRATOU, Z. et al. Linking cocoa varietals and microbial diversity of Nicaraguan fine cocoa bean fermentations and their impact on final cocoa quality appreciation. **International Journal of Food Microbiology**, v. 304, p. 106–118, 2019.

PAPALEXANDRATOU, Z.; DE VUYST, L. Assessment of the yeast species composition of cocoa bean fermentations in different cocoa-producing regions using denaturing gradient gel electrophoresis. **FEMS Yeast Research**, v. 11, n. 7, p. 564–574, 2011.

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

PATEL, A. et al. An overview of potential oleaginous microorganisms and their role in biodiesel and omega-3 fatty acid-based industries. **Microorganisms**, v. 8, n. 3, p. 1–40, 2020.

PENTJUSS, A. et al. Biotechnological potential of respiring *Zymomonas mobilis*: A stoichiometric analysis of its central metabolism. **Journal of Biotechnology**, v. 165, n. 1, p. 1–10, 2013.

PEREIRA, A. P. M.; SANT'ANA, A. S. Diversity and fate of spore forming bacteria in cocoa powder, milk powder, starch and sugar during processing: A review. **Trends in Food Science & Technology**, v. 76, p. 101–118, 2018.

PEREIRA, F. B. et al. Industrial robust yeast isolates with great potential for fermentation of lignocellulosic biomass. **Bioresource Technology**, v. 161, p. 192–199, 2014a.

PEREIRA, G. V. DE M. et al. Microbiological and physicochemical characterization of small-scale socoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture. **Applied and Environmental Microbiology**, v. 78, n. 15, p. 5395–5405, 2012.

PEREIRA, G. V. DE M. et al. Spontaneous cocoa bean fermentation carried out in a novel-design stainless steel tank: Influence on the dynamics of microbial populations and physical-chemical properties. **International Journal of Food Microbiology**, v. 161, n. 2, p. 121–133, 2013a.

PEREIRA, G. V. DE M. et al. Isolation, selection and evaluation of yeasts for use in fermentation of coffee beans by the wet process. **International Journal of Food Microbiology**, v. 188, p. 60–66, 2014b.

PEREIRA, G. V. DE M. et al. How to select a probiotic? A review and update of methods and criteria. **Biotechnology Advances**, v. 36, n. 8, p. 2060–2076, 2018.

PEREIRA, G. V. DE M. et al. Lactic acid bacteria: what coffee industry should know? **Current Opinion in Food Science**, v. 31, p. 1–8, 2020.

PEREIRA, G. V. DE M.; MAGALHÃES-GUEDES, K. T.; SCHWAN, R. F. RDNA-based DGGE analysis and electron microscopic observation of cocoa beans to monitor microbial diversity and distribution during the fermentation process. **Food Research International**, v. 53, n. 1, p. 482–486, 2013b.

PEREIRA, G. V. DE M.; SOCCOL, V. T.; SOCCOL, C. R. Current state of research on cocoa and coffee fermentations. **Current Opinion in Food Science**, v. 7, p. 50–57, 2016.

PEREIRA, G. V. M. et al. Great intraspecies diversity of *Pichia kudriavzevii* in cocoa fermentation highlights the importance of yeast strain selection for flavor modulation of cocoa beans. **LWT - Food Science and Technology**, v. 84, p. 290–297, 2017.

PERELMUTER, K.; FRAGA, M.; ZUNINO, P. In vitro activity of potential probiotic *Lactobacillus murinus* isolated from the dog. **Journal of Applied Microbiology**, v. 104, n. 6, p. 1718–1725, 2008.

PESSÔA, M. G. et al. Newly isolated microorganisms with potential application in biotechnology. **Biotechnology Advances**, v. 37, n. 2, p. 319–339, 2019.

PHAM, V. H. T.; KIM, J. Cultivation of unculturable soil bacteria. **Trends in Biotechnology**, v. 30, n. 9, p. 475–484, 2012.

PINILLA, L.; TORRES, R.; ORTIZ, C. Bioethanol production in batch mode by a native strain of *Zymomonas mobilis*. **World Journal of Microbiology and Biotechnology**, v. 27, n. 11, p. 2521–2528, 2011.

QUAST, C. et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. **Nucleic Acids Research**, v. 41, n. D1, p. 590–596, 2013.

QUIGLEY, L. et al. High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. **Applied and Environmental Microbiology**, v. 78, n. 16, p. 5717–5723, 2012.

RAMOS, C. L. et al. Strain-specific probiotics properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. **Food Microbiology**, v. 36, n. 1, p. 22–29, 2013.

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

RAPPÉ, M. S. et al. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. **Nature**, v. 418, p. 630–633, 2002.

RODRIGUEZ-CAMPOS, J. et al. Dynamics of volatile and non-volatile compounds in cocoa (*Theobroma cacao* L.) during fermentation and drying processes using principal components analysis. **Food Research International**, v. 44, n. 1, p. 250–258, 2011.

ROELOFSEN, P. A. Fermentation, drying, and storage of cacao Beans. **Advances in Food Research**, v. 8, n. C, p. 225–296, 1958.

ROMANENS, E. et al. A lab-scale model system for cocoa bean fermentation. **Applied Microbiology and Biotechnology**, v. 102, n. 7, p. 3349–3362, 2018.

ROMBOUTS, J. E. Observations on the microflora of fermeting cacao beans in Trinidad. **Proc. Soc. Appl. Bacteriol.**, v. 15, p. 103–111, 1952.

ROMERO-CORTES, T. et al. Relationship between fermentation index and other biochemical changes evaluated during the fermentation of Mexican cocoa (*Theobroma cacao*) beans. **Journal of the Science of Food and Agriculture**, v. 93, n. 10, p. 2596–

2604, 2013.

ROTTIERS, H. et al. Dynamics of volatile compounds and flavor precursors during spontaneous fermentation of fine flavor Trinitario cocoa beans. **European Food Research and Technology**, v. 245, n. 9, p. 1917–1937, 2019.

SAHA, B. C. et al. Production of D-arabitol by a newly isolated *Zygosaccharomyces rouxii*. **Journal of Industrial Microbiology & Biotechnology**, v. 34, p. 519-523.

SAICHANA, N. et al. Acetic acid bacteria: A group of bacteria with versatile biotechnological applications. **Biotechnology Advances**, v. 33, n. 6, p. 1260–1271, 2015.

SANCHEZ, J.; GUIRAUD, J. P.; GALZY, P. A study of the polygalacturonase activity of several yeast strains isolated from cocoa. **Applied Microbiology and Biotechnology**, v. 20, p. 262–267, 1984.

SANDHYA, M. V. S. et al. Inoculum of the starter consortia and interactive metabolic process in enhancing quality of cocoa bean (*Theobroma cacao*) fermentation. **LWT - Food Science and Technology**, v. 65, p. 731–738, 2016.

SANTOS, A. et al. (1-6)-β-D-Glucan as cell wall receptor for *Pichia membranifaciens* killer toxin. **Applied and Environmental Microbiology**, v. 66, n. 5, p. 1809–1813, 2000.

SARBU, I.; CSUTAK, O. The microbiology of cocoa fermentation. In: **Caffeinated and Cocoa Based Beverages: Volume 8. The Science of Beverages**. Elsevier Inc., 2019. p. 423–446.

SARKAR, A.; RAO, K. V. B. Marine yeast: A potential candidate for biotechnological applications - A review. **Asian Journal of Microbiology, Biotechnology and Environmental Sciences**, v. 18, n. 3, p. 627–634, 2016.

SATPUTE, S. K. et al. Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. **Biotechnology Advances**, v. 28, n. 4, p. 436–450, 2010.

SCHRÖDER, J. et al. Complete genome sequence of *Corynebacterium* variabile DSM 44702 isolated from the surface of smear-ripened cheeses and insights into cheese ripening and flavor generation. **BMC Genomics**, v. 12, n. 545, p. 1–23, 2011.

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

SCHWAN, R.F.; PEREIRA, G.V.M.; FLEET, G.H. Microbial activities during cocoa fermentation. In: **Cocoa and Coffee Fermentations**. SCHWAN, R.F.; FLEET, G.H. (eds). CRC Press: Boca Raton, p. 129-192, 2015.

SCHWAN, R. F.; VANETTI, C. D.; SILVA, D. Characterization and distribution of aerobic, spore-forming bacteria from cacao fermentations in Bahia. **Journal of Food Science**, v. 51, n. 6, p. 1583–1584, 1986.

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

SEONG, C. N. et al. Taxonomic hierarchy of the phylum Firmicutes and novel Firmicutes species originated from various environments in Korea. **Journal of Microbiology**, v. 56, n. 1, p. 1–10, 2018.

SERRA, J. L. et al. Determination of the microbial community in Amazonian cocoa bean fermentation by Illumina-based metagenomic sequencing. **LWT - Food Science and Technology**, v. 106, p. 229–239, 2019.

SHARMA, D.; SHARMA, B.; SHUKLA, A. K. **Biotechnological approach of microbial lipase: A reviewBiotechnology**, 2011.

SIEUWERTS, S.; BRON, P. A.; SMID, E. J. Mutually stimulating interactions between lactic acid bacteria and *Saccharomyces cerevisiae* in sourdough fermentation. **LWT - Food Science and Technology**, v. 90, p. 201–206, 2018.

SIM, I.; PARK, K.-T.; LIM, Y.-H. Characterization and immunomodulation activity of *Lactobacillus sakei* L2 and L8 isolated from chicken cecum. **Microbiology and Biotechnology Letters**, v. 44, n. 2, p. 201–207, 2016.

SINGH, R. et al. Microbial enzymes:Industrial progress in 21st century. **3 Biotech**, v. 6, n. 174, p. 1–15, 2016.

SINGH, R. S.; SINGH, T.; PANDEY, A. Microbial enzymes — An overview. In: **Advances in Enzyme Technology**. Elsevier B.V., 2019. p. 1–40.

SMID, E. J.; KLEEREBEZEM, M. Production of aroma compounds in lactic fermentations. **Annual Review of Food Science and Technology**, v. 5, p. 313–326, 2014.

SONG, J.; OH, H.-M.; CHO, J.-C. Improved culturability of SAR11 strains in dilution-to-extinction culturing from the East Sea, West Pacific Ocean. **FEMS Microbiology Letters**, v. 295, n. 2, p. 141–147, 2009.

SONG, Z. et al. Unraveling core functional microbiota in traditional solid-state fermentation by high-throughput amplicons and metatranscriptomics sequencing. **Frontiers in Microbiology**, v. 8, n. July, p. 1–14, 2017.

SOUZA, P. M.; OLIVEIRA E MAGALHÃES, P. Application of microbial α-amylase in industry - a review. **Brazilian Journal of Microbiology**, v. 41, n. 4, p. 850–861, 2010.

SPOHNER, S. C. et al. *Kluyveromyces lactis*: An emerging tool in biotechnology. **Journal of Biotechnology**, v. 222, p. 104–116, 2016.

STASOFT, INC. **Statistica (data analysis software system)**, version 7. Disponível em: <www. statsoft.com>.

STATISTA. **Per capita chocolate consumption worldwide in 2017, by country**. Disponpivel em: <https://www.statista.com/statistics/819288/worldwide-chocolate-

consumption-by-country/#:~:text=Chocolate%20consumption%20continues%20to%20grow,over%20the%20last%20five%20years.>. Acesso: 28 out. 2020.

SUN, S. et al. Salicylate and phthalate pathways contributed differently on phenanthrene and pyrene degradations in *Mycobacterium* sp. WY10. **Journal of Hazardous Materials**, v. 364, n. October 2018, p. 509–518, 2019.

SUNDARRAM, A.; MURTHY, T. P. K. α-Amylase production and applications: A review. **Journal of Applied & Environmental Microbiology**, v. 2, n. 4, p. 166–175, 2014.

SUZUKI, S.; HIRAISHI, A. *Novosphingobium naphthalenivorans* sp. nov., a naphthalene-degrading bacterium isolated from polychlorinated-dioxin-contaminated environments. **Journal of General and Applied Microbiology**, v. 53, n. 4, p. 221–228, 2007.

TELES SANTOS, T. et al. Characterization of lactobacilli strains derived from cocoa fermentation in the south of Bahia for the development of probiotic cultures. **LWT - Food Science and Technology**, v. 73, p. 259–266, 2016.

URDACI, M. C.; BRESSOLLIER, P.; PINCHUK, I. *Bacillus clausii* probiotic strains. **Journal of Clinical Gastroenterology**, v. 38, n. Supplement 2, p. S86–S90, 2004.

UTRILLA-VÁZQUEZ, M. et al. Analysis of volatile compounds of five varieties of Maya cocoa during fermentation and drying processes by Venn diagram and PCA. **Food Research International**, v. 129, p. 108834, 2020.

VAN DE GUCHTE, M. et al. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. **PNAS**, v. 103, n. 24, p. 7300–7305, 2006.

VÁSQUEZ, Z. S. et al. Biotechnological approaches for cocoa waste management: A review. **Waste Management**, v. 90, p. 72–83, 2019.

VERSALOVIC, J. et al. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. **Methods in Molecular and Cellular Biology**, v. 5, n. 1, p. 25–40, 1994.

VIESSER, J. A. et al. Exploring the contribution of fructophilic lactic acid bacteria to cocoa beans fermentation: isolation, selection and evaluation. **Food Research International**, v. 136, p. 109478, 2020.

VIESSER, J. A. et al. Co-culturing fructophilic lactic acid bacteria and yeast enhanced sugar metabolism and aroma formation during cocoa beans fermentation. **International Journal of Food Microbiology**, v. 339, p. 109015, 2021.

VISINTIN, S. et al. Molecular identification and physiological characterization of yeasts, lactic acid bacteria and acetic acid bacteria isolated from heap and box cocoa bean fermentations in West Africa. **International Journal of Food Microbiology**, v. 216, p.

69–78, 2016.

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

VOIGT, J. et al. The proteolytic formation of essential cocoaspecific aroma precursors depends on particular chemical structures of the vicilin-class globulin of the cocoa seeds lacking in the globular storage proteins of coconuts, hazelnuts and sunflower seeds. **Food Chemistry**, v. 51, n. 2, p. 197–205, 1994.

WANG, Y. et al. Isolation and characterization of low nucleic acid (LNA)-content bacteria. **ISME Journal**, v. 3, n. 8, p. 889–902, 2009.

WEI, Y. et al. High-throughput sequencing of microbial community diversity in soil, grapes, leaves, grape juice and wine of grapevine from China. **PLoS ONE**, v. 13, n. 3, p. 1–17, 2018.

WOOD, G. A. .; LASS, R. A. **Cocoa**. Fourth ed. Oxford: Longman Group Ltd., 1985.

XIAO, H. et al. Exploiting *Issatchenkia orientalis* SD108 for succinic acid production. **Microbial Cell Factories**, v. 13, n. 121, p. 1–11, 2014.

XU, J. et al. Microbial dynamics and metabolite changes in Chinese Rice Wine fermentation from sorghum with different tannin content. **Scientific Reports**, n. March, p. 1–11, 2018.

XU, Y. et al. *Zygosaccharomyces bailii* is a potential producer of various flavor compounds in Chinese Maotai-flavor liquor fermentation. **Frontiers in Microbiology**, v. 8, p. 1–9, 2017.

XU, Z. Biological production of hydrogen from renewable resources. In: YANG, S.-T. (Ed.). **Bioprocessing for Value-Added Products from Renewable Resources**. Elsevier B.V., 2007. p. 527–557.

XUE, S. J. et al. Fatty acids from oleaginous yeasts and yeast-like fungi and their potential applications. **Critical Reviews in Biotechnology**, v. 38, n. 7, p. 1049–1060, 2018.

YAMADA, Y.; YUKPHAN, P. Genera and species in acetic acid bacteria. **International Journal of Food Microbiology**, v. 125, p. 15–24, 2008.

YANASE, H. et al. Ethanol production from wood hydrolysate using genetically engineered *Zymomonas mobilis*. **Applied Microbiology and Biotechnology**, v. 94, n. 6, p. 1667–1678, 2012.

ZENGLER, K. et al. Cultivating the uncultured Karsten. **PNAS**, v. 99, n. 24, p. 15681–15686, 2002.

ZHANG, G.; HE, G. Predominant bacteria diversity in Chinese traditional sourdough. **Journal of Food Science**, v. 78, n. 8, p. 1218–1223, 2013.

ZHANG, Q. et al. Draft genome sequence of *Komagataeibacter maltaceti* LMG 1529T, a vinegar-producing acetic acid bacterium isolated from malt vinegar brewery acetifiers. **Genome Annoucements**, v. 6, n. 16, p. 4–5, 2018.

ZHANG, X.-X. et al. Microbial PAH-Degradation in soil: Degradation pathways and contributing factors. **Pedosphere**, v. 16, n. 5, p. 555–565, 2006.

ZHENG, J. et al. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. **International Journal of Systematic and Evolutionary Microbiology**, v. 70, n. 4, p. 2782–2858, 2020.

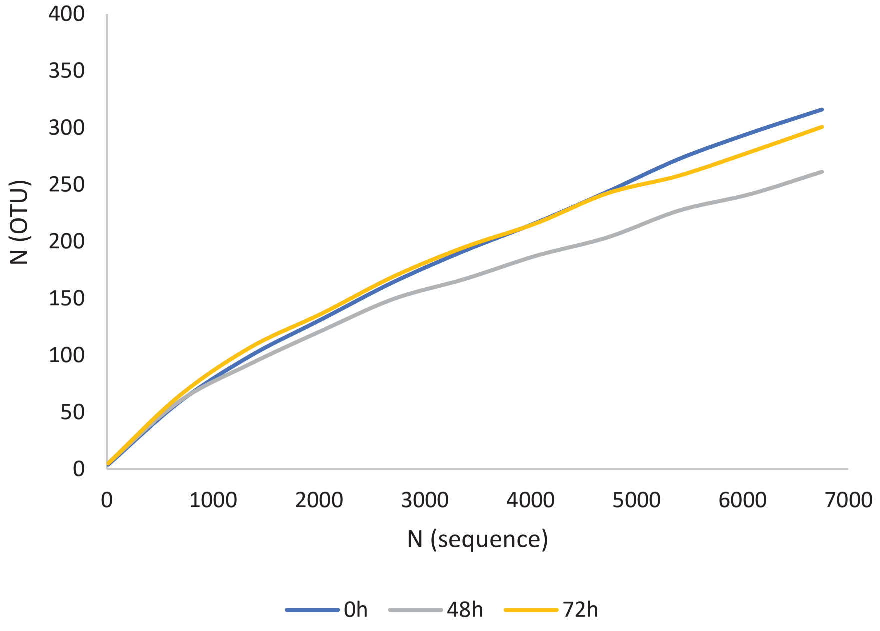
ZOTTA, T.; PARENTE, E.; RICCIARDI, A. Aerobic metabolism in the genus *Lactobacillus*: impact on stress response and potential applications in the food industry. **Journal of Applied Microbiology**, v. 122, n. 4, p. 857–869, 2017.

ZUCKO, J. et al. Probiotic – friend or foe? **Current Opinion in Food Science**, v. 32, p. 45–49, 2020.

ANNEXES

CHAPTER II

FIGURE A2.1 - RAREFACTION CURVES FOR THE BACTERIAL COMMUNITY CORRESPONDING EACH FERMENTED COCOA PULP BEAN MASS SAMPLES AT 0, 48 AND 72 H.



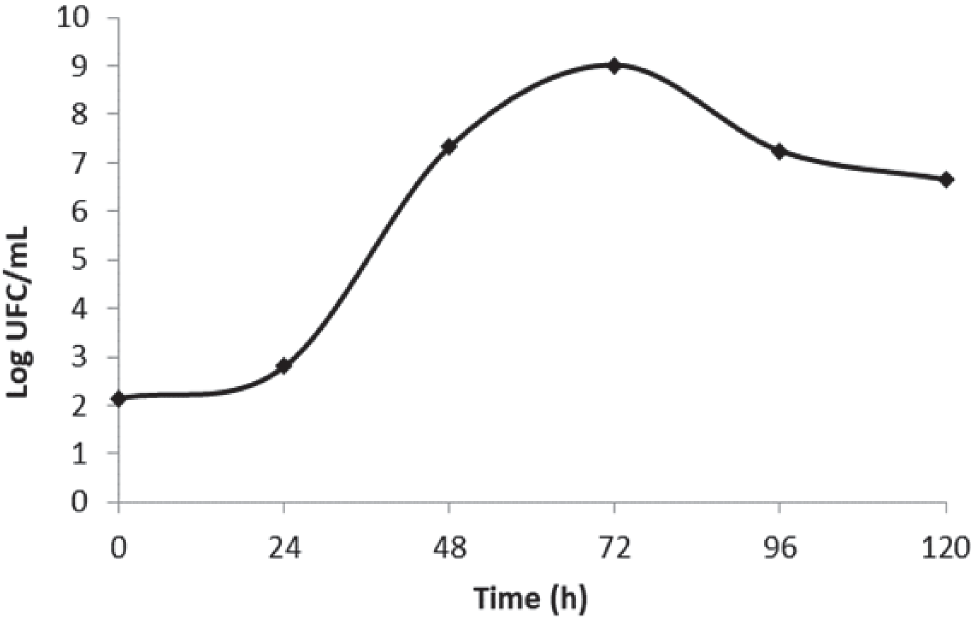
Source: The author (2020).

TABLE A2.1 - LIST OF BACTERIA GENERA FOUND DURING SPONTANEOUS COCOA BEANS FERMENTATION.

Time (h)	**Genera**	**Relative abundance OTU (%)**
0	*Pediococcus, Pseudomonas, Sphingomonas, Paenibacillus, Stenotrophomonas, Actinomycetospora, Brevundimonas, Methylobacterium, Acinetobacter, Agrobacterium, Sphingobium, Fructobacillus, Bradyrhizobium, Burkholderia, Bacillus, Staphylococcus, Lactobacillus, Gluconobacter, Serratia, Haemophilus, Corynebacterium, Rhodococcus, Bifidobacterium, Acetobacter, Salinispora, Novosphingobium, Prevotella*	30.260, 18.086, 8.347, 2.434, 2.260, 2.086, 1.565, 1.217, 0.869, 0.521, 0.521, 0.695, 0.695, 0.695, 0.695, 0.347, 0.347, 0.347, 0.347, 0.347, 0.173, 0.173, 0.173, 0.173, 0.173, 0.173, 0.173
48	*Pediococcus, Erwinia, Klebsiella, Stenotrophomonas, Pseudomonas, Serratia, Lactobacillus, Blautia, Actinomycetospora, Bacteroides, Prevotella, Leuconostoc, Faecalibacterium, Ruminococcus, Allobaculum, Coprobacillus, Mycoplana, Sphingomonas, Enterobacter, Pantoea, Collinsella, Bacillus, Coprococcus, Dorea, Oscillispora,*	10.914, 0.422, 0.204, 0.204, 0.129, 0.027, 0.020, 0.050, 0.006, 0.006, 0.006, 0.006, 0.006, 0.006, 0.006, 0.006, 0.006, 0.006, 0.006, 0.006, 0.013, 0.013, 0.013, 0.013, 0.013
72	*Pediococcus, Pseudomonas, Erwinia, Sphingomonas, Stenotrophomonas, Lactobacillus, Klabsiella, Paenibacillus, Faecalibacterium, Oscillospira, Brevundimonas, Bacillus, Dorea, Ruminococcus, Acinetobacter, Actinomycetospora, Turicibacter, Coprobacillus, Methylobacterium, Salinispora, Serratia, Corynebacterium, Allobaculum, Bradyrhizobium, Achromobacter, Streptococcus, Parabacteroides, Staphylococcus, Enterococcus, Blautia, Peptococcus, Phascolarctobacterium, Catenibacterium, Rhizobium, Gluconobacter, Novosphingobium, Burkholderia, Ralstonia, Pantoea*	15.753, 1.496, 0.286, 0.240, 0.209, 0.186, 0.155, 0.085, 0.062, 0.062, 0.054, 0.038, 0.038, 0.038, 0.038, 0.031, 0.031, 0.023, 0.023, 0.023, 0.023, 0.015, 0.015, 0.015, 0.015, 0.015, 0.007, 0.007, 0.007, 0.007, 0.007, 0.007, 0.007, 0.007, 0.007, 0.007, 0.007, 0.007, 0.007

The author (2020).

FIGURE A2.2 - POPULATION OF LACTIC ACID BACTERIA (LAB) IN FYP AGAR DURING LABORATORY-SCALE SPONTANEOUS COCOA BEANS FERMENTATION.



Source: The author (2020).

TABLE A2.2 - EVALUATION AND IDENTIFICATION OF PRESUMPTIVE FLAB ISOLATES FROM LABORATORY-SCALE COCOA BEANS FERMENTATION.

		O.D (600 nm)				FRUCTOSE (g/L)			
	ISOLATE	**0h**	**12h**	**24h**	**36h**	**0h**	**12h**	**24h**	**36h**
FYP	*Pediococcus pentosaceus* LPBF01	0.050	0.317 ± 0.007	0.595 ± 0.007	0.849 ± 0.018	10.0	7.445 ± 0.167	5.523 ± 0.126	4.297 ± 0.120
	Pediococcus acidilactici LPBF02	0.050	0.297 ± 0.007	0.568 ± 0.009	0.826 ± 0.033	10.0	7.606 ± 0.143	5.566 ± 0.140	4.419 ± 0.184
	Pediococcus acidilactici LPBF03	0.050	0.545 ± 0.022	0.828 ± 0.041	0.978 ± 0.075	10.0	5.690 ± 0.033	4.403 ± 0.087	3.867 ± 0.109
	Pediococcus acidilactici LPBF04	0.050	0.340 ± 0.054	0.675 ± 0.050	0.868 ± 0.010	10.0	7.284 ± 0.138	5.097 ± 0.170	4.241± 0.198
	Pediococcus acidilactici LPBF05	0.050	0.524 ± 0.021	0.819 ± 0.081	0.958 ± 0.013	10.0	5.986 ± 0.078	4.458 ± 0.090	3.966 ± 0.093
	Pediococcus acidilactici LPBF06	0.050	0.536 ± 0.019	0.812 ± 0.024	0.956 ± 0.012	10.0	5.929 ± 0.131	4.496 ± 0.107	3.979 ± 0.125
	Pediococcus acidilactici LPBF07	0.050	0.552 ± 0.050	0.823 ± 0.056	0.976 ± 0.049	10.0	5.635 ± 0.132	4.433 ± 0.135	3.881 ± 0.180
	Pediococcus pentosaceus LPBF08	0.050	0.315 ± 0.012	0.596 ± 0.052	0.856 ± 0.012	10.0	7.496 ± 0.038	5.234 ± 0.080	4.266 ± 0.082
	Pediococcus acidilactici LPBF09	0.050	0.308 ± 0.008	0.585 ± 0.042	0.832 ± 0.024	10.0	7.549 ± 0.101	5.556 ± 0.122	4.358 ± 0.036
	Pediococcus acidilactici LPBF10	0.050	0.362 ± 0.009	0.685 ± 0.110	0.883 ± 0.080	10.0	7.082 ± 0.125	5.036 ± 0.143	4.213 ± 0.157
	Pediococcus acidilactici LPBF11	0.050	0.429 ± 0.073	0.773 ± 0.060	0.892 ± 0.052	10.0	6.526 ± 0.071	4.704 ± 0.090	4.176 ± 0.052
	Bacillus subtilis LPBF12	0.050	0.263 ± 0.009	0.516 ± 0.081	0.809 ± 0.019	10.0	7.812 ± 0.094	6.019 ± 0.091	4.511 ± 0.113
	Bacillus subtilis LPBF13	0.050	0.372 ± 0.022	0.675 ± 0.096	0.826 ± 0.020	10.0	6.950 ± 0.033	5.097 ± 0.118	4.419 ± 0.127

Pediococcus acidilactici LPBF14	0.050	0.536 ± 0.048	0.823 ± 0.073	0.974 ± 0.115	10.0	5.929 ± 0.113	4.433 ± 0.126	3.893 ± 0.101
Lactobacillus plantarum LPBF15	0.050	0.537 ± 0.016	0.820 ± 0.085	0.969 ± 0.060	10.0	5.713 ± 0.091	4.445 ± 0.089	3.920 ± 0.185
Pediococcus acidilactici LPBF16	0.050	0.420 ± 0.027	0.782 ± 0.092	0.875 ± 0.084	10.0	6.583 ± 0.086	4.676 ± 0.060	4.235 ± 0.055
Lactobacillus plantarum LPBF17	0.050	0.412 ± 0.036	0.769 ± 0.059	0.935 ± 0.046	10.0	6.673 ± 0.037	4.712 ± 0.049	4.038 ± 0.068
Lactobacillus plantarum LPBF18	0.050	0.551 ± 0.010	0.816 ± 0.087	0.966 ± 0.056	10.0	5.646 ± 0.031	4.472 ± 0.058	3.392 ± 0.076
Pediococcus pentosaceus LPBF19	0.050	0.485 ± 0.052	0.722 ± 0.062	0.875 ± 0.020	10.0	6.151 ± 0.059	4.928 ± 0.074	4.235 ± 0.060
Pediococcus acidilactici LPBF20	0.050	0.439 ± 0.061	0.785 ± 0.018	0.911 ± 0.095	10.0	6.475 ± 0.075	4.645 ± 0.047	4.091 ± 0.010
Bacillus subtilis LPBF21	0.050	0.273 ± 0.022	0.536 ± 0.045	0.809 ± 0.092	10.0	7.716 ± 0.012	5.929 ± 0.121	4.511 ± 0.127
Pediococcus acidilactici LPBF22	0.050	0.413 ± 0.037	0.830 ± 0.016	0.956 ± 0.080	10.0	6.624 ± 0.019	4.372 ± 0.112	3.979 ± 0.100
Pediococcus acidilactici LPBF23	0.050	0.502 ± 0.063	0.788 ± 0.044	0.924 ± 0.099	10.0	6.116 ± 0.060	4.631 ± 0.094	4.058 ± 0.104
Pediococcus pentosaceus LPBF24	0.050	0.361 ± 0.005	0.702 ± 0.070	0.832 ± 0.054	10.0	7.137 ± 0.037	4.993 ± 0.110	4.358 ± 0.048
Pediococcus acidilactici LPBF25	0.050	0.350 ± 0.012	0.782 ± 0.030	0.939 ± 0.056	10.0	7.188 ± 0.033	4.676 ± 0.034	4.025 ± 0.077
Pediococcus acidilactici LPBF26	0.050	0.426 ± 0.023	0.828 ± 0.076	0.984 ± 0.072	10.0	6.538 ± 0.066	4.403 ± 0.110	3.844 ± 0.115
Pediococcus pentosaceus LPBF27	0.050	0.503 ± 0.007	0.766 ± 0.049	0.956 ± 0.077	10.0	6.100 ± 0.048	4.729 ± 0.038	3.979 ± 0.078
Bacillus subtilis LPBF28	0.050	0.384 ± 0.015	0.545 ± 0.091	0.812 ± 0.090	10.0	6.877 ± 0.054	5.690 ± 0.106	4.496 ± 0.097
Pediococcus acidilactici LPBF29	0.050	0.511 ± 0.068	0.830 ± 0.044	0.970 ± 0.050	10.0	6.033 ± 0.074	4.372 ± 0.063	3.907 ± 0.100

Lactobacillus plantarum LPBF30	0.050	0.536 ± 0.006	0.849 ± 0.032	0.978 ± 0.049	10.0	5.929 ± 0.022	4.297 ± 0.086	3.867 ± 0.065
Pediococcus acidilactici LPBF31	0.050	0.368 ± 0.027	0.715 ± 0.062	0.865 ± 0.081	10.0	7.029 ± 0.063	4.971 ± 0.086	4.250 ± 0.113
Pediococcus pentosaceus LPBF32	0.050	0.361 ± 0.017	0.691 ± 0.141	0.830 ± 0.140	10.0	7.137 ± 0.014	5.020 ± 0.096	4.372 ± 0.040
Pediococcus acidilactici LPBF33	0.050	0.420 ± 0.100	0.717 ± 0.069	0.935 ± 0.053	10.0	6.583 ± 0.125	4.955 ± 0.076	4.038 ± 0.132
Pediococcus acidilactici LPBF34	0.050	0.425 ± 0.033	0.751 ± 0.117	0.889 ± 0.110	10.0	6.550 ± 0.059	4.788 ± 0.095	4.189 ± 0.126
Lactobacillus plantarum LPBF35	0.050	0.473 ± 0.018	0.803 ± 0.062	0.958 ± 0.089	10.0	6.184 ± 0.012	4.537 ± 0.055	3.966 ± 0.026
Pediococcus acidilactici LPBF36	0.050	0.509 ± 0.105	0.865 ± 0.118	0.976 ± 0.035	10.0	6.057 ± 0.098	4.250 ± 0.032	3.881 ± 0.051
Lactobacillus plantarum LPBF37	0.050	0.460 ± 0.093	0.766 ± 0.120	0.895 ± 0.150	10.0	6.218 ± 0.114	4.729 ± 0.059	4.158 ± 0.067
Lactobacillus plantarum LPBF38	0.050	0.536 ± 0.011	0.830 ± 0.087	0.966 ± 0.132	10.0	5.929 ± 0.054	4.372 ± 0.112	3.932 ± 0.064
Pediococcus acidilactici LPBF39	0.050	0.445 ± 0.010	0.756 ± 0.040	0.924 ± 0.072	10.0	6.440 ± 0.163	4.755 ± 0.099	4.058 ± 0.011
Lactobacillus plantarum LPBF40	0.050	0.431 ± 0.024	0.769 ± 0.069	0.970 ± 0.116	10.0	6.502 ± 0.117	4.712 ± 0.058	3.907 ± 0.016
Pediococcus acidilactici LPBF41	0.050	0.335 ± 0.082	0.516 ± 0.106	0.868 ± 0.147	10.0	7.313 ± 0.029	6.019 ± 0.041	4.241 ± 0.050
Lactobacillus plantarum LPBF42	0.050	0.409 ± 0.019	0.797 ± 0.056	0.939 ± 0.027	10.0	6.711 ± 0.044	4.560 ± 0.077	4.025 ± 0.060
Pediococcus pentosaceus LPBF43	0.050	0.452 ± 0.090	0.828 ± 0.117	0.966 ± 0.105	10.0	6.314 ± 0.012	4.403 ± 0.042	3.932 ± 0.122
Pediococcus acidilactici LPBF44	0.050	0.457 ± 0.075	0.791 ± 0.028	0.906 ± 0.114	10.0	6.271 ± 0.127	4.604 ± 0.109	4.117 ± 0.132
Pediococcus acidilactici LPBF45	0.050	0.379 ± 0.034	0.734 ± 0.026	0.832 ± 0.029	10.0	6.901 ± 0.008	4.877 ± 0.122	4.358 ± 0.070

	Lactobacillus plantarum LPBF46	0.050	0.469 ± 0.021	0.801 ± 0.041	0.976 ± 0.061	10.0	6.200 ± 0.050	4.553 ± 0.060	3.881 ± 0.124
	Pediococcus acidilactici LPBF47	0.050	0.508 ± 0.006	0.749 ± 0.023	0.889 ± 0.078	10.0	6.067 ± 0.033	4.804 ± 0.037	4.189 ± 0.054
	Pediococcus acidilactici LPBF48	0.050	0.541 ± 0.044	0.788 ± 0.019	0.939 ± 0.010	10.0	5.701 ± 0.090	4.631 ± 0.060	4.025 ± 0.062
	Pediococcus acidilactici LPBF49	0.050	0.413 ± 0.056	0.764 ± 0.027	0.889 ± 0.128	10.0	6.624 ± 0.020	4.741 ± 0.088	4.189 ± 0.016
	Pediococcus acidilactici LPBF50	0.050	0.335 ± 0.007	0.638 ± 0.010	0.844 ± 0.050	10.0	7.313 ± 0.022	5.210 ± 0.031	4.307 ± 0.085
	Pediococcus acidilactici LPBF51	0.050	0.457 ± 0.015	0.791 ± 0.100	0.915 ± 0.123	10.0	6.271 ± 0.062	4.604 ± 0.071	4.080 ± 0.100
	Pediococcus acidilactici LPBF52	0.050	0.397 ± 0.039	0.766 ± 0.090	0.856 ± 0.076	10.0	6.783 ± 0.031	4.729 ± 0.114	4.266 ± 0.066
	Pediococcus acidilactici LPBF53	0.050	0.343 ± 0.016	0.645 ± 0.070	0.865 ± 0.054	10.0	7.219 ± 0.040	5.179 ± 0.107	4.250 ± 0.081
	Pediococcus acidilactici LPBF54	0.050	0.445 ± 0.033	0.739 ± 0.032	0.856 ± 0.028	10.0	6.440 ± 0.052	4.845 ± 0.080	4.266 ± 0.040
	Pediococcus acidilactici LPBF55	0.050	0.309 ± 0.081	0.585 ± 0.023	0.841 ± 0.070	10.0	7.531 ± 0.024	5.556 ± 0.039	4.333 ± 0.061
30 FYP	*Leuconostoc pseudomesenteroides* LPBF56	0.050	0.445 ± 0.033	0.739 ± 0.032	0.856 ± 0.028	10.0	6.440 ± 0.052	4.845 ± 0.080	4.266 ± 0.040
	Leuconostoc pseudomesenteroides LPBF57	0.050	0.309 ± 0.081	0.585 ± 0.023	0.841 ± 0.070	10.0	7.531 ± 0.024	5.556 ± 0.039	4.333 ± 0.061
	Leuconostoc pseudomesenteroides LPBF58	0.050	0.547 ± 0.076	0.755 ± 0.095	0.883 ± 0.036	10.0	5.678 ± 0.027	4.771 ± 0.038	4.213 ± 0.064
	Pediococcus pentosaceus LPBF59	0.050	0.507 ± 0.080	0.813 ± 0.031	0.969 ± 0.015	10.0	6.082 ± 0.049	4.486 ± 0.035	3.920 ± 0.065
	Leuconostoc pseudomesenteroides LPBF60	0.050	0.368 ± 0.029	0.720 ± 0.078	0.865 ± 0.065	10.0	7.029 ± 0.045	4.940 ± 0.093	4.250 ± 0.092
	Pediococcus pentosaceus LPBF61	0.050	0.377 ± 0.031	0.727 ± 0.050	0.849 ± 0.032	10.0	6.925 ± 0.025	4.906 ± 0.080	4.297 ± 0.066

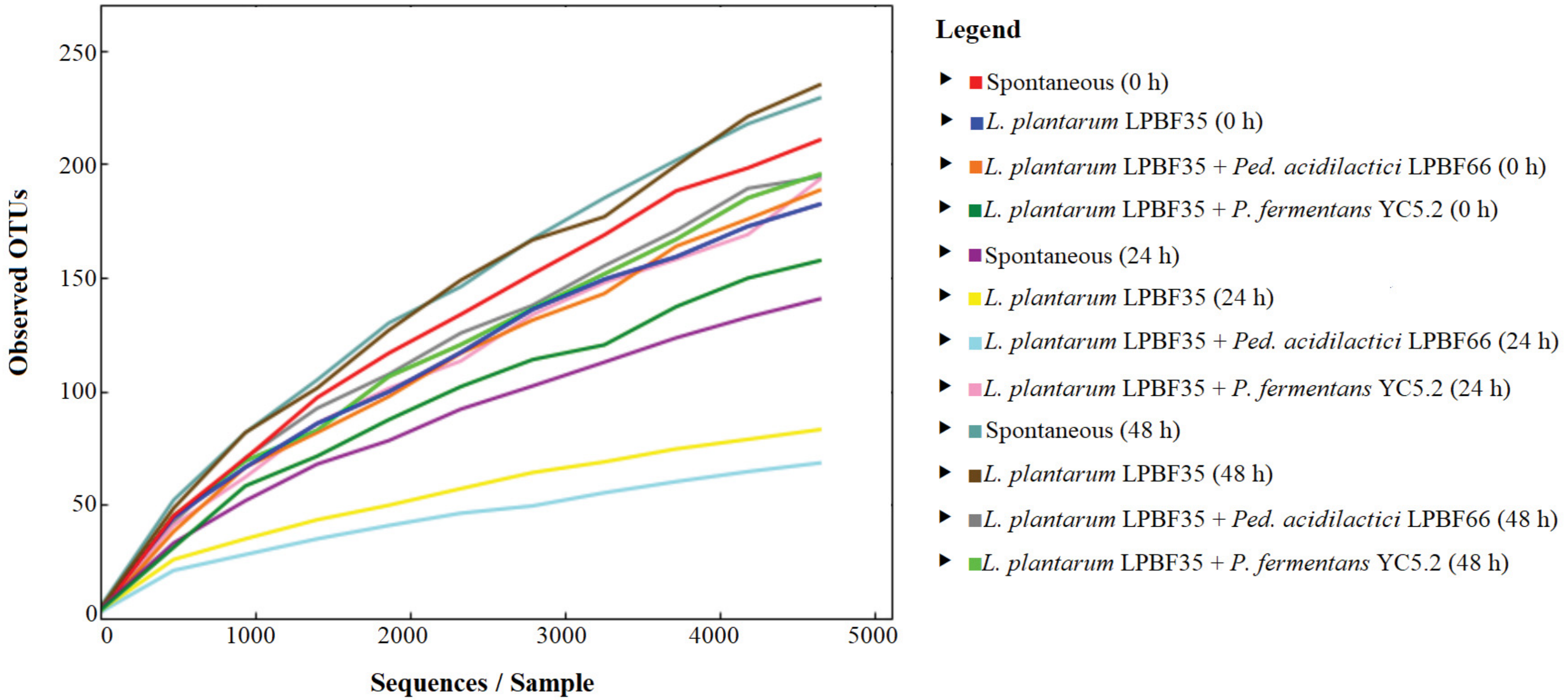
Pediococcus acidilactici LPBF62	0.050	0.592 ± 0.012	0.830 ± 0.008	0.988 ± 0.071	10.0	5.536 ± 0.085	4.372 ± 0.090	3.830 ± 0.104
Pediococcus pentosaceus LPBF63	0.050	0.264 ± 0.009	0.509 ± 0.017	0.784 ± 0.013	10.0	7.743 ± 0.115	6.057 ± 0.113	4.653 ± 0.088
Pediococcus acidilactici LPBF64	0.050	0.251 ± 0.005	0.480 ± 0.035	0.795 ± 0.014	10.0	7.853 ± 0.091	6.617 ± 0.092	4.588 ± 0.077
Pediococcus acidilactici LPBF65	0.050	0.306 ± 0.007	0.681 ± 0.039	0.797 ± 0.062	10.0	7.573 ± 0.104	5.059 ± 0.139	4.560 ± 0.125
Pediococcus acidilactici LPBF66	0.050	0.263 ± 0.020	0.297 ± 0.068	0.308 ± 0.046	10.0	7.812 ± 0.066	7.606 ± 0.083	7.549 ± 0.080
Pediococcus acidilactici LPBF67	0.050	0.242 ± 0.070	0.507 ± 0.059	0.744 ± 0.089	10.0	7.893 ± 0.060	6.082 ± 0.044	4.816 ± 0.117
Pediococcus acidilactici LPBF68	0.050	0.236 ± 0.024	0.455 ± 0.053	0.657 ± 0.107	10.0	7.957 ± 0.097	6.298 ± 0.113	5.144 ± 0.104
Pediococcus acidilactici LPBF69	0.050	0.361 ± 0.031	0.680 ± 0.044	0.734 ± 0.080	10.0	7.137 ± 0.085	5.069 ± 0.128	4.877 ± 0.117
Pediococcus acidilactici LPBF70	0.050	0.549 ± 0.046	0.680 ± 0.076	0.751 ± 0.045	10.0	5.664 ± 0.089	5.069 ± 0.117	4.788 ± 0.075
Pediococcus acidilactici LPBF71	0.050	0.404 ± 0.009	0.545 ± 0.067	0.715 ± 0.096	10.0	6.752 ± 0.085	5.690 ± 0.121	4.971 ± 0.072
Pediococcus acidilactici LPBF72	0.050	0.258 ± 0.019	0.294 ± 0.014	0.322 ± 0.025	10.0	7.838 ± 0.036	7.635 ± 0.029	7.380 ± 0.042
Pediococcus acidilactici LPBF73	0.050	0.317 ± 0.008	0.530 ± 0.040	0.681 ± 0.073	10.0	7.445 ± 0.064	5.939 ± 0.077	5.059 ± 0.092
Pediococcus acidilactici LPBF74	0.050	0.212 ± 0.021	0.323 ± 0.083	0.350 ± 0.077	10.0	8.091 ± 0.045	7.347 ± 0.102	7.188 ± 0.081
Pediococcus acidilactici LPBF75	0.050	0.290 ± 0.005	0.317 ± 0.038	0.340 ± 0.050	10.0	7.663 ± 0.036	7.445 ± 0.067	7.284 ± 0.083
Pediococcus acidilactici LPBF76	0.050	0.525 ± 0.035	0.728 ± 0.097	0.803 ± 0.108	10.0	5.968 ± 0.071	4.888 ± 0.108	4.537 ± 0.088
Pediococcus acidilactici LPBF77	0.050	0.368 ± 0.028	0.552 ± 0.055	0.681 ± 0.090	10.0	7.029 ± 0.023	5.635 ± 0.099	5.059 ± 0.127

	Pediococcus acidilactici LPBF78	0.050	0.240 ± 0.004	0.321 ± 0.039	0.396 ± 0.050	10.0	7.918 ± 0.026	7.410 ± 0.068	6.838 ± 0.098
	Pediococcus acidilactici LPBF79	0.050	0.335 ± 0.027	0.558 ± 0.053	0.788 ± 0.079	10.0	7.313 ± 0.040	5.599 ± 0.088	4.631 ± 0.115
	Pediococcus acidilactici LPBF80	0.050	0.384 ± 0.015	0.744 ± 0.060	0.797 ± 0.030	10.0	6.877 ± 0.034	4.816 ± 0.095	4.560 ± 0.051

Source: The author (2020).

CHAPTER III

FIGURE A3.1. ALPHA RAREFACTION CURVES OF OBSERVED BACTERIAL OTUS (OPERATIONAL TAXONOMIC UNITS) USING ILLUMINA-BASED AMPLICON SEQUENCING FROM TEMPORAL SAMPLES OF SPONTANEOUS AND INOCULATED COCOA BEAN FERMENTATION PROCESSES.



Source: The author (2020).

TABLE A3.1. RELATIVE ABUNDANCE (%) OF LOW BACTERIAL DIVERSITY AT FAMILY AND GENUS LEVEL DURING 0, 24, AND 48 H OF SPONTANEOUS AND INOCULATED COCOA BEAN FERMENTATION PROCESSES.

	Cocoa bean treatment											
	Spontaneous			***L. plantarum* LPBF35**			***L. plantarum* LPBF35 + *Ped. acidilactici* LPBF66**			***L. plantarum* LPBF35 + *P. fermentans* YC5.2**		
Families / Genera	**0 h**	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**	**0 h**	**24 h**	**48 h**
Acidobacteriaceae	-	-	<0.01	-	-	-	-	-	-	-	-	-
Ellin6075	-	-	<0.01	-	-	-	-	-	-	-	-	-
EB1017	-	0.01	<0.01	-	-	-	-	-	-	-	-	<0.01
mb2424	-	-	-	-	-	-	-	-	-	0.01	-	-
Candidatus koribacter	-	-	-	-	-	<0.01	-	-	-	-	-	<0.01
JdFBGBact	-	-	-	-	-	<0.01	-	-	-	-	-	-
Actinomyces	-	-	-	-	-	-	-	-	-	-	<0.01	-
Brevibacterium	-	-	-	-	-	<0.01	-	-	-	-	-	-
Corynebacterium	0.05	<0.01	0.02	-	-	0.01	-	-	0.01	-	0.01	0.01
Brachybacterium	-	-	-	-	-	-	-	-	-	-	<0.01	-
Janibacter	0.01	-	-	-	-	-	-	-	-	-	-	-
Kineosporiaceae	0.02	-	-	-	-	-	-	-	-	-	-	-
Micrococcaceae	0.03	0.01	0.02	-	<0.01	0.01	-	0.01	<0.01	-	-	<0.01
Micrococcus	-	-	-	-	-	<0.01	-	-	<0.01	-	-	-
Mycobacterium	-	<0.01	-	-	-	-	-	<0.01	-	-	-	-
Nocardioidaceae	-	-	-	-	-	-	-	-	-	-	-	<0.01
Nocardioides	0.01	-	-	-	-	-	-	-	-	-	-	-
Propionibacterium	-	-	<0.01	-	-	<0.01	-	-	<0.01	-	-	<0.01
Actinomycetospora	0.03	-	<0.01	<0.01	-	-	-	-	-	-	-	-
Pseudonocardia	-	<0.01	-	<0.01	-	-	-	-	<0.01	-	-	-
Streptomyces	-	-	-	-	<0.01	-	-	<0.01	-	-	-	-
Bifidobacteriaceae	0.05	-	-	-	-	-	-	-	-	-	-	-

Coriobacteriaceae	-	-	-	-	-	-	-	-	-	-	-	<0.01
Collinsella	-	-	-	<0.01	-	-	-	-	<0.01	-	<0.01	<0.01
Slackia	<0.01	-	<0.01	-	-	<0.01	<0.01	-	-	-	<0.01	<0.01
Gaiellaceae	-	-	-	-	-	<0.01	-	-	-	-	-	<0.01
Bacteroides	0.09	0.04	0.03	0.04	0.01	0.03	0.03	0.03	0.03	0.02	0.03	0.03
Prevotella	0.07	0.03	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01
S24-7	-	-	-	<0.01	-	<0.01	-	-	<0.01	-	<0.01	<0.01
CF231	<0.01	-	<0.01	-	-	<0.01	<0.01	-	<0.01	-	<0.01	<0.01
[Paraprevotellaceae]	-	-	-	-	-	-	<0.01	<0.01	<0.01	-	<0.01	<0.01
Cyclobacteriaceae	-	-	-	-	-	-	-	-	-	-	0.01	-
Spirosoma	-	-	0.02	-	-	-	-	-	-	-	-	-
Flavobacteriaceae	-	-	-	-	-	-	-	-	<0.01	-	-	<0.01
Flavobacterium	-	-	<0.01	-	-	-	-	-	-	-	-	-
Pedobacter	-	-	-	<0.01	-	-	-	-	-	-	-	<0.01
Sediminibacterium	-	-	-	-	-	-	-	-	<0.01	-	-	<0.01
Saprospiraceae	-	-	-	-	-	<0.01	-	-	-	-	-	-
Caldilineaceae	-	-	<0.01	-	-	<0.01	-	<0.01	-	-	<0.01	<0.01
Thermogemmatisporaceae	-	-	-	-	-	-	-	-	<0.01	-	-	-
Pseudanabaenaceae	-	-	-	-	-	<0.01	-	-	-	-	-	-
Bacillaceae	0.11	0.03	0.02	<0.01	0.02	0.01	<0.01	0.05	0.01	-	<0.01	0.01
Bacillus	0.26	0.01	<0.01	<0.01	0.02	0.02	0.02	0.05	0.01	0.01	<0.01	<0.01
Geobacillus	-	-	-	-	-	-	-	-	-	-	-	<0.01
Paenibacillus	0.04	-	-	<0.01	-	<0.01	-	-	0.01	-	0.01	<0.01
Planococcaceae	-	<0.01	-	-	-	<0.01	<0.01	-	-	-	-	<0.01
Rummeliibacillus	-	-	-	-	<0.01	-	-	<0.01	-	-	-	-
Staphylococcus	0.08	<0.01	0.02	<0.01	<0.01	0.03	-	-	0.01	0.01	<0.01	0.01
Gemellaceae	-	-	-	<0.01	-	<0.01	<0.01	-	<0.01	-	<0.01	<0.01
Aerococcaceae	<0.01	-	<0.01	-	-	<0.01	<0.01	-	-	-	-	-
Aerococcus	<0.01	-	-	<0.01	-	<0.01	<0.01	-	<0.01	0.01	<0.01	<0.01
Carnobacterium	-	-	-	-	-	<0.01	-	-	<0.01	-	-	-
Enterococcaceae	<0.01	-	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01
Enterococcus	-	-	0.01	<0.01	-	<0.01	0.01	0.01	<0.01	0.01	<0.01	<0.01
Leuconostocaceae	-	0.57	0.34	-	-	-	-	-	-	-	-	-
Lactococcus	0.03	0.12	0.24	0.02	<0.01	<0.01	0.01	0.01	0.02	0.01	0.01	0.01

Streptococcus	-	-	0.01	<0.01	-	0.01	-	-	-	-	<0.01	<0.01
Turicibacter	0.02	-	0.01	0.01	-	<0.01	0.01	<0.01	<0.01	-	0.01	0.01
Clostridiaceae	0.13	<0.01	0.04	0.04	0.01	0.03	0.03	0.02	0.03	0.02	0.03	0.04
Clostridium	0.01	-	0.01	0.01	-	<0.01	0.01	-	0.01	-	<0.01	<0.01
Lachnospiraceae	0.01	<0.01	0.01	0.02	0.01	0.02	0.02	-	0.02	0.02	0.01	0.01
Blautia	0.03	0.01	0.01	0.02	-	0.01	0.02	0.01	0.01	-	0.02	0.02
Coprococcus	<0.01	-	<0.01	<0.01	-	<0.01	<0.01	-	-	-	<0.01	<0.01
Dorea	<0.01	-	0.01	<0.01	-	<0.01	<0.01	-	<0.01	0.01	<0.01	0.01
[Ruminococcus]	0.05	-	0.03	0.04	-	0.03	0.03	-	0.02	0.02	0.02	0.03
Peptococcus	0.01	-	<0.01	<0.01	-	<0.01	<0.01	-	-	-	<0.01	-
Peptostreptococcaceae	<0.01	-	<0.01	-	-	<0.01	<0.01	-	<0.01	-	<0.01	<0.01
Ruminococcaceae	0.04	-	0.04	0.03	-	0.03	0.03	-	0.01	0.01	0.02	0.03
Faecalibacterium	0.04	<0.01	0.01	0.01	<0.01	<0.01	0.01	<0.01	0.01	0.01	0.01	0.01
Oscillospira	0.02	-	0.02	0.01	-	<0.01	0.01	-	0.01	0.01	0.01	0.01
Ruminococcus	0.02	-	<0.01	<0.01	-	<0.01	<0.01	-	<0.01	-	<0.01	<0.01
Veillonellaceae	-	-	-	-	-	-	-	-	-	-	<0.01	-
Megamonas	0.01	<0.01	<0.01	<0.01	-	<0.01	<0.01	-	<0.01	0.01	<0.01	<0.01
Phascolarctobacterium	<0.01	-	-	<0.01	-	<0.01	<0.01	-	<0.01	-	<0.01	<0.01
Veillonella	-	-	-	-	-	-	-	-	-	-	<0.01	-
Anaerococcus	0.01	-	-	-	-	0.01	-	-	-	-	<0.01	-
Finegoldia	-	-	-	-	-	<0.01	-	-	-	-	-	-
Erysipelotrichaceae	<0.01	-	<0.01	<0.01	-	<0.01	<0.01	-	<0.01	0.01	0.01	<0.01
Allobaculum	0.01	-	<0.01	<0.01	-	<0.01	<0.01	-	-	-	<0.01	<0.01
Catenibacterium	-	-	-	<0.01	-	-	-	-	-	-	<0.01	<0.01
Coprobacillus	-	-	<0.01	<0.01	-	<0.01	<0.01	-	<0.01	0.01	<0.01	<0.01
[Eubacterium]	-	-	<0.01	<0.01	<0.01	-	<0.01	<0.01	<0.01	-	<0.01	<0.01
cc_115	-	-	<0.01	<0.01	-	-	<0.01	-	-	-	<0.01	<0.01
Fusobacterium	0.11	0.06	0.04	0.04	0.03	0.03	0.05	0.05	0.02	0.01	0.03	0.03
Leptotrichia	-	-	0.01	-	-	-	-	-	-	-	-	-
Phycisphaeraceae	-	0.01	-	-	-	-	-	-	-	-	-	-
Gemmataceae	0.01	-	-	-	-	-	-	-	-	-	-	<0.01
Gemmata	-	-	-	-	-	-	-	-	-	-	-	<0.01
Planctomyces	-	-	-	-	-	<0.01	-	-	-	-	-	-
Caulobacteraceae	-	-	-	-	-	-	-	-	-	-	-	<0.01

Aurantimonadaceae	0.01	-	<0.01	-	0.01	-	-	-	<0.01	-	<0.01	-
Bradyrhizobiaceae	0.02	0.03	<0.01	<0.01	0.02	<0.01	<0.01	0.04	<0.01	-	<0.01	<0.01
Bradyrhizobium	0.01	-	-	<0.01	-	-	<0.01	-	-	-	<0.01	-
Hyphomicrobiaceae	-	-	-	-	-	<0.01	-	-	-	-	-	-
Devosia	-	-	-	-	-	-	-	-	-	-	<0.01	-
Hyphomicrobium	0.01	-	-	-	-	-	-	-	-	-	-	-
Pedomicrobium	-	<0.01	-	-	0.03	-	-	<0.01	-	-	-	-
Rhodoplanes	-	0.01	-	-	0.02	<0.01	-	0.03	-	-	-	-
Methylobacteriaceae	-	-	-	-	-	<0.01	-	-	-	-	-	<0.01
Methylobacterium	0.03	-	0.01	<0.01	<0.01	0.01	-	0.01	0.01	-	-	-
Mesorhizobium	0.03	-	-	-	-	-	-	-	-	-	<0.01	-
Agrobacterium	-	-	0.01	-	-	0.01	-	-	-	-	<0.01	<0.01
Labrys	-	-	-	-	-	-	-	<0.01	-	-	-	-
Rhodobacteraceae	<0.01	-	0.02	-	-	0.02	-	-	<0.01	-	<0.01	<0.01
Paracoccus	-	-	0.01	-	-	-	-	-	-	-	<0.01	-
Acetobacteraceae	0.01	0.16	<0.01	-	-	-	-	<0.01	-	-	-	<0.01
Acetobacter	-	-	0.02	-	-	<0.01	-	-	-	-	-	-
Roseomonas	-	-	-	-	-	<0.01	-	-	-	-	-	-
Rhodospirillaceae	-	-	-	-	-	-	-	-	-	-	-	<0.01
Azospirillum	0.06	0.08	-	-	0.06	-	-	0.13	<0.01	-	-	-
Anaplasma	-	-	-	-	-	-	-	-	-	-	<0.01	<0.01
Erythrobacteraceae	-	-	-	-	-	<0.01	-	-	-	-	<0.01	<0.01
Sphingomonadaceae	0.04	-	<0.01	<0.01	-	<0.01	<0.01	-	<0.01	-	<0.01	<0.01
Kaistobacter	-	-	-	-	-	<0.01	-	-	-	-	-	-
Novosphingobium	-	-	-	-	-	-	-	-	<0.01	-	-	-
Sphingobium	-	-	0.01	-	-	-	-	-	-	-	-	-
Sphingomonas	0.13	-	-	<0.01	-	0.05	-	-	<0.01	-	-	0.01
Achromobacter	-	-	<0.01	-	-	-	-	-	-	-	-	<0.01
Sutterella	0.01	<0.01	0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	0.01	0.01	0.01
Burkholderia	<0.01	0.01	0.01	-	<0.01	-	-	<0.01	0.01	-	<0.01	<0.01
Comamonadaceae	0.03	-	0.02	<0.01	-	0.01	<0.01	-	<0.01	-	<0.01	<0.01
Comamonas	0.01	-	-	-	-	-	-	-	<0.01	-	<0.01	-
Delftia	0.01	-	<0.01	<0.01	-	<0.01	<0.01	-	<0.01	-	<0.01	<0.01
Tepidimonas	-	-	-	-	-	-	-	-	<0.01	-	<0.01	0.01

Oxalobacteraceae	-	-	0.01	<0.01	<0.01	-	-	<0.01	<0.01	-	<0.01	<0.01
Janthinobacterium	-	-	0.01	<0.01	-	0.01	-	-	-	-	-	0.05
Ralstonia	0.01	-	-	-	-	-	-	-	-	-	<0.01	<0.01
Neisseria	-	-	-	-	-	-	-	-	<0.01	-	-	-
Rhodocyclaceae	<0.01	-	<0.01	<0.01	-	-	<0.01	-	<0.01	-	<0.01	<0.01
Dechloromonas	<0.01	-	-	<0.01	-	-	-	-	-	-	<0.01	<0.01
Hydrogenophilus	-	-	-	-	-	0.01	-	-	-	-	-	-
Plesiocystis	-	-	-	-	-	-	-	-	-	-	<0.01	-
Syntrophobacteraceae	0.05	-	-	-	-	-	-	-	-	-	-	-
Aeromonadaceae	0.04	<0.01	0.01	-	-	-	-	-	<0.01	-	<0.01	<0.01
Succinivibrionaceae	-	-	-	-	<0.01	-	-	<0.01	-	-	-	-
Alteromonadaceae	<0.01	-	-	-	-	<0.01	-	-	-	-	-	-
Alteromonas	0.04	-	<0.01	-	-	0.01	-	-	<0.01	-	<0.01	<0.01
Glaciecola	-	-	-	-	-	<0.01	-	-	-	-	-	-
[Chromatiaceae]	<0.01	-	-	-	-	-	-	-	-	-	-	<0.01
Enterobacter	<0.01	-	-	-	-	-	-	-	-	-	<0.01	-
Klebsiella	0.22	0.32	0.08	<0.01	<0.01	-	-	<0.01	-	-	<0.01	-
Pantoea	0.20	0.24	0.06	<0.01	<0.01	-	-	-	<0.01	-	-	-
Serratia	0.03	0.03	0.02	<0.01	-	-	<0.01	<0.01	<0.01	-	<0.01	<0.01
Trabulsiella	-	<0.01	-	-	-	-	-	-	-	-	-	-
Coxiellaceae	-	-	-	-	-	-	-	-	-	-	<0.01	-
Alcanivorax	-	-	-	-	-	-	-	-	<0.01	-	-	<0.01
Halomonadaceae	-	-	-	-	-	<0.01	-	-	-	-	<0.01	<0.01
Candidatus portiera	-	-	-	<0.01	-	-	-	-	-	-	-	-
Reinekea	-	-	-	-	-	<0.01	-	-	-	-	<0.01	-
Haemophilus	0.01	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter	0.11	0.03	0.02	<0.01	<0.01	0.01	<0.01	-	0.01	0.01	0.01	0.01
Enhydrobacter	-	-	-	-	-	-	-	-	-	-	-	<0.01
Pseudomonas	0.37	0.03	0.03	0.01	-	0.06	<0.01	<0.01	0.01	0.01	0.03	0.01
Thiohalorhabdaceae	-	-	-	-	-	-	-	-	-	-	<0.01	-
Pseudoalteromonadaceae	0.01	-	-	-	-	-	-	-	-	-	-	<0.01
Pseudoalteromonas	0.04	-	-	-	-	-	-	-	-	-	-	-
Vibrio	0.26	-	0.04	<0.01	-	<0.01	-	-	0.01	-	0.01	0.01
Nevskia	-	-	-	-	-	<0.01	-	-	-	-	-	-

Steroidobacter	-	-	-	-	-	-	-	-	-	-	<0.01	<0.01
Xanthomonadaceae	-	-	-	-	-	<0.01	-	-	-	-	-	-
Luteibacter	-	<0.01	-	-	<0.01	-	-	-	-	-	-	-
Stenotrophomonas	-	-	-	-	-	-	-	-	<0.01	-	<0.01	-
Anaeroplasma	<0.01	-	<0.01	<0.01	-	<0.01	<0.01	-	-	-	<0.01	<0.01
Anaeroplasmataceae	-	-	-	-	-	-	-	-	-	-	-	-
Mycoplasma	-	-	-	-	-	-	-	-	<0.01	-	-	-
Ellin515	-	-	-	-	-	-	<0.01	-	-	-	-	-
Ellin517	-	-	-	-	-	-	-	-	<0.01	-	-	-
DA101	0.05	<0.01	-	-	-	-	-	-	-	-	-	-

Source: The author (2020).