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FERMENTED COCOA MICROBIOME: LINKING MICROBIAL AND QUALITY ASPECTS

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Fermented cocoa microbiome:

Linking microbial and quality aspects

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Fermented cocoa microbiome:

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With references, with summaries in English

“The subject of this thesis was inspired by

seeking the essential of the invisible

tended to ignore to the human eye”

SUMMARY

Cocoa bean fermentation is a complex process address by a vast number of autochthonous microorganisms. This microbial diversity has a significant impact on the physicochemical, microbiological, safety and sensorial quality of fermented cocoa beans and their products. Nowadays, big efforts have been done to determine effective parameters for optimizing the fermentation process. However, the inline measurements (pH, temperature, cut test and fermentation index) use to evaluate the cocoa fermentation are easy to perform but these tests are not accurate. In contrast, microbiological tests are not used as a parameter to evaluate the sucess of the cocoa fermentation in the chocolate industry regardless of the great impact the microbial communities have on the quality of fermented cocoa. This thesis mainly deals with the microbial ecology of two cocoa varieties: *Criollo* and *Forastero.* Cocoa beans were fermented using different methods (Box and Heap) in Cameroon and Mexico. The aims of this thesis are to 1) explore the microbial communities on fermented cocoa beans, 2) correlate the concentration of metabolites produced during cocoa fermentation and the presence of microbial communities, 3) investigate the potential associations between bacterial communities and the metabolic pathways activated during cocoa fermentation. This thesis comprises five stages describe as following, an extensive literature review, detailed experimental study, assessment and development of new primers set for amplicon sequencing, quality and microbial assessment and, lastly the perspectives and challenges related to the selection of starter cultures and microbial identification. In detail, chapters 1 and 2 provide an overview of critical literature of microbial fermented cocoa beans over the last decade

and the potential of fungi to produce functional volatile compounds. Chapter 3 describes the dynamics of microbial communities and chemical compounds formation of inoculated and non-inoculated cocoa fermentation to improve our understanding to explore starter cultures with greater specificity and sensitivity. The results found in the previous chapter shaped chapter 4, that describes the limitations and errors of using amplicon sequencing to explore fungal dynamics within fermented cocoa beans. Considering the findings from the previous studies chapter 5 focused on the quality assessment of fermented cocoa beans discriminated by the variety and describes the role of cocoa variety in microbial composition. The final chapter of this thesis discusses the challenges and perspectives on the identification of microbial communities in fermented cocoa beans and identifies further research need. Furthermore, it also discussed the legislation and consumer acceptance of using starter cultures during the chocolate process. Most of this thesis comprises previously peer-reviewed, published work with the contribution of many collaborators. This thesis will be of benefit for both present and future chocolate research in the field of microbial ecology and food quality control.

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MICROBES

IN COCOA BEANS

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1.1 FROM COCOA TREE TO COCOA BEAN

Theobroma cacao L. is a perennial tree native to the South American tropical region and is cultivated only in the equatorial zone. Nowadays, this agricultural product serves as one of the main incomes for farmers in world tropical countries.

Figure 1.1 Most produced cocoa varieties

In general, *Forastero*, *Criollo*, and *Trinitario* are the three main varieties of cocoa produce worldwide, and its pods are approximately 12 to 30 cm in length, composed of 30 to 40 seeds cover by a mucilaginous white pulp. Interestingly, 95% of the world's cocoa production comes from the *Forastero* and its varietal hybrids cross [1].

Cocoa trees have been intensively grown in various parts of the globe. According to recently published figures, in 2015-2016 the annual production of cocoa of the eight main producing countries, Ivory Coast, Ghana, Indonesia, Cameroon, Brazil, Nigeria, Ecuador, and Peru, was estimated at 3,985 thousand tones, of which 37% of the total cocoa was produced in Ivory Coast. Besides the most important cocoa producing-countries, cocoa beans are also produces in Uganda, Malaysia, India, Mexico, Venezuela and Colombia [2].

1.1.1 Nutritional composition of cocoa beans

Cocoa bean is composed of two cotyledons and an embryo enclose by a seed coat, envelope in a sweet, white mucilaginous pulp. This pulp is approximately 40% of the weight of the fresh seed and holds 10 - 15% sugars,

mainly glucose, fructose, and sucrose [3,4]. However, when cocoa is unripe, the pulp has a higher proportion of sucrose compare to a ripe pulp, which is composed mainly of fructose and glucose [5]. Besides sugars, the cocoa pulp contains pectin and other polysaccharides, proteins, amino acids, minerals, and vitamins, mainly vitamin C [6].

From the nutritional point of view, cocoa beans are composed mainly by water (32 - 39%), total fat (30 - 32%, of which 65% is saturated fat), proteins (8 - 10%), cellulose (2 - 3%), starch (4 - 6%), pentoses (4–6%), sucrose (2 - 3%), polyphenols (5 - 6%), organic acids, mainly citric, oxalic, and malic (1%), theobromine (1 - 3%) and caffeine (0.2 - 0. 1%) [7,8]. This chemical composition of cocoa beans and pulp can be considered an appropriate medium for microbial growth [3,9]. Once the pulp and the cocoa beans are removed from the pod (mechanically or manually), they interact with a vast variety of microorganisms from the environment.

1.2 COCOA BEAN FERMENTATION

Fermentation is one of the oldest food-manufacturing methodologies used to add value to the raw materials. This process involves a metabolic route to obtain energy from organic compounds, without the participation of an oxidizing agent of exogenous origin [10]. Some of the advantages of a successful fermentation are that it allows perishable products such as milk, meat, and vegetables to have higher microbiological stability and safety [11]. Besides the bio-preservation of foodstuff, the microbes involved during fermentation have a significant impact on the physicochemical, microbiological, safety and sensorial quality of the products [12]. In food production, three kinds of fermentation are used such as lactic, alcoholic and acetic fermentation [13]. These can be

performed simultaneously in some food matrices, such as cocoa beans for the production of chocolate [8,14–18].

The cocoa fermentation process is regulated mainly by yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and members of the *Bacillaceae* (especially *Bacillus)* that use the pulp as a growing substrate. During the microbial cocoa fermentation, several molecules are released, which give the chocolate its characteristic aromatic profile, reducing the bitter taste and astringency and finally kill the embryo to avoid its germination [19,20]. Thus, most efforts have been focused on the identification of the microbiota involved during fermentation in the last decade, there are still some discrepancies between the relative abundance of microbial communities reported in fermented cocoa beans from different origins and fermented from different types of fermentation (box, heap) [21].

The contrasting values on the relative abundance of microbial communities might be due to the use of different identification methods. The "cocobiota" has been studied since the '60s, however, the use of DNA sequencing technologies to profile the microbial ecosystems of foodstuff has been conducted only during the last 10 years, while DNA based fingerprinting such as denaturing gradient gel electrophoresis (DGGE) or restriction fragment polymorphism (RFLP) have been widely used to identify and quantify microbial species involved in food matrices [22]. In this chapter, a total of 47 available published studies are discussed in deep to describe the main microbial populations belonging to yeasts, LAB, AAB, and *Bacillaceae* involved in different varieties of fermented cocoa beans from different origins, using different fermentation methods, mainly box, heap or tray. In addition, a brief description of the methods that have been used to identified microbial communities in spontaneous fermentation is discussed.

1.2.1 Microbial succession and biochemical changes in fermented cocoa beans

Cocoa fermentation is a complex process involving autochthonous microorganisms [23,24] originated from post-harvest procedures (from knives, banana or plantain leaves), pods surfaces, worker's hands and from the surface of the containers where the fermentation process is carried out and were yeasts, LAB and AAB play the leading role [8,24–28]. During cocoa bean fermentation, three main stages can be distinguished. An overview of the microbial and biochemical reactions involved in cocoa bean fermentation is shown in Figure 1.2.

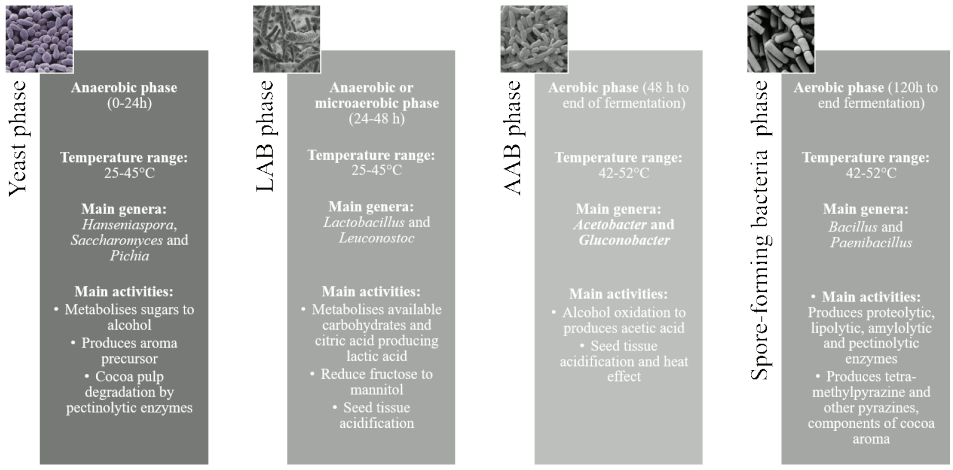

Figure 1.2 Physical requirements and biochemical characteristics of the microbial growth of yeasts, LAB, AAB, and *Bacillaceae* genus during cocoa bean fermentation. Adapted from [20]

The first stage involves the growth of yeasts which mainly belongs to *Saccharomycetaceae* family, including *Hanseniaspora*, *Saccharomyces*, *Kluyveromyces* and *Pichia* [29], these genera produce ethanol, and its growth is favored by the higher concentration of glucose and citric acid and the low availability of oxygen. However, yeasts can also produce other compounds such

as carbon dioxide, organic acids (acetic and succinic acid) and glycerol [3,4,30–35]. Yeasts are also involved in the degradation of pectin found in the cocoa pulp, and in the production of a large number of aroma compounds precursors, such as higher alcohols and esters, that contribute significantly to the development of chocolate aroma profile [23,36].

The second stage is characterized by the increment of lactic acid concentrations, as a consequence of the increase of LAB populations and the decrease of yeasts. Noteworthy, the importance of the role of LAB during the fermentation of cocoa has been controversial. According to Ho *et al.* (2015) LAB may not be essential in cocoa bean fermentation [15]. However, several studies support the presence of a limited diversity of LAB, mainly heterofermentative, such as *Lactobacillus fermentum,* that produces volatile compounds such as diacetyl, acetoin, and 2,3-butanediol, supports bacterial growth and allows a slight increase of pH in cocoa pulp [4,26,35,37–39].

Finally, in the third stage, LAB population begins to decrease and simultaneously increase the population of AAB, which are responsible for the simultaneous oxidation of ethanol produced by yeasts and the conversion of lactic acid produced by LAB to acetic acid and acetoin [25,40]. Subsequently, acetic acid can be overoxidized to carbon dioxide and water. The rise in temperature, the decrease in pH from 6.5 to 4.8 and the penetration of acetic acid and ethanol to the cocoa bean are the cause of the death of the embryo [3,6,31,41–43]. Besides the well-known bacteria described above, *Bacillus* has been often isolated from fermented cocoa beans, however, its contribution to this process remains to be elucidated. According to Ouattara *et al.* (2011), *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus fusiformis* isolated from cocoa fermentation were the main producers of pectin lyase during cocoa fermentation [44]. In addition, it seems that *Bacillus* could be implicated on the production of other pectinolytic enzymes such as polygalacturonase during cocoa fermentation [45]. However it should be pointed

out that members of the *Bacillus* genus can release off- flavor on fermented cocoa beans due to the production of short-chain fatty acids [8,18].

Despite the biochemical changes described above, a cell disruption occurs inside the cocoa bean. This phenomenon causes the release of cellular components of the cotyledon and produces favorable environments to develop taste precursors and pigment degradation by the enzymatic action of invertases, glycosidases, and proteases as endoproteinases, carboxylases, aminopeptidases and polyphenol oxidases [46]. Also, enzymatic hydrolysis of cocoa bean proteins, mainly vicilin and albumins, occurs during this process. These biochemical reactions can produce peptides with a wide range of molecular weights, amino acids and reducing sugars that are substrates needed for Maillard reactions which are necessary for the full development of aromas and flavors during drying and roasting of cocoa bean [47–49].

1.2.2 Main microorganisms involved in cocoa bean fermentation

The study of microbial diversity during the fermentation process of cocoa has been addressed in many studies using culture-dependent and independent methods. Microbial diversity of fermented cocoa beans in the last ten years has been carried out in Ivory Coast [12,33,43–45,50–54], Ghana [12,23,41,42,55], Brazil [8,32,33,42,56–62], Malaysia [33,35,43,63], Mexico [64], Honduras [65] Ecuador [32,33], Cuba [66], Cameron [12,21], Bolivia [67], Nigeria [68], Philippines [69], and Australia [15,36] as shown in Table 1.1.

Table 1.1 Overview of the studies addressing microbial diversity involved in spontaneous cocoa fermentation from main studies performed over the last decade

Publication year	A	B	Br	C	Cu	E	G	H	I	M	Me	N	P	**Total**
2008			1				1					1		3
2009							2							2
2010			1				1							2
2011			3			2			3	1				9

2012			1						1	1				3
2013			3				1			2				6
2014	1													1
2015	1		1						1		1		1	5
2016		1	3	1	1		1		3					10
2017			2						1					3
2018			1	1				1						3
Total	**2**	**1**	**16**	**2**	**1**	**2**	**6**	**1**	**9**	**4**	**1**	**1**	**1**	**47**

Abbreviations: A: Australia, **B:** Bolivia, **Br:** Brazil, **C:** Cameron, **Cu**: Cuba, **E:** Ecuador, **G:** Ghana. **H:** Honduras, **I:** Ivory Coast, **M:** Malaysia, **Me:** Mexico, **N:** Nigeria, **P:** Philippines

In detail, the most representative microbial species of fermented cocoa beans from 13 different geographical locations include a total of 274 microbes, comprising 125 yeasts, 66 LAB and 49 AAB and 11 *Bacillus* as shown in Table 1.2. One notes, that the yeast community showed greater diversity compared with bacteria (Table 1.2). Overall, the most commonly identified microbial species involved in fermented cocoa include yeasts (*Pichia kudriavzevii*, *Hanseniaspora opuntiae*, and *Saccharomyces cerevisiae)*, LAB (*Lactobacillus fermentum*, and *Lactobacillus plantarum)*, and AAB (*Acetobacter pasteurianus)*. Additionally, in some studies, the presence of a member of the *Bacillus* genus, *Enterobacteriaceae*, and filamentous fungi have also been reported [42,70,71]. Concerning the microbial fermented cocoa beans species found by country, interesting data are driving to the conclusion that minor microbial species discriminate cocoa beans by origin (Table 1.2).

Table 1.2 Frequency distribution of main microbial species found in fermented cocoa beans

Main microbes	A	B	Br	C	Cu	E	G	H	I	M	Me	N	P	**Total**
Acetobacter calcoacetis								1						1
Acetobacter cerevisiae								1						1
Acetobacter fabarum		1	2					1						4
Acetobacter ghanensis		1	2				2	1						6
Acetobacter lovaniensis			1											1
Acetobacter pasteurianus	2	1	7	1		2		1	1	3				18
Acetobacter pomorum			1							1				2
Acetobacter senegalensis		1	2					1						4
Acetobacter sp.			1						1					2
Acetobacter syzygii			1	1			2		1					5
Acetobacter tropicalis			1											1
Acinetobacter sp.			1											1
Bacillus cereus			3											3
Bacillus fusiformis									1					1
Bacillus pumilus									1				1	2
Bacillus sp.				2										2

Main microbes	A	B	Br	C	Cu	E	G	H	I	M	Me	N	P	**Total**
Bacillus subtilis			2						1					3
Candida krusei			1											1
Candida magnoliae			1											1
Candida nitrativorans									1					1
Candida pseudotropicalis			1											1
Candida sorbosivorans						2								2
Candida tropicalis			1						3					4
Enterobacteriaceae									1					1
Enterococcus italicus										1				1
Fructobacillus sp.										1				1
Fructobacillus pseudoficulneus			1											1
Fructobacillus tropaeoli						2								2
Galactomyces geotrichum									3					3
Gluconobacter frateurii	2													2
Gluconobacter oxydans			2											2
Hanseniaspora guilliermondii	2		1			1		1	1	2				8
Hanseniaspora opuntiae		1	3	2	1	1	1	1	2	2				14
Hanseniaspora sp.			2								1			3
Hanseniaspora thailandica							1							1
Hanseniaspora uvarum			7			1		1	1	2				12
Klebsiella sp.				1										1
Kluyveromyces marxianus	2			2										4
Lactobacillus brevis												2		2
Lactobacillus cacaonum								1						1
Lactobacillus casei			1											1
Lactobacillus curieae									1					1
Lactobacillus durianis			1											1
Lactobacillus fermentum	2	1	10	3		2	2	1	1	3		2		27
Lactobacillus mali			1											1
Lactobacillus nagelii			2											2
Lactobacillus pentosus	2									1				3
Lactobacillus plantarum	2	1	9					1	1	2		2		18
Lactobacillus rhamnosus			1											1
Lactobacillus sp.								1						1
Leuconostoc mesenteroides									1					1
Leuconostoc pseudomesenteroides			1			2			1	1				5
Lysinibacillus fusiformis			3											3
Pediococcus acidilactici		1	2									2		5
Pichia caribbica			3											3
Pichia galeiforms									3					3
Pichia kluyveri			4											4
Pichia kudriavzevii	2		2		1	3			6	2				16
Pichia manshurica					1	2	1							4
Pichia membranifaciens			1											1
Pichia mexicana													1	1
Pichia pijperi				2										2
Pichia sp.			2											2
Saccharomyces cerevisiae	2	1	12	2		2	1	1	4	4	1			30
Schizosaccharomyces pombe										1				1
Tatumella punctata						2								2
Tatumella saanichensis						2								2
Torulaspora delbrueckii			2											2
Wickerhamomyces anomalus									3					3

Abbreviations: A: Australia, **B:** Bolivia, **Br:** Brazil, **C:** Cameron, **Cu**: Cuba, **E:** Ecuador, **G:** Ghana. **H:** Honduras, **I:** Ivory Coast, **M:** Malaysia, **Me:** Mexico, **N:** Nigeria, **P:** Philippines

Interestingly, it appears that the dominating microbial species are not at all present in every country studied, as observed in Figure 1.3. The following results indicate that microbial composition can be discriminated by geographical location. In detail, Malaysia and Brazil reported the presence of all the most commonly identified microorganisms involved in cocoa bean fermentation (*P. kudriavzevii*, *H. opuntiae*, *H. uvarum*, *S. cerevisiae*, *L. fermentum*, *L. plantarum*, and *A. pasterianus)*, while Cuba and Mexico reported only the presence of yeasts. In general, further studies are needed to elucidate the bacterial ecology in fermented cocoa beans from the American and Asian continent to provide a better knowledge of the cocobiota in this region.

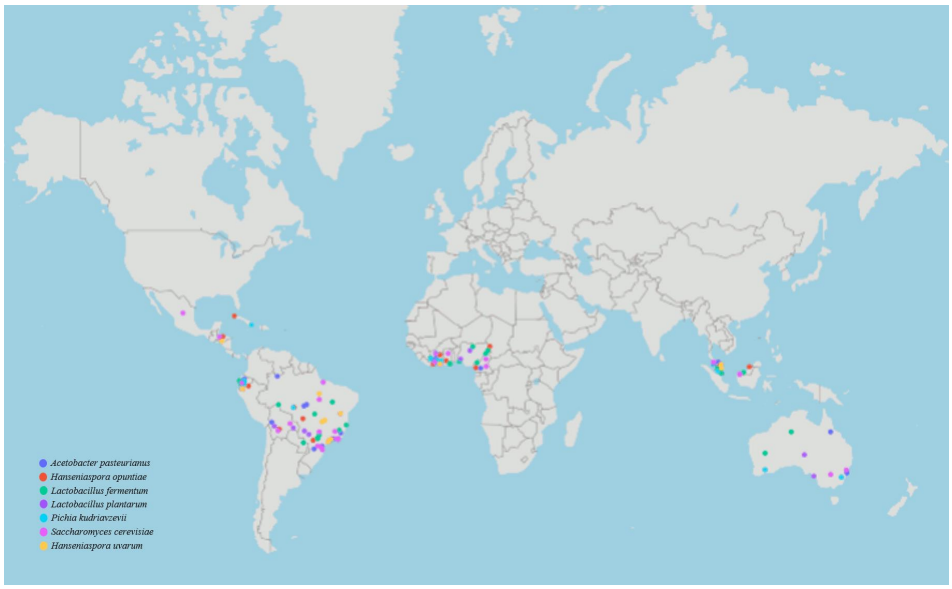

Figure 1.3 Occurrence of dominating microbial species over the last decade in fermented cocoa beans

1.2.3 Geographical location of microbial fermented cocoa communities

Over the last decade, there has been a growing interest in studying fermented cocoa beans from leading producing countries, except Indonesia and

Peru, from which to our best knowledge, no update or none information has been generated concerning microbial diversity. In contrast, we observed that over the last decade the microbial community of fermented cocoa beans of Brazil has been extensively studied followed by Ivory Coast, and Malaysia (Table 1.1).

1.2.4 Fermented cocoa varieties studied in food microbiology

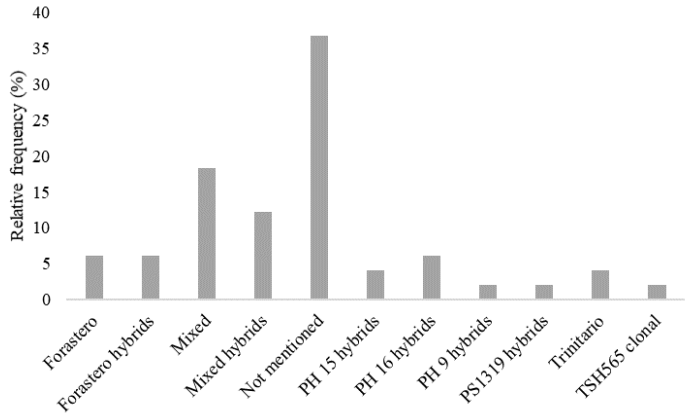

Figure 1.4 Percentage by the fermented cocoa varieties studied from 2008 to 2018

Thus, fermented cocoa beans have been extensively studied; it is alarming that 37% of the total amount of studies published do not mention the type of cocoa variety investigated as shown in Figure 1.4. Excluding the studies that did not mention the type of cocoa variety one also notes that the mixed of cocoa varieties (*Trinitario, Criollo* and/or *Forastero*) have been more investigated rather than single varieties (Figure 1.4).

1.2.5 Fermentation method and duration

There are several methods of fermentation employed for cocoa bean, and these can vary according to the region where the cocoa is fermented. The most frequent methods studied over the last decade are the following: (i) heap [52,55], (ii) trays [23,68,72], (iii) box [34,35,64], (iv) basket [8], and (v) platform (Figure 1.5). While the quantity of cocoa beans process varies between 5 and 2,000 kg

[8]. One notes that this amount of beans depends on the production capacity of the local cocoa farm.

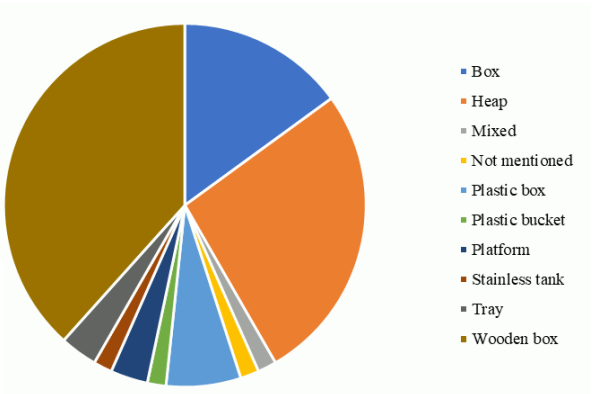

Figure 1.5
Percentage by the type of cocoa fermentation method used

1.3 IDENTIFICATION METHODS USED TO ASSESS MICROBIAL DIVERSITY IN FERMENTED COCOA BEANS

Methods for investigating the cocobiota have been either studied through culture-dependent or culture-independent methods. The traditional microbial culturing approach has been extensively used for enumeration and identification of microbial communities in fermented cocoa beans [21,33,34,68]. However, this technique fails to reproduce the ecological niches and symbiotic relationships encountered in a complex natural ecosystem [73]. Through culture-independent methods, molecular techniques including direct cloning or sequencing of DNA fragments of fingerprinting methods [restriction fragment polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP) and denaturing high-performance liquid chromatography (DHPLC)] has been used to identify the microbial community [74]. The microbial ecology of fermented cocoa beans over the last decade have been mostly assessed through culture-independent technique such as PCR-DGGE [32–36,50,64,75], while only a few studies have used amplicon sequencing to analyze microbial communities as shown in Figure 1.6 [12,55,63,66,76].

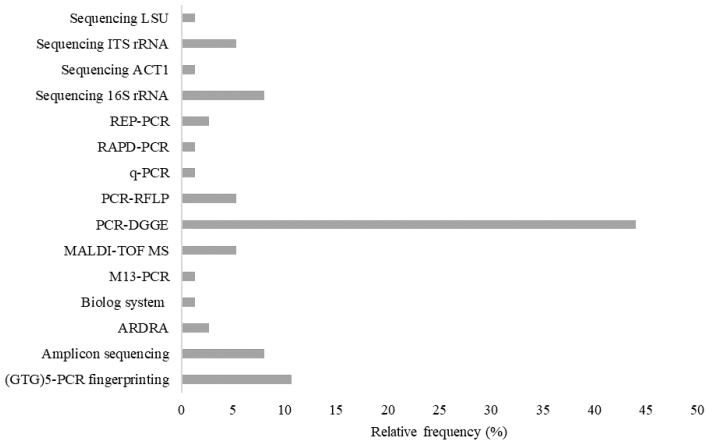

Figure 1.6 Percentage by the type of independent methodology used to identify fermented cocoa microbial diversity

Mis-identification of microbial species is a limiting factor that also influences in all independent and dependent techniques [9,76]. Therefore, the assessment of both dependent and independent culture methods has been extensively used to reveal the microbial ecology in fermented cocoa beans [21,24,26,32,34,55,63,77]. Overall, the study of microbial diversity through culture-dependent methodologies is an essential step to select potential starter culture with relevant biochemical or physiological features as high aromatic compounds producing, tolerant to heat, acid, ethanol, and phenolic compounds, and pectin and citric acid metabolizing strains. These two methodologies complement and provide a better understanding of the complexity of the cocoa bean fermentation process, as well as the growth kinetics and presence of the microorganisms involved. Therefore, it is highly recommended to use an integrated approach to study the microbial ecology to decrease the risk of mis-identification of microorganisms.

Besides molecular biological techniques, the identification of microbes using either intact cells or cell extracts have been also detected by Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) and Biolog system. In addition to these techniques, conventional

morphological and biochemical assessments, have been used to profile the microbial diversity in fermented cocoa beans [78].

1.3.1 Challenges and perspectives

The use of genomics and metabolomics techniques have generated a great amount of knowledge to elucidate the cocobiota ecosystem. In detail, the -omics sciences seem to be an excellent alternative to understand the diversity and functionality of the microorganisms involved in the cocoa ecosystem. Illeghems *et al.* (2015) reconstructed the microbial meta-pathways based on metagenomic data of a fermented cocoa bean from Brazil after 30 h [79]. The analysis of bacterial functionality showed that the central metabolic pathways associated with LAB were heterolactic fermentation and citrate assimilation, while AAB, was only partially reconstructed and was involved in responses toward stress. However, these methods are characterized to be computationally intensive and are again challenged by sequencing bias as well as under/overestimations [76]. Despite these challenges, culture-independent methods have revealed greater microbial compared with culturing, provide novel insights into many food ecosystems and hold great promising data access [21,32,36,64,78]. However, culture-dependent studies are necessary to achieve the proper selection of the strains that will be part of starter culture.

1.4 STARTER CULTURES USED DURING COCOA FERMENTATION

Several attempts on designing a microbial starter culture have been addressed for cocoa fermentation [21,23,38,43,58,60,61,72,80–84]. Interestingly, the first evaluation of the performance of a mixed starter culture composed of *S. cerevisiae* var. *chevalieri*, *Lactococcus lactis*, *L. plantarum*, *Acetobacter aceti*, and *Gluconobacter oxydans* subsp. *suboxydans* for fermented cocoa beans was

assessed in 1998 [80]. According to Schwan (1998), it was concluded that starter cultures are a viable option to obtain a reliable fermentation process and consequently desirable characteristics of chocolate [80]. However, the use of *Gluconobacter* species has also been known as producers of acids and off-flavors and cause a late yeast development into cocoa fermentation [14,25,34,40].

Over the last decade, *Kluyveromyces marxianus*, *P. kluyveri*, *S. cerevisiae*, *T. delbrueckii*, *L. fermentum*, and *A. pasteurianus* have also been extensively studied. In detail, Leal *et al.* (2008) demonstrated the capacity of a hybrid strain of *K. marxianus* to produce a chocolate with more acceptability by the sensorial panelist in comparison with those obtained from a spontaneous fermentation process [60]. Besides enhancing the organoleptic characteristics of cocoa beans, the inoculated fermentation with *K. marxianus* increased the volume of the sweating and modified the microorganism's dynamics and protein degradation.

P. kluyveri a recognized strain for its high production capacity of aromatic compounds was also evaluated as inoculum to conduct this process. The use of this yeast pronounced differences in the composition of volatile organic compounds (VOCs) in roasted cocoa liquors and chocolate when compared with those obtained from a spontaneous fermentation process. The chocolate produced with the inoculated cocoa beans were described as fruity, acid, yogurt and balsamic flavors, whereas the spontaneously fermented chocolates were described as sweet, cocoa and caramel flavors. However, despite these differences, the perception by consumers did not show a significant change [72].

The differences between the composition of VOCs between inoculated and non-inoculated cocoa beans is also supported by Visintin *et al.* (2017). According to this study, a positive impact on the aromatic profile of the chocolate produced with beans inoculated with a mixed starter culture composed by *S. cerevisiae* and *T. delbrueckii* and a monoculture of *T. delbrueckii* was observed

in comparison to those obtained through spontaneous fermentation [82]. However, a recent study reported no significant difference in the composition of VOCs at the end of the fermentation between inoculated and non-inoculated cocoa beans using the same inoculated yeast strains [21].

On the other hand, Crafack *et al.* (2013), studied the potential impact of *P. kluyveri* (aromatic) and *K. marxianus* (pectinolytic) on cocoa flavor during fermentation coupled with *L. fermentum* L18 or with *A. pasteurianus* A149 [23]. They concluded that the starter culture composed of *P. kluyveri* CH/*Lb. fermentum* L18/*A. pasteurianus* A149 seemed to have a positive effect on the flavor profile compared with spontaneously fermented cocoa bean by obtaining the highest scores for sensorial descriptors such as sweetness, fruitiness, cocoa aroma and general liking from a panel of an un-trained panelist. Besides the most studied microorganisms also *Bacillus pumilus* and *Pichia mexicana* have shown to shorten the fermentation process from 6 to 4 days [69].

Based on the knowledge of bacterial metabolism and physiology as on the microbial diversity within fermented cocoa beans, two bacterial species, *L. fermentum* and *A. pasteurianus* can be considered good candidates for starter cultures. In detail, the heterofermentative metabolism, fructose-growth capacity, citrate conversion, mannitol production and acid, heat, and ethanol tolerance of *Lb. fermentum* are suitable characteristics of a starter strains in cocoa fermentation [14,26,37–39], while *A. pasteurianus*, a relevant AAB in the cocoa bean fermentation process, shows ethanol-, mannitol-, and lactic acid- oxidizing capacity and heat-tolerance [3,14,35,40]. Finally, the great diversity of yeasts involved in the cocoa bean fermentation process has a pronounced effect on the fermentation efficiency and the quality of the fermented cocoa bean. The most outstanding yeast species, such as *Saccharomyces*, *Pichia*, and *Hanseniaspora* have suitable characteristics, such as the production of aromatic compounds, high growth capacity and pectinolytic activity [14,36,63]. In general, we can conclude that a proper starter culture can direct the cocoa fermentation process and must

be composed of at least one strain of each microorganism's group (yeast, LAB, and AAB). However, it is necessary to select the strain of LAB properly to avoid the overproduction of lactic acid during this process and therefore having a negative impact on cocoa bean quality.

1.4.1 Application of starter cultures in fermented cocoa beans at industrial level

A few companies have promoted the use of starter cultures for cocoa fermentation. In short, the company Barry Callebaut executed a project called "Cocoa Horizons" in Ivory Coast promoting the use of a yeast starter culture, mainly *Saccharomyces* strains, to conduct the fermentation process of cocoa to obtain chocolate with a high content of aromatic compounds [85]. Lallemand, a starter culture company and a French chocolate producer (Cémoi) together with an agriculture research Centre (CIRAD) launched a research program in 2016 focused on conducting the cocoa bean fermentation process by using starter cultures to obtain a consistent quality of chocolate. To achieve this objective, commercial yeast strains used in wine production, as well as commercial AAB, were used [86]. The complexity of microbial ecology during cocoa fermentation is currently undergoing a revolution. The use of starter cultures provides a positive effect on the flavor profile, but there are some discrepancies on the performance of the strains, consequently, it has been difficult to standardize the performance of starter culture in terms of repetition to obtain high-quality chocolate. For this reason, when introducing starter cultures to dominate a cocoa ecosystem, research on microbial ecology, as well as quality assurance in cocoa fermentation, should be expanded.

1.5 OBJECTIVE OF THIS THESIS

In the framework of continuing the optimization of the fermented cocoa processes, the aim of this thesis was to assess the structure and function of microbial communities of fermented cocoa beans to determine effective parameters for quality control. This thesis focus on three microbial ecological challenges. First, when fermented cocoa beans are exposed to starter cultures, an improvement in the quality of the end product could occur if the environmental condition is properly managed. Environmental conditions affect the performance of starter cultures efficiency, when predictable characteristics, reproducible rate of a specific feature and the absence of pathogen or spoilage microorganism contributes to a more efficient production process. Therefore, this thesis aimed to introduce a starter culture to evaluate the production of desirable and reproducible features exposed to different fermentation methods to guarantee quality. Second, since the microbial ecology is essential to ensure a successful fermentation and flavor development; it is important to understand how the composition of the cocoa microbial ecosystem is established and their mechanism of action, the second aim of this thesis focus on advance the functions encoded at the level of DNA by estimating the strength of the relationship between microbial associations and how they regulate the aroma development of fermented cocoa beans and the fermentation process. Third, despite the importance of microbial communities during cocoa fermentation, fungal biodiversity has been studied far less than bacteria, therefore, the last aim of this thesis was to investigate the potential bias associated with the amplification of fungal communities to assess reliable and accurate ecological populations.

1.5.1 Thesis outline

Chapter 2 is a compilation of available literature found on the formation of volatile organic compounds (VOCs) during inoculated cocoa fermentation with yeasts and the potential health benefits of selected VOCs. The use of yeast

species as starter during cocoa fermentation and the quality evaluation of the chocolate produced from inoculated cocoa beans were investigated and the development of VOCs during fermentation, roasting and final product was tracked.

Chapter 3 focusses on describing the microbial ecology of fermented cocoa beans and the effect of yeast starter cultures as a possible enhancer of chocolate quality. Fermented cocoa beans were exposed to *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* at the beginning of the fermentation. The possibility of yeast starter cultures to enhance cocoa aroma was assessed by analyzing volatile and non-volatile compounds in fermented cocoa beans and the microbial ecology were assessed by using amplicon-sequencing. In addition, the influence of the fermentation processing method used on the formation of all parameters was determined.

Chapter 4 explores the critical role of primer selection to assess reliable and accurate ecological populations. Fungal communities within fermented cocoa beans and a mock community were evaluated using high-throughput sequencing tools targeting two different nuclear ribosomal RNA regions to indicate possible preferential amplification phenomenon.

Chapter 5 expands the previous studies by describing the microbial ecology and the potential metabolic pathways and interactions between bacterial communities and the aroma and metabolite profile of two cocoa varieties during fermentation. The predicted metagenomes were determined by using the PICRUSt analysis. The possibility to differentiate organoleptic characteristics between cocoa varieties during fermentation was assessed by a trained panelist and chromatography techniques.

Chapter 6 provides a general conclusion based on the investigation presented in this thesis. The most relevant findings and the future research needed

on the use of starter cultures as well as on the microbial ecology concerns are discussed.

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TRACEBILITY OF VOCs

GENERATED ON FERMENTED COCOA BEANS

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ABSTRACT

Microbial communities are responsible for the unique functional properties of chocolate. During the microbial growth, several antimicrobial and antioxidant metabolites are produced and can influence human wellbeing. In the last decades, the use of starter cultures in cocoa fermentation has been pushed to improve nutritional value, quality, and overall product safety. However, it must be noted that unpredictable changes in cocoa flavor have been reported between the different strains from the same species used as a starter, causing a loss of desirable notes and flavors. Thus, the importance of an appropriate selection of the starter cultures based on the biogenic effect that optimize chocolate quality has become a major interest in the chocolate industry. This chapter aimed to review the microbial communities identified from spontaneous cocoa fermentation and focused on the yeast starters strain used in cocoa beans and their sensorial and flavor profile. The potential compounds that could have health-promoting benefits like limonene, benzaldehyde, 2-phenylethanol, 2-methylbutanal, phenylacetaldehyde, and 2-phenylethyl acetate were evaluated as these compounds were formed during fermentation and their presence remained constant after roasting. Further research is needed to highlight the future perspectives of microbial volatile compounds as biomarkers to warranty food quality and safety.

2.1 INTRODUCTION

Certainly, people have been changing their food consumption patterns and lifestyle over the last decade. To counteract unhealthy food choices, functional food has emerged as a strategy to increase the consciousness of the relationship between diet and disease/health to consumers. The objective of a successful functional food is to target a specific group of consumers and to meet their health demands without compromising flavor, taste, and color. In this context, the most use bioactive compounds in the food industry include alkaloids, anthocyanins, carotenoids, flavonoids, glucosinolates, isoflavones, phenolic acids, hydrolysate proteins, tannins, and phytochemicals terpenes [1].

Volatile organic compounds (VOCs) are organic molecules that include esters, alcohols, aldehydes, ketones, phenols, terpenes, etc. Recently, it has been demonstrated that VOCs provide health benefits to consumers [2]. These VOCs are synthesized naturally by a broad number of plants or microorganisms (as secondary metabolites) to enable interactions with their environment. Health benefits provide by microbial communities can be either direct or indirect. The difference between these two concepts relies on the difference in the ingestion of a live microorganism (direct) or microbial metabolites (indirect or biogenic effect) [3]. Undeniably, a biogenic effect is commonly observed in fermented foods such as chocolate.

The production of microbial metabolites in cocoa beans begins during fermentation, in this process, microorganisms, encompassing bacteria and yeasts, serve to confer taste, texture and desirable aromas to the final product. An effective cocoa fermentation develops when a correct microbial succession of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) takes place [4,5]. The success of these dynamics is due to the nutrient content of the cocoa pulp that is used as an optimal substrate for the microbial growth, and yeasts are considered the first microorganisms growing at the beginning of the fermentation

process, producing ethanol, organic acids, and VOCs, that contribute as precursors of chocolate flavor [6]. For those reasons, yeasts have been widely used as starter cultures in cocoa beans with the aim to enrich the sensorial quality of chocolate. However, the modulation of the remarkable complexity of microbial communities in cocoa beans to obtain an optimal flavor fingerprinting as well as understanding the metabolic and regulatory networks, concerning the production of secondary metabolites are still not clear. In this context, the present chapter aims to describe the individual capacities of yeast species to produce aroma compounds to enhance flavor perception and nutritional or healthy values. More importantly, it assesses the most frequently VOCs identified during the three different steps of chocolate elaboration, including fermentation, roasting and the final product, chocolate (Figure 2.1). It's important to clarify, that the VOCs identified in the fermentation and final product were the most frequently identified VOCs in inoculated cocoa beans with yeasts, while the most frequently identify VOCs during roasting were assessed from non-inoculated cocoa beans.

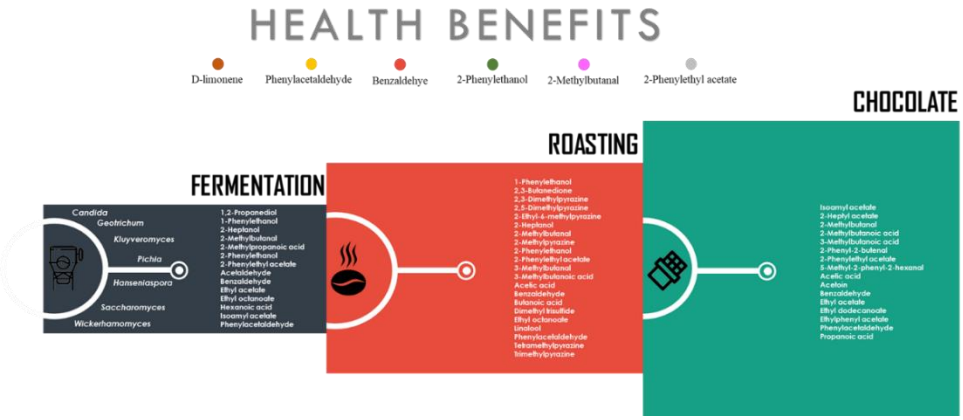

Figure 2.1 Tracking volatile compounds from chocolate

2.2 YEAST SPECIES USED AS STARTER DURING COCOA FERMENTATION

Yeasts are considered a safe source of ingredients and additives for food processing because they have a positive image with consumers [7]. The interest in yeasts as starter cultures has arisen in recent years especially in relation to the addition of *Saccharomyces, Pichia, Kluyveromyces, Candida*, and *Torulaspora* to fermented cocoa beans [5,8–16]. Yeasts are considered a safe source of ingredients and additives for food processing because they have a positive image with consumers [7]. The interest in yeasts as starter cultures has arisen in recent years especially in relation to the addition of *Saccharomyces, Pichia, Kluyveromyces, Candida*, and *Torulaspora* to fermented cocoa beans [5,8–16]. Yeasts that are being used to ferment cocoa beans are shown in Table 2.1. It should be noted that the starter cultures used to drive cocoa fermentation process have been applied only in few cocoa-producing countries such as Brazil, Malaysia, Indonesia, and Cameroon [5,8–15,17]. The biggest challenge of standardizing the cocoa fermentation process is to control the environmental conditions of the process. Therefore, the choice of selecting cocoa fermenting starters should be based on the environmental conditions of the cocoa-producing country and the microbial communities involved during the process. Up to date the most frequently yeast culture used in fermented cocoa beans is *Saccharomyces cerevisiae*. This yeast has the capability to assimilate and ferment reducing sugars and citric acid, produce aroma substances and killer-like toxins, it has a high pectinolytic activity and can prevent microbial pathogen growth [4,6,18–22]. Despite the well-known *Saccharomyces*, non-*Saccharomyces* yeast (*Kluyveromyces, Hanseniaspora, Pichia* and *Torulaspora)* have been also showed a relevant pectinolytic activity and increased the aroma complexity in wine [23,24]. However, these species exhibit a lower ethanol yield, and sugar consumption compared to *S. cerevisiae* [25]. Regardless of the limitations of non-

Saccharomyces yeast to metabolize ethanol, several studies have inoculated cocoa beans during fermentation with mixed yeast cultures (Table 2.1) [5,11,26]. However, the combination of different yeast species often results in unpredictable compounds production and/or different microbial communities, which can affect both the chemical and ecological population of fermented cocoa beans. The unpredictable changes specifically from the ecological point of view might be explained by the antagonist activity of yeast species. *S. cerevisiae* is well known to inhibit the growth of non-*Saccharomyces* species (*Hanseniaspora guilliermondii, Torulaspora delbrueckii, Kluveromyces marxianus*, and *Lachancea thermotolerants)* by producing antimicrobial peptides with a 4.0, 4.5 and 6.0 kDa [27]. Therefore, the selection of starter cultures to produce chocolate plays an important role not only in the modulation of the microbial communities but rather to achieve optimal sensorial properties, such as cocoa, malty and fruity flavors [5,11,28]. In this regard, future research is needed to elucidate the variability at the strain level that contributes to an added value to the cocoa fermentation [8].

Table 2.1 Functional yeasts used as starters in cocoa fermentation

Genera/species	Year	Country	Type of cocoa bean	Type of fermentation	Amount	VOCs		SA	Reference
						F	C		
Kluyveromyces marxianus MMIII-41	2008	Brazil	NM	Plastic basket	45 kg	-	-	+	Leal *et al.*, 2008[9]
Saccharomyces cerevisiae UFLA CA11	2014	Brazil	PH 16	Wooden box	60 kg	+	-	-	Ramos *et al.*, 2014[12]
Saccharomyces cerevisiae UFLA CA11	2014	Brazil	PS1030	Wooden box	60 kg	+	-	-	Ramos *et al.*, 2014[12]
Saccharomyces cerevisiae UFLA CA11	2014	Brazil	FA13	Wooden box	60 kg	+	-	-	Ramos *et al.*, 2014[12]
Saccharomyces cerevisiae UFLA CA11	2014	Brazil	PS1319	Wooden box	60 kg	+	-	-	Ramos *et al.*, 2014[12]
Saccharomyces cerevisiae, Pichia kluyveri and Hanseniaspora uvarum	2015	Brazil	PS1319	Wooden box	100 kg	-	-	+	Batista *et al.*, 2015[10]
Candida sp.	2015	Malaysia	NM	Basket	5 kg	-	-	-	Mahazar *et al.*, 2015[15]
Saccharomyces cerevisiae H19	2016	Malaysia	NM	Basket	50 kg	-	+	+	Meersman *et al.*, 2016[16]
Saccharomyces cerevisiae H28	2016	Malaysia	NM	Basket	50 kg	-	+	+	Meersman *et al.*, 2016[16]
Saccharomyces cerevisiae H37	2016	Malaysia	NM	Basket	50 kg	-	+	+	Meersman *et al.*, 2016[16]
Saccharomyces cerevisae var. Chevalieri	2014	Indonesia	*Forastero*	Plastic bags	NM	-	-	-	Cempaka *et al.*, 2014[14]
Saccharomyces cerevisiae	2016	Brazil	CCN51	Wooden box	100 kg	-	+	+	Menezes *et al.*, 2016[13]
Saccharomyces cerevisiae	2016	Brazil	CEPEC2004	Wooden box	100 kg	-	+	+	Menezes *et al.*, 2016[13]
Saccharomyces cerevisiae	2016	Brazil	FA13	Wooden box	100 kg	-	+	+	Menezes *et al.*, 2016[13]
Saccharomyces cerevisiae	2016	Brazil	PS1030	Wooden box	100 kg	-	+	+	Menezes *et al.*, 2016[13]
Torulaspora delbrueckii	2017	Brazil	PS1319	Wooden box	300 kg	-	+	+	Visintin *et al.*, 2017[11]
T. delbrueckii	2017	Brazil	SJ02	Wooden box	300 kg	-	+	+	Visintin *et al.*, 2017[11]
S. cerevisiae and T. delbrueckii	2017	Brazil	PS1319	Wooden box	300 kg	-	+	+	Visintin *et al.*, 2017[11]
Pichia kudriavzevii LPB06	2017	Brazil	NM	Lab scale	400 g	+	-	-	Pereira *et al.*, 2017[8]
Pichia kudriavzevii LPB07	2017	Brazil	NM	Lab scale	400 g	+	-	-	Pereira *et al.*, 2017[8]
Saccharomyces cerevisiae	2018	Cameroon	*Forastero*	Wooden box	200 kg	+	-	-	Mota *et al.*, 2018[5]
Saccharomyces cerevisiae	2018	Cameroon	*Forastero*	Heap	100 kg	+	-	-	Mota *et al.*, 2018[5]
Saccharomyces cerevisiae and T. delbrueckii	2018	Cameroon	*Forastero*	Wooden box	200 kg	+	-	-	Mota *et al.*, 2018[5]
Saccharomyces cerevisiae and T. delbrueckii	2018	Cameroon	*Forastero*	Heap	100 kg	+	-	-	Mota *et al.*, 2018[5]

Abbreviations: SA, Sensorial analysis; NM, not mentioned; F, Fermented cocoa volatile compounds profile; C, Chocolate volatile compounds profile from inoculated cocoa beans; +, Presence; -, Absence

2.2.1 Quality evaluation of the chocolate produced from inoculated cocoa beans

Contrasting findings on the sensory analysis of chocolate produced from cocoa beans inoculated with yeasts has been recently assessed (Table 2.1) [9–11,13,17]. In detail, the consumer panel from Brazil and Malaysia had described chocolates inoculated with *K. marxianus* (Brazil), *S. cerevisiae* (Malaysia and Brazil), *T. delbrueckii* and a mixed culture of *S. cerevisiae* and *T. delbrueckii* (Brazil) with better desirable notes, flavor attributes, and global acceptability compared with chocolate produced from spontaneous cocoa beans fermentation [9,11,17]. In contrast, coffee and sour attributes with not better acceptance were described from the chocolate produced in Brazil inoculated with a mixture of three yeast starters (*S. cerevisiae*, *P. kluyveri* and *H. uvarum*) during cocoa fermentation [10]. Interestingly, the chocolate produced from different cocoa varieties originated from Brazil inoculated with *S. cerevisiae* during fermentation were clearly discriminated based on the perceptible attributes of each variety [11,13]. However, the lack of the small number of published studies regarding the sensory analysis of chocolate produced from inoculated cocoa beans with yeast species during fermentation from different countries to improve sensorial attributes is not conclusive.

2.3 CHANGES IN THE NUTRIENT COMPOSITION FROM FERMENTED TO ROASTED COCOA BEANS

The transformation of the nutrient content of cocoa beans during fermentation plays an important role in the development of selected attributes in the final product (chocolate). Fats, proteins, and carbohydrates are the main macronutrients found in cocoa seeds (Table 2.2) [29–33]. Beans also contain

amines that are already present in the unfermented dried cocoa and as expected their amount increase after fermentation and decrease after thermal cocoa processing [34,35]. The first step in processing cocoa beans is to ferment amino acids, oligopeptides and reducing sugars (Table 2.2). This step is crucial for the development of the quality cocoa flavor that depends on the balance of organic compounds. In general, the biochemical processes involve over the fermentation and roasting of cocoa beans comprise the hydrolysis of sucrose and proteins, oxidation and hydrolysis of phenolic compounds, biosynthesis of alkaloids, amino acids, release of alcohols (that are also oxidize to acetic and lactic acid) and the breakdown of fatty acids [5,29,30,36].

Table 2.2 Nutritional composition of cocoa beans expressed as g/kg

Source	Origin	Variety	Genetic material	Carbohydrates				Lipids	Proteins
				Sucrose	Fructose	Glucose	Total carbohydrates		
Afoakwa *et al.*, 2013[36]	Ghana	NM	Unfermented				155.00	552.00	216.00
Efraim *et al.*, 2010[30]	Brazil	*Forastero*	Unfermented					548.20	238.80
Afoakwa *et al.*, 2013[36]	Ghana	NM	Fermented				210.00	534.00	188.00
Efraim *et al.*, 2010[30]	Brazil	*Forastero*	Fermented					556.00	169.90
Redgwell *et al.*, 2003[32]	Ghana	NM	Dry cocoa beans	1.58	4.18	0.62			
Redgwell *et al.*, 2003[32]	Ivory Coast	NM	Dry cocoa beans	1.55	2.80	0.80			
Redgwell *et al.*, 2003[32]	Ecuador	NM	Dry cocoa beans	4.83	1.72	0.84			
Gu *et al.*, 2013[33]	Papua New Guinea	*Trinitario*	Roasted						458.60
Gu *et al.*, 2013[33]	Indonesia	*Trinitario*	Roasted						498.50
Gu *et al.*, 2013[33]	China	*Trinitario*	Roasted					392.40	
Gu *et al.*, 2013[33]	China	*Trinitario*	Roasted					434.40	
Redgwell *et al.*, 2003[32]	Ghana	NM	Roasted	1.41	0.60	0.05			
Redgwell *et al.*, 2003[32]	Ivory Coast	NM	Roasted	2.03	0.44	0.05			134.40
Redgwell *et al.*, 2003[32]	Ecuador	NM	Roasted	6.24	0.61	0.11			181.70

Abbreviations: NM, not mentioned

2.3.1 Composition of volatile compounds from cocoa beans

More than 600 different VOCs have been identified in chocolate. Substances such as aliphatic esters, polyphenols, unsaturated aromatic carbonyls, diketopiperazines, pyrazines, and theobromine are developed and these compounds provide the characteristic chocolate flavor [29].

2.3.1.1 VOCs associated with inoculated cocoa beans

A total of twenty VOCs profiles from inoculated cocoa beans with yeast starters (n=10) and chocolate produced from inoculated cocoa beans also with yeasts (n=10) have been recently reported from nine different cocoa varieties using fourteen different yeast strains over the world (Table 2.1). The identified VOCs from five different cocoa varieties inoculated with different yeasts during fermentation originated from Cameroon and Brazil was used to create a list of all the identified compounds. The data were treated as dummy variables indicating whether the VOCs were identified and only the most frequently reported were used to increase our knowledge of the probable VOCs formed when cocoa beans are inoculated with yeasts [5,8,12]. As expected, esters, alcohols, and aldehydes were the three major VOCs groups identified in fermented cocoa beans inoculated with yeast (Figure 2.2).

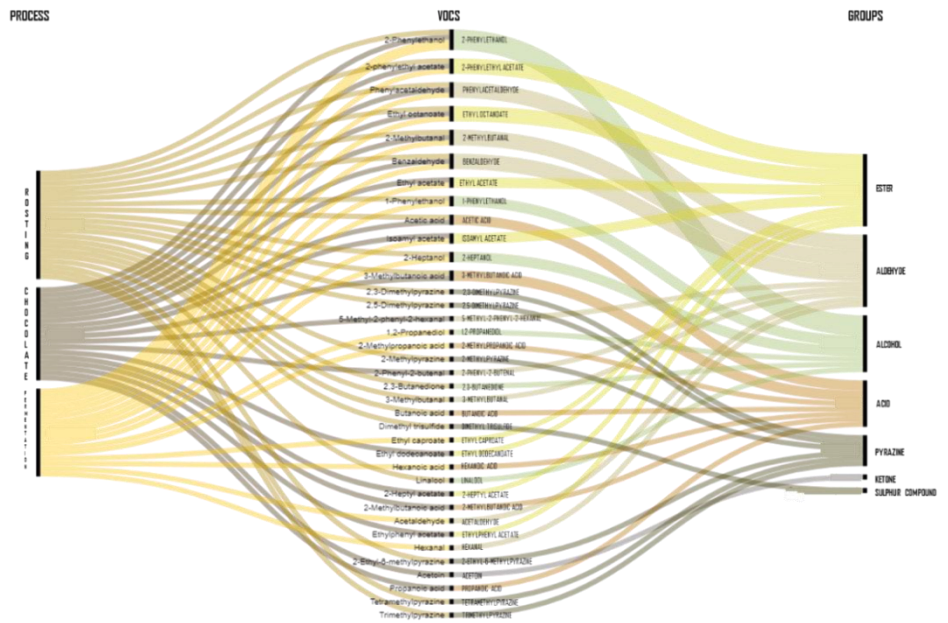

Figure 2.2 Most identified and abundant volatile compounds in fermented and roasted cocoa beans and chocolate

In detail, the most predominant VOCs among the three studies were ethyl acetate, benzaldehyde, hexanoic acid, and the key aromatic markers for chocolate (2-heptanol, 2-phenylethanol, 2-phenylethyl acetate and phenylacetaldehyde), while the most abundant compounds at the end of the fermentation were ethyl octanoate, 1-butanol, 1-pentanol, phenylacetaldehyde, ethyl acetate, isoamyl acetate, limonene and acetic acid (Table 2.3) [5,8,12]. It is important to highlight that recently, it has been demonstrated that the volatilome profile of cocoa beans fermented in boxes increased the production of alcohols and esters compared to heap fermentations [5]. However, not only the type of fermentation could influence the volatilome profile. It was shown that the effect of the yeast starter on different cocoa variety also influence the relative percentage of VOCs, such as 2-phenylethanol and ethyl acetate [12]. Concerning, the dynamics of VOCs, it was reported that the concentrations of limonene-epoxide and 1-butanol decreased over the fermentation time, while ethyl acetate, limonene, benzaldehyde, benzyl alcohol, acetoin, 3-methyl-1-butanol, acetic acid and the

key-aroma markers (phenylacetaldehyde, 2-heptanol and, 2-phenylethanol) increased (Table 2.3). The development of VOCs during cocoa fermentation and the appropriate selection of starter culture play a crucial role especially for consumers that follow the raw-food diet. Fermented cocoa beans are a suitable food product for this new trend towards raw foods and desirable attributes should also be met after fermentation [37].

Table 2.3 Concentration ranges (µg/kg) of volatile compounds of raw, fermented and roasted cocoa beans and chocolate

Volatile compounds	Raw beans [5, 12]	End of fermentation [5, 12]	Roasting [39-42, 44]	Chocolate [11, 13, 17]
Aldehydes				
2-Methylbutanal	0.70 - 1.24	0.49 - 1.46	111.00 - 4500.00	0.21 - 38.30
Acetaldehyde	0.02 - 0.85	0.00 - 0.18	285.00 - 285.00	0.60 - 41.70
Benzaldehyde	0.21 - 0.55	0.59 - 0.75	28.00 - 895.00	2.77 - 53.50
Decanal	0.03 - 0.06	0.02 - 0.04		1.00 - 1.00
Dodecanal	0.00 - 0.02	0.00 - 0.01		0.10 - 0.50
Furfural	0.00 - 0.24	0.00 - 0.25	26.00 - 87.00	
Hexanal	0.02 - 3.65	0.01 - 6.55		
Nonanal	0.14 - 0.19	0.09 - 0.14	46.00 - 46.00	0.05 - 1.52
Phenylacetaldehyde	4.06 - 6.09	3.49 - 12.37	60.00 - 5500.00	0.06 - 0.15
(E)-2-Undecenal	0.00 - 0.01	0.00 - 0.05		
2-Phenyl-2-butenal	0.00 - 0.00	0.00 - 0.05		
Alcohols				
(Z)-3-Hexen-1-ol	0.00 - 37.65	0.01 - 0.02		
1,2-Propanediol	0.00 - 0.00	0.07 - 0.35		1.10 - 1.70

1-Butanol	3.20	-	33.26	-	10.50						
1-Decanol	0.01	-	0.01	-	0.01						
1-Dodecanol	0.02	-	0.17	-	0.38						
1-Heptadecanol	0.03	-	0.10	-	0.21						
1-Heptanol	0.04	-	0.05	-	0.00				0.03	-	0.05
1-Hexanol	0.21	-	0.43	-	0.22						
1-Octanol	0.06	-	0.09	-	0.17						
1-Octen-3-ol	0.03	-	0.05	-	0.18						
1-Pentanol	0.13	-	0.83	-	0.14						
1-Phenylethanol	0.29	-	0.55	-	0.34						
1-Propanol	0.00	-	1.01	-	1.02						
2,3-Butanediol	0.00	-	9.60	-	2.07	62.00	-	356.00	35.40	-	65.35
2-Ethyl-1-hexanol	0.31	-	0.49	-	0.34				0.37	-	0.71
Furfuryl alcohol	0.00	-	0.00	-	10.71				0.49	-	0.90
2-Heptanol	0.35	-	0.54	-	8.97	32.00	-	1070.00	0.00	-	0.00
2-Hexanol	0.42	-	1.13	-	0.18						
2-Methyl-1-butanol	0.00	-	3.36	-	2.75				0.10	-	3.70
2-Methyl-1-propanol	0.00	-	0.22	-	10.33						
2-Nonanol	0.04	-	0.06	-	0.78				1.00	-	1.00
2-Pentanol	25.70	-	47.70	-	4.32				0.47	-	0.47
2-Phenylethanol	0.31	-	0.55	-	6.87	63.00	-	7500.00	3.60	-	142.00
3-Methyl-1-butanol	1.09	-	1.30	-	1.86	27.00	-	238.00	0.10	-	27.10
3-Methyl-1-pentanol	0.63	-	7.64	-	3.08						

Benzyl alcohol	0.04	-	0.05	0.03	-	0.07	104.00	-	104.00	0.20	-	0.23
Ethanol	2.17	-	3.89	1.25	-	3.81	124.00	-	124.00	4.06	-	6.71
Isobutanol	0.10	-	1.54	0.06	-	0.14						
Methanol	0.00	-	15.74	0.00	-	24.41	9068.00	-	9068.00			
(E)-3-Hexen-1-ol				0.00	-	43.75						
Acids												
2-Methylpropanoic acid	0.00	-	0.00	0.00	-	0.60	79.00	-	79.00	7.70	-	48.80
3-Methylbutanoic acid	0.05	-	0.10	3.51	-	9.20	86.00	-	9700.00	0.10	-	48.10
Acetic acid	0.68	-	1.30	4.33	-	28.40	5.60	-	330000.00	734.00	-	2555.70
Butanoic acid	0.00	-	7.36	0.00	-	13.10	21.00	-	570.00	1.30	-	2555.70
Decanoic acid	0.00	-	1.32	0.00	-	0.00						
Heptanoic acid	0.00	-	9.79	0.00	-	0.09	31.00	-	31.00			
Hexanoic acid	0.16	-	2.71	0.00	-	0.50	116.00	-	116.00	0.40	-	1.47
Nonanoic acid	0.00	-	10.28	0.00	-	0.00				0.10	-	0.10
Octanoic acid	0.03	-	0.06	0.11	-	0.27						
Ketones												
2-Heptanone	0.66	-	1.28	0.88	-	3.61	85.00	-	140.00	1.10	-	5.20
2-Pentanone	1.55	-	9.73	1.01	-	2.23						
2-Undecanone	0.04	-	0.05	0.00	-	0.03				1.00	-	1.00
Acetoin	0.38	-	0.47	1.23	-	5.98	14.00	-	1143.00	1.99	-	505.20

Acetophenone	1.17 - 3.06	0.81 - 2.31	14.00 - 225.00	
Esters				
1,2-Propanediol diacetate	6.50 - 8.11	1.21 - 2.53		
Isoamyl acetate	0.00 - 56.50	0.00 - 17.65		
2,3-Butanediol diacetate	0.15 - 0.30	0.03 - 1.20		
2-Pentanol acetate	1.42 - 2.55	1.78 - 3.93		
Diethyl malate	0.00 - 0.00	0.18 - 0.44		
Diethyl succinate	0.06 - 11.65	0.00 - 0.93		
Ethyl acetate	0.00 - 18.45	0.00 - 22.82	66.00 - 66.00	1.40 - 28.90
Ethyl benzoate	0.00 - 0.02	0.13 - 0.24	2.10 - 2.10	
Ethyl butanoate	0.26 - 3.99	0.06 - 4.18		
Ethyl caproate	0.17 - 0.22	0.43 - 0.94		
Ethyl dodecanoate	0.00 - 1.64	0.38 - 1.77	24.00 - 24.00	
Ethyl octanoate	0.00 - 0.03	0.00 - 74.29	3.30 - 143.00	0.09 - 19.10
Ethyl pyruvate	0.00 - 0.88	1.78 - 20.88		
Ethyl-o-toluate	0.00 - 0.00	0.33 - 0.62		
Furfuryl acetate	1.59 - 27.04	0.13 - 3.57		
Hexyl acetate	0.00 - 0.01	0.00 - 0.04		
Isoamyl benzoate	0.10 - 0.19	0.02 - 0.56		
Isobutyl acetate	0.14 - 1.97	0.06 - 1.98		
Methyl octanoate	0.10 - 0.15	0.00 - 0.00		
Mono-ethyl succinate	0.00 - 1.98	0.00 - 0.52		
Hexyl butanoate	0.00 - 0.05	0.00 - 0.00		

Phenyl acetate	0.00 - 0.54	0.00 - 0.14		
α-Phenylethyl acetate	0.00 - 0.45	0.17 - 0.89	34.00 - 930.00	2.60 - 37.10
Propyl acetate	0.00 - 0.09	0.00 - 1.34		
β-Phenylethyl acetate	0.03 - 0.12	0.73 - 1.69		
Terpenes				
Carveol	0.01 - 0.05	0.00 - 0.00		
(Z)-Linalool oxide pyranoid	0.10 - 0.15	0.06 - 0.17		
(Z)-Linalool oxide furanoid	0.03 - 0.10	0.00 - 0.10	21.00 - 21.00	
Nerylacetone	0.02 - 0.04	0.00 - 0.00		
Limonene	6.65 - 12.37	6.43 - 30.60		
Geraniol	0.00 - 0.31	0.00 - 0.00		
Limonene epoxide	0.29 - 0.90	0.00 - 0.02		
Sabinene	0.05 - 0.17	0.05 - 0.30		
α-Caryophyllene	0.08 - 0.09	0.07 - 0.18		
α-Citral	0.03 - 0.10	0.02 - 0.15		
α-Limonene diepoxide	0.00 - 0.02	0.00 - 0.02		
β-Caryophyllene	0.01 - 0.02	0.01 - 0.03		
β-Citronellol	0.00 - 0.00	0.00 - 0.41		
β-Myrcene	1.98 - 2.32	0.96 - 3.14	66.00 - 66.00	
(E)- β -ocimene	0.08 - 0.34	0.06 - 0.51		

Lactones			
Δ-Decalactone		0.00 - 0.20	
Other compounds			
1,1-Diethoxyethane	0.06 - 21.65	0.12 - 5.83	
o-Guaiacol	0.00 - 0.01	0.02 - 0.62	230.00 - 230.00
Phenol	0.02 - 0.03	0.02 - 0.37	7.00 - 7.00
trans-Methyl dihydrojasmonate	0.02 - 0.04	0.02 - 0.04	

Values are expressed as concentration ranges (μg/kg). Different colour showed a decrease (light blue) or increase (light green) of selected VOCs concentrations

Despite the development of VOCs in inoculated cocoa fermentations, the volatilome profile of chocolate produced from cocoa beans originated from Brazil and Malaysia, inoculated with *S. cerevisiae* and *T. delbrueckii* and a mixed culture of these two yeasts at the beginning of the fermentation has been assessed (Table 2.1) [11,13,17]. Interesting observations can be made regarding the most frequently identified VOCs in the fermented cocoa beans inoculated with yeasts and the chocolate produced also from inoculated cocoa beans with yeasts, which support the idea that some VOCs produced during fermentation can remain after processing (Figure 2.2). Remarkably, acetic acid was the most abundant VOCs during fermentation and remained the most abundant VOCs in chocolate followed by acetoin and 2-phenylethanol (Table 2.3) [11,13,16].

Several limitations were noted during the collection of the reported VOCs in both the inoculated fermented cocoa beans and the chocolate produced from different inoculated cocoa beans. The first limitation is related to the incongruency of the total number of VOCs and terpenoids reported, whereas some studies do not report any terpenoid and the total number of VOCs identified varies from 34 to 72 compounds [5,11–13,16,38]. Second, studies that identified VOCs in inoculated fermented cocoa beans and chocolate are limited. Although there are no studies that have tracked the presence of VOCs over the whole chocolate process, this chapter provides us an idea of which VOCs are only formed during the fermentation of cocoa beans inoculated with yeast species and could probably remain in the end product. It is worth noting that future research in the identification of VOCs may further increase our knowledge on the role of yeasts, particularly if they increase the production of esters, aldehydes, and terpenoids. This could heighten the positive impacts of yeasts during cocoa fermentation.

2.3.1.2 Dynamics of VOCs during roasting

Cocoa beans are roast to diminish moisture and acidity by reducing concentrations of volatile acids such as acetic acid and water [29]. However, the degree of the reduction of volatile acid dependens on time/temperature conditions used [39]. Several chemical reactions such as Maillard and Strecker reactions play an important role during roasting to develop the characteristic aroma and flavor of chocolate [35]. These reactions reduce sugars and amino acids to produce mainly heterocyclic groups such as aldehydes and pyrazines. Indeed, the roasting process has been showed to be a more effective amine generator than the fermentation process. However, the fermentation process supplied precursors for Strecker aldehyde formation. Overall, these reactions also depend on temperature and pH, in which higher temperatures increase the amine generation [29,35,39].

Enormous progress has been currently made in the identification of VOCs during roasting [28,39–45]. In detail, a total of 243 VOCs has been recently reported from three different cocoa varieties originating from ten different countries (Table 2.4). The most frequently identified and abundant VOCs in roasted cocoa beans were acetic acid, 3-methylbutanoic acid, benzaldehyde, and the key aromatic compounds (2-heptanol, 2-phenylethanol, phenylacetaldehyde, and 2-methylbutanal, Table 2.3) [39–42,44,45]. Interestingly, we observed that the key aromatic compounds (2-phenylethyl acetate, phenylacetaldehyde and, 2-heptanol), benzaldehyde, acetic acid, as well as trimethylpirazine, 3-methylbutanal were formed during inoculated fermentations and still present after the roasting process [28,39–45].

Table 2.4 Overview of the volatile organic compounds of roasted cocoa beans from different origins under different roasting conditions

Source	Country	Variety	Equipment	Roasting conditions	
				(°C)	min
Bonhevi *et al.*, 2005[40]	Ghana, Cameroon, Ivory Coast, Brazil, and Ecuador	NM	GC-MS	130	48
Ramli *et al.*, 2006[39]	Malaysia	NM	GC-MSD	150	30
Frauendorfer and Shieberle, 2008[44]	Grenada	*Criollo*	HRGC-MS	95	14
Huang and Barringer, 2011[41]	Ecuador	NM	SIFT-MS	150	30
Van Durme *et al.*, 2016[42]	Ghana and Tanzania	NM	HS-SPME-GC-MS	150	30
Magagna *et al.*, 2018[43]	Mexico	NM	HS-SPME-GCxGC-MS	100-130	20-40
Tan and Kerr, 2018[45]	United States of America	*Forastero*	GC-MS and ANN-based-e-nose	135	0-40
Magagna *et al.*, 2017[28]	Ecuador and Mexico	*Trinitario* hybrids	GCxGC-MS, GCx2GC-MS/FID	nm	nm

Abbreviations: NM: Not mentioned, **SIFT-MS:** Selected Ion Flow Tube-Mass Spectrometry, **GC-MS:** Gas Chromatograph-Mass Spectrometer, **ANN:** Artificial neural network, **GC-MSD:** Gas chromatography-Mass selective detector, **HRGC-MS:** High-Resolution Gas Chromatography-Mass Spectrometry, **HS-SPME**: Head Space Solid Phase Micro-Extraction, **FID:** Flame Ionization Detector

In terms of the concentration changes of VOCs during roasting, it has been shown that the key odorants formed during fermentation 2-heptanol, 2-phenylethyl acetate, 2-phenylethanol, butanoic acid, and ethyl 2-methylbutanoate remained nearly constant during the roasting process, while the formation of pyrazines, a by-product of Maillard reaction, mainly occurs during roasting [28,44,45]. It should be also noted that the loss and development of limonene, ethyl acetate, benzaldehyde, and 2-methylbutanal after thermal processing remains still unclear.

2.4 SYNTHESIS OF VOCS BY FUNGAL COMMUNITIES AND THEIR POTENTIAL HEALTH BENEFITS

Research on microbial flavor generation has been tremendously increased over the last two decades and special attention was given to understand the microbial processes or microbial strategies to produce flavor compounds [8,46–50]. Interestingly, VOCs have been traditionally used and added to food products more for pleasure and consumers acceptability than for nutritional reasons. However, microorganisms and their metabolites produced have been also exploited for their tremendous potential to provide health benefits in humans. In fact, it has been recently pointed out the potential health benefits contributed mainly by VOCs in plant foods [2].

VOCs can be synthesized by biological process (microorganisms during fermentation), chemical reactions (synthetic and semi-synthetic) or plant extracts, depending on the type of compound that need to be synthesized. Concerning the biological processes, the microbial metabolism includes the transformation of natural precursor (sugars, organic acids, amino acids, and fatty acids) to a wide range of flavor molecules such as aliphatic, aromatics, terpenes, lactones, O-heterocycles, S- and N-containing compounds [49]. This chapter focusses on the formation of six VOCs (2-phenylethanol, phenylacetaldehyde, 2-methylbutanal, benzaldehyde, limonene, and 2-phenylethyl acetate) that are formed during inoculated cocoa fermentation with yeasts, and remained present after roasting, and the potential health benefits of these VOCs. Overall, a total of 36 fungi are known as producers of the selected VOCs (Figure 2.3). In detail, it has been demonstrated that the majority of the selected VOCs were produced by *S. cerevisiae, H. uvarum, H. guilliermondii, Galactomyces geotrichum* and a high variation between species of *Candida* and *Pichia* (Figure 2.3).

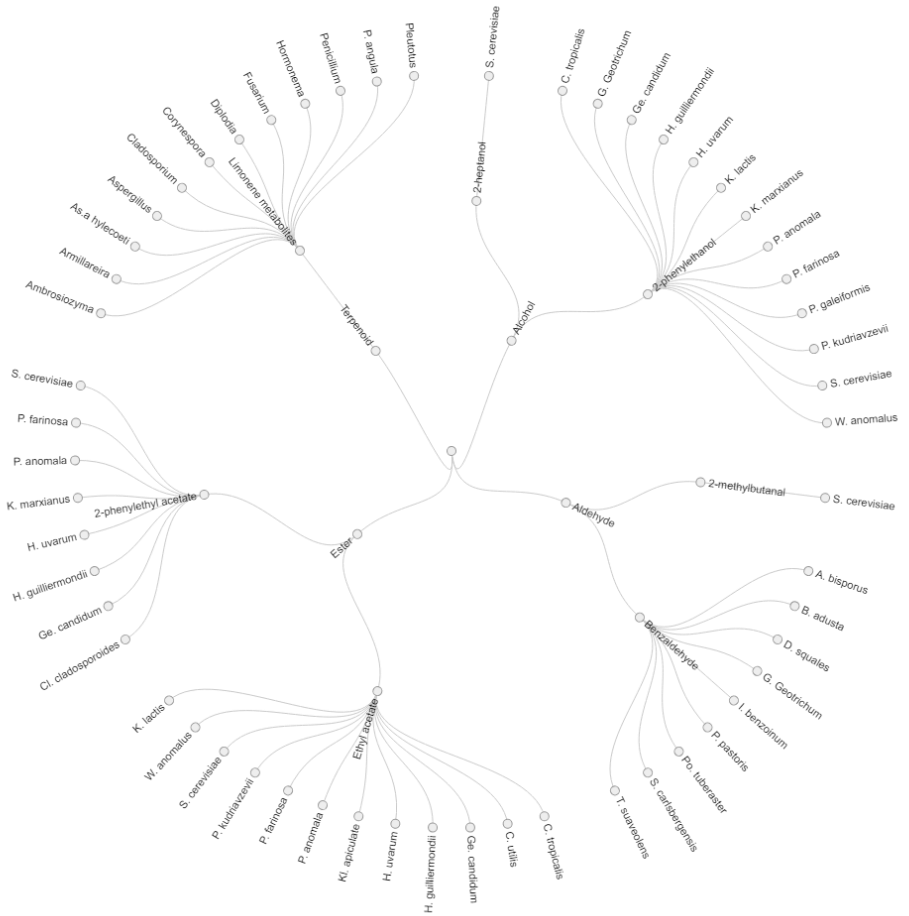

Figure 2.3 Yeast producer of selected key aromatic compounds in cocoa beans. **Abbreviations:** S: *Saccharomyces*, P: *Pichia*, C: *Candida*, G: *Galactomyces*, Ge: *Geotrichum*, H: *Hanseniaspora*, K: *Kluyveromyces*, W: *Wickerhamomyces*, A: *Agaricus*, B: *Bjerkandera*, D: *Dichomitus*, I: *Ischnoderma*, Po: *Polyporus*, T: *Trametes*. Kl: *Kloeckera*, Cl: *Cladosporium*, As: *Ascoid*

Fusel alcohols are generally synthesize using the yeast's Ehrlich pathway, this pathway contains three-enzyme cascade that converts valine, leucine, and isoleucine into their corresponding alcohols [49]. The microbial production of L-phenylalanine to **2-phenylethanol** (a rose-like odor) involve the transamination of the amino acid to phenylpyruvate, decarboxylation to **phenylacetaldehyde**

and reduction to the alcohol by yeast species [46,48,51–55], while the synthesis of secondary alcohols, such as 2-heptanol can be obtained from 2-heptanone (Table 2.5) [56]. Regarding the potential health benefit 2-phenylethanol, this VOC inhibits the growth of Gram-negative bacteria and filamentous fungi [57,58].

Table 2.5 Summary table of the yeast producer of selected key aromatic compounds in cocoa beans

Group	VOCs	Microorganism	Reference
Alcohol	2-heptanol	*Saccharomyces cerevisiae*	Cappaert and Laroche, 2004 [56]
	2-phenylethanol	*Candida tropicalis*	Koné *et al.*, 2016 [48]
		Galactomyces geotrichum	Koné *et al.*, 2016 [48]
		Geotrichum candidum	Janssens *et al.*, 1992 [46]
		Hanseniaspora guilliermondii	Moreira *et al.*, 2005 [73]
		Hanseniaspora uvarum	Moreira *et al.*, 2005 [73]
		Kluyveromyces lactis	Janssens *et al.*, 1992 [46], Fabre *et al.*, 1997 [55]
		Kluyveromyces marxianus	Janssens *et al.*, 1992 [46], Whittmann *et al.*, 2002 [53], Etschman *et al.*, 2005 [52], Fabre *et al.*, 1997 [55]
		Pichia anomala	Janssens *et al.*, 1992 [46]
		Pichia farinosa	Janssens *et al.*, 1992 [46]
		Pichia galeiformis	Koné *et al.*, 2016 [48]
		Pichia kudriavzevii	Koné *et al.*, 2016 [48]
		Saccharomyces cerevisiae	Kim *et al.*, 2014 [51], Koné *et al.*, 2016 [48], Schwan and Wheals, 2004 [6], Moreira *et al.*, 2005 [54], Fabre *et al.*, 1997 [55]
		Wickerhamomyces anomalus	Koné *et al.*, 2016 [48]
Aldehydes	2-methylbutanal	*Saccharomyces cerevisiae*	Janssens *et al.*, 1992 [46], Larroy *et al.*, 2002 [67]
	Benzaldehyde	*Agaricus bisporus*	Janssens *et al.*, 1992 [46]
		Bjerkandera adusta	Lapadatescu *et al.*, 1997 [64]
		Dichomitus squales	Lapadatescu *et al.*, 1997 [64]
		Galactomyces geotrichum	Koné *et al.*, 2016 [48]

		Ischnoderma benzoinum	Lapadatescu *et al.*, 1997 [64]
		Pichia pastoris	Berger, 2007 [49]
		Polyporus tuberaster	Kawabe and Morita, 1994 [65]
		Saccharomyces carlsbergensis	Pal *et al.*, 2009 [66]
	Phenylacetaldehyde	*Kluyveromyces marxianus*	Etschman *et al.*, 2005 [52]
		Acetobacter	Berger, 2007 [49]
Ester	Ethyl acetate	*Candida tropicalis*	Koné *et al.*, 2016 [48]
		Candida utilis	Janssens *et al.*, 1992 [46]
		Geotrichum candidum	Janssens *et al.*, 1992 [46]
		Hanseniaspora guilliermondii	Rojas *et al.*, 2001 [78]
		Hanseniaspora uvarum	Rojas *et al.*, 2001 [78]
		Kloeckera apiculate	Schwan and Wheals, 2004 [6]
		Pichia anomala	Janssens *et al.*, 1992 [46], Rojas *et al.*, 2001 [78]
		Pichia farinosa	Janssens *et al.*, 1992 [46]
		Pichia kudriavzevii	Koné *et al.*, 2016 [48], Pereira *et al.*, 2017 [8]
		Saccharomyces cerevisiae	Janssens *et al.*, 1992 [46], Koné *et al.*, 2016 [48], Rojas *et al.*, 2001 [78], Schwan and Wheals, 2004 [6]
		Wickerhamomyces anomalus	Koné *et al.*, 2016 [48]
		Kluyveromyces lactis	Van Laere *et al.*, 2008 [79]
	2-Phenylethyl acetate	*Cladosporium cladosporoides*	Janssens *et al.*, 1992 [46]
		Geotrichum candidum	Janssens *et al.*, 1992 [46]
		Hanseniaspora guilliermondii	Rojas *et al.*, 2001 [78], Moreira *et al.*, 2005 [54]
		Hanseniaspora uvarum	Rojas *et al.*, 2001 [78]
		Kluyveromyces marxianus	Janssens *et al.*, 1992 [46], Whittmann et al., 2002 [56], Etschman *et al.*, 2005 [52]
		Pichia anomala	Janssens *et al.*, 1992 [66], Rojas *et al.*, 2001 [78]
		Pichia farinosa	Janssens *et al.*, 1992 [46]
		Saccharomyces cerevisiae	Kone *et al.*, 2016 [48], Rojas *et al.*, 2001[78]
Terpenoid	Limonene	*Ascoidea hylecoeti*	Janssens *et al.*, 1992 [46]
	Limonene metabolites (terpineol, verbenol)	*Armillareira*, *Aspergillus Cladosporium*	Duetz *et al.*, 2003 [68], Janssens *et al.*, 1992 [46]

Limonene metabolites (limonene-1,2-epoxide)	*Corynespora* *Diplodia*	Duetz *et al.*, 2003 [68]
Limonene metabolites (verbenone)	*Hormonema*	Berger, 2007 [49]
Limonene metabolites (carvone, carveol)	*Penicillium* *Pleutotus*	Janssens *et al.*, 1992 [46], Duetz *et al.*, 2003 [68]
Limonene metabolites	*Pichia angula* *Ambrosiozyma* *Fusarium*	Janssens *et al.*, 1992 [46] Berger, 2007 [49]

Besides 2-phenylethanol, other VOCs such as benzaldehyde and its derivates are used as preservatives [59,60]. However, this compound is also capable to induce antitumor activity in human cells [59–62] and inhibit the oxidation [33,63]. Concerning the conversion of benzyl alcohol or L-phenylalanine into **benzaldehyde,** this conversion has been attributed not only to yeasts but also to the basidiomycetes presence (Table 2.5) [46,48,64–66]. In general, aldehydes can be produced by the metabolism of yeast. Short-chain aliphatic aldehydes such as acetaldehyde, 2-methyl-1-propanal, 2-methylbutanal and 3-methylbutanal are produced by the oxidation of alcohols such as 2-methylbutanol, 3-methylbutanol and 2-methyl-1-propanol [46,67].

Regarding the biosynthesis and conversion of monoterpenes by microbial metabolism, the formation of this VOC group has been associated with the basidiomycetes metabolism. Limonene is produced by the plants as a defense for pathogens, and his transformations into other monoterpenoids such as carvone, terpineol, perillyl alcohol, limonene epoxide, and verbenone have been associated with the activity of several fungal species (Table 2.5) [46,49,68]. Interestingly, recent *in vivo* and *in vitro* studies have been reported as an anticarcinogenic and the antinociceptive activity of limonene [69–75], and this compound has been also used as a preservative [76].

Last but not least, the well-known ester **2-phenylethyl acetate** is recognized for its antimicrobial activity [77]. In general, the biotransformation of esters includes a more complex catabolic reaction, and it comprises the esterification of amino acids or short-chain aliphatic fatty acid and terpenyl alcohol into the desired flavor ester. The transformation of **2-phenylethyl acetate** is usually metabolized from amino acids, such as phenylalanine and/or phenylpyruvic acid also from yeast species (Table 2.5) [46,54,78]. Besides 2-phenylethyl acetate, ethyl acetate is formed from the esterification of leucine, isoleucine or valine and natural aliphatic alcohol. This ethyl acetate production has been attributed to the activity of yeast (Table 2.5) [6,8,48,54,78,79].

Overall, the potential health effect of the selected VOCs synthesize by chemical reactions or biological processes has been linked to prevent or delay diseases or the growth of undesirable microorganisms. In summary, it has been reported from *in vivo* and *in vitro* studies the anticarcinogenic and the antinociceptive activity of limonene, [69–75], the antitumor activity of benzaldehyde [59–62] and antioxidant activity of benzaldehyde and its derivates [80,81]. In addition, 2-phenylethanol [57,58,82], 2-phenylethyl acetate [77], limonene [76] benzaldehydes and derivates [63,80,81,83] have been widely used as preservatives.

While most of the studies have been focusing on describing the capacity of VOCs on preventing, slowing or inhibiting microorganisms, tumors, or cells growth to provide health benefits, recent literature has been demonstrated the capacity of these compounds to stimulate through oral routes and the olfactory receptors in the nose that communicates with the limbic system of the brain via neurons, to change the mood and emotions by creating a sedative effect for reduction of stress and anxiety and finally reducing the pain perception [84]. On the other hand, the dysfunction of the chemosensory activities is highly related to differences in dietary behaviors, including loss of appetite, unintended weight

loss, malnutrition and well-known psychiatric and neurological disorders [85–90]. More important is the fact that this loss has been reported to affect the general population and it remains undiagnosed in some patients [85,91]. In this regard, **2-phenylethanol** has been used to counteract the olfactory dysfunction due to multiple etiologies [92–98]. However, the mechanism of action of the improvements of the smell progress and the association of chemosensory function with dietary and health outcomes remains still unclear. There is no doubt that individuals with this dysfunction, highly observed in neurological diseases such as Parkinson's, are more likely to experience a hazardous event and are the major concern for public health. Considering the positive effect of the single compounds, also produced by microbial communities during cocoa fermentation, it might be possible that the consumption of chocolate produced from inoculated cocoa beans with yeasts could provide a positive health effect to the consumers. However, more comprehensive studies are required to confirm the potential effect of VOCs from chocolate in human health. In terms of international legal regulations, according to the European Committee, all the VOCs proposed in this review are categorized as flavoring agents and does not represent a safety concern since they are predictably metabolized efficiently to innocuous products and its estimated daily intake are below the threshold for daily human intake [99].

Microbial communities in, on and around our food are essential for exploring the interaction between the food system and its inter-connectedness with human health. Tracking the production of functional compounds produced by microbes will serve to improve the formation of desirable compounds. Future perspectives on the selection of the best candidate starter cultures possessing genes coding for oral usage to acquire desirable compounds and the mechanism underlying flavor perception linked to the nutritional or health values need to be assessed. The findings of the present chapter and future analyses of VOCs may help to inform researchers, policymakers, the chocolate industry and the general

public to explore yeasts as proper producers of important VOCs to improve the quality and health.

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DYNAMICS AND BIODEVERSITY DURING

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ABSTRACT

Forastero hybrid cocoa bean fermentations have been carried out in a box (B) and in a heap (H), with or without the inoculation of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* as starter cultures. The bacteria, yeasts, and microbial metabolites (volatile and non-volatile organic compounds) were monitored during fermentation to assess the connection between microbiota and the release of metabolites during this process. The presence of starter cultures was detected, by means of culture-dependent analysis, during the first 2 days of both fermentations. However, no statistical difference was observed in any of the physicochemical or microbiological analyses. Plate counts revealed the dominance of yeasts at the beginning of both fermentations, and these were followed by acetic acid bacteria and lactic acid bacteria. *Hanseniaspora opuntiae*, *S. cerevisiae*, *Pichia pijperi*, *Acetobacter pasteurianus*, and *Lactobacillus fermentum* were the most abundant operational taxonomic units (OTUs) during both fermentation processes (B and H), although different relative abundances were observed. Only the diversity of the fungal species indicated a higher level of complexity in the B than in the H fermentations ($P < 0.05$), and a statistically significant difference between the initially inoculated starter cultures was observed ($P < 0.01$). However, the microbial metabolite analysis indicated different distributions of the volatile and non-volatile compounds between the two procedures, that is, B and H ($P < 0.05$), rather than between the inoculated and non-inoculated fermentations. The box fermentations showed faster carbohydrate metabolism and greater production of organic acid compounds, which boosted the formation of alcohols and esters, than did the heap fermentations. Overall, the microbial dynamics and associations between the bacteria, yeasts, and metabolites were found to depend on the type of fermentation. In spite of the limited effectiveness of the considered inoculated starter strains, this study provides new information on the microbial development of B and H cocoa fermentations, under inoculated and non-inoculated conditions,

as we coupled yeast/bacterial amplicon-based sequencing data with microbial metabolite detection. The information so far available suggests that microbial communities have played an important role in the evolution of aroma compounds. Understanding the pathways that microorganisms follow during the formation of aromas could be used to improve the fermentation processes and to enhance chocolate quality

3.1 INTRODUCTION

Cocoa (*Theobroma cacao* L.) is an important plant crop throughout the world, and its production serves as a main source of income in several developing countries [1]. Chocolate production begins with the harvesting of the cocoa fruit, where cocoa beans and the surrounding mucilaginous pulp inside the pods are removed. At this point, the product has an astringent characteristic and needs to be ferment, dry, and roast to acquire the optimal features of cocoa flavor and taste [2]. Spontaneous fermentation normally lasts from 3 to 10 days in heaps, boxes, baskets, or trays.

According to Schwan and Fleet [3], the microbiota present during cocoa fermentation is composed of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Two important stages are present during cocoa fermentation; in the first stage, yeasts proliferate in the reducing sugars and citric acid from the pulp and produce ethanol and carbon dioxide. At the same time, the temperatures and pH increase, due to aerobic and oxidative reactions, thus allowing LAB and AAB to grow [3]. LAB mainly transforms sugars and organic acids into lactic acid, and, under aerobic conditions, AAB converts ethanol to acetic acid [2]. The second stage involves the death of the seed embryo, due to the high concentrations of ethanol and acetic acid, and an increase in temperatures [2]. The quality of the end product chocolate depends on the three previously cited groups of microorganisms since they are able to produce metabolites and flavor precursors [3].

Although the importance of yeasts during cocoa fermentation has emerged in recent studies [4,5], fungal biodiversity in fermented food has been studied far less than bacteria. In spite of the use of high-throughput sequencing (HTS) this decade, this new technology has mainly been used to obtain new insights into the domain of fermented foods, as it enables the genetic variants of a complex ecosystem to be discovered, validated, and screened [6]. The importance of identifying the microbial composition of food ecosystems involves

finding appropriate starter cultures that enhance a particular aspect of the product. *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* have recently been detected and used as starter cultures in cocoa fermentation, and they have shown a positive impact on the aroma profile of the end product [7,8]. However, there has been much controversy concerning the choice of the starter cultures used in cocoa fermentation to improve the quality of the end. This chapter explores the impact of the use of starter cultures on cocoa fermentation to guarantee the production of cocoa beans with specific and reproducible features exposed to different fermentation methods. We point out the challenges related to the reproducibility of the effect of the starter cultures on cocoa bean fermentation and their correlations with the initial microbial populations and importance for aroma development.

The aimed of this chapter is to describe the dynamics and biodiversity of both bacteria and yeasts by means of amplicon-based sequencing of the 16S rRNA genes and the Internal Transcribed Spacer 2 (ITS2) gene, respectively, during cocoa bean fermentation carried out both spontaneously and in the presence of yeast starter cultures, in both boxes and heaps, in order to acquire more detailed knowledge about the relationship between microorganisms and their surroundings. The non-volatile and volatile organic compounds were also assessed with the aim of investigating how the use of cultures can affect the volatilome profile of fermented cocoa from the two different fermentation processes. In this chapter, we have also proposed the measurements of associations between microbial communities and the development of microbial volatile and non-volatile compounds. A better understanding of the microbial communities and physicochemical dynamics during box and heap fermentations will undoubtedly help the development of new management procedures for the production of high-quality cocoa.

3.2 MATERIALS AND METHODS

3.2.1 Cocoa bean fermentations

The lyophilized *S. cerevisiae* ID67 and *T. delbrueckii* ID103 strains were provided by Lallemand (Montreal, Quebec, Canada) and were used as starter cultures in farmer-scale cocoa bean fermentations carried out in Ngoumou (Yaoundé, Cameroon) at the end of the mid-crop in 2016 (September to October 2016). The strains were chosen according to the study by Visintin *et al.* [8]. Briefly, cocoa pods of the *Forastero* hybrid were harvested by traditional methods and stored on the ground for 2 to 3 days before opening the pods. The cocoa pods were cut with non-sterile machetes, and the beans and the adhering pulp were removed by hand. Approximately 3 h after breaking the pods, the cocoa bean pulp was grouped into two independent batches (for the box and heap processes). Approximately 200 kg of fresh cocoa bean pulp was placed in a wooden box (0.06 m^3), covered with banana leaves, and closed with a wooden lid to protect it from the open air. The heap fermentations were set up with smaller amounts of beans than the box fermentations, due to the fact that an adult can manually turn no more than 100 kg of bean pulp. These beans were piled on top of banana leaves and covered with other banana leaves and jute rags. The field experiment involved inoculating the cocoa bean pulp with *S. cerevisiae* ID67 (S) or with *S. cerevisiae* ID67 in co-cultures with *T. delbrueckii* ID103 (ST) in a 1:1 ratio (wt/vol) at the beginning of both fermentation processes (B and H). The lyophilized starter cultures were revitalized in a sterile saline solution for 30 min at room temperature and were progressively added and mixed with the cocoa pulp mass to final concentrations of 7.0 ± 0.2 log CFU g^{-1}. Non-inoculated fermentations were carried out, without adding any starter culture to either fermentation process (B and H) and were used as a control (Figure 3.1). All trials were performed in duplicate (n = 12). According to the local agricultural practices; the cocoa bean pulp mass was turned manually at 48 and 96 h, and the

fermentations were stopped after 120 h by spreading the beans on a drying platform. An aliquot of 1 to 1.5 kg of cocoa pulp was collected in sterile bags after 0, 48, 96, and 120 h for each of the six experimental trials. The pulp was taken randomly from at least five different zones of the fermentative mass in both the B and H fermentations. It should be noted that sampling was performed at 48 and 96 h before mixing the mass. Approximately 20 g of sample was collected, stored at −20°C, and transported on dry ice to the department of agriculture, forestry and food sciences (University of Turin, Ital) for further metabolite analysis. Aliquots of 25 g of each sample were subjected to microbiological analyses at an experimental laboratory that was set up on-site. The pH values and temperatures were measured at the same sampling times during fermentation considering an average of five random zones of the cocoa bean pulp mass, and using a pH thermometer (Crison, Modena, Italy).

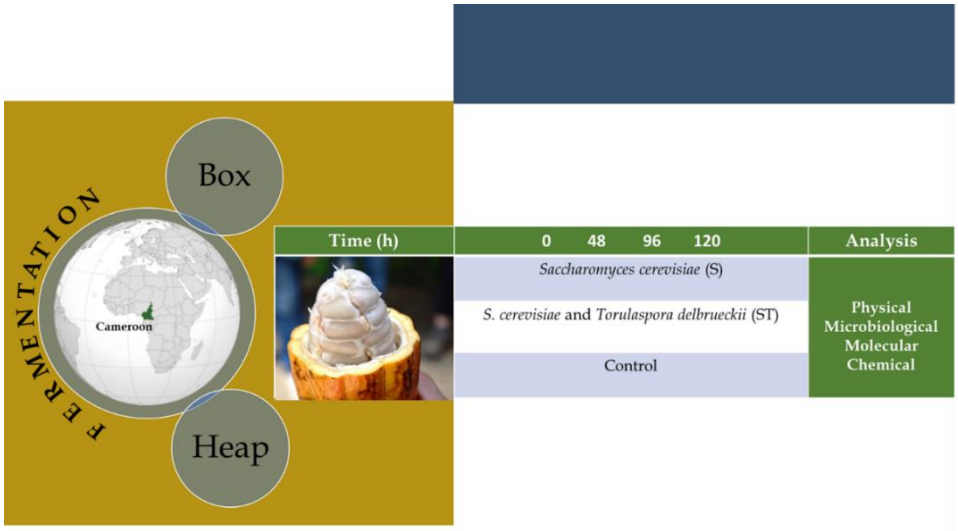

Figure 3.1 General methodology of cocoa fermentation study

3.2.2 Culture-dependent microbial community dynamics

A classical microbiological analysis was performed on samples recovered at 0, 48, 96, and 120 h. Twenty-five grams of cocoa beans and the adhering pulp were homogenized with 225 ml of ringer's solution (Oxoid, Milan,

Italy). Decimal dilutions were prepared in quarter-strength ringer's solution. Aliquots of 0.1 ml of the appropriate dilutions were spread in triplicate on the following media: WL nutrient agar (WLN; Lab M, Heywood, Lancashire, UK) plus 1 μg/ml tetracycline (Sigma-Aldrich, Milan, Italy) to count the total yeasts incubated for 5 days at 30°C; de Man-Rogosa-Sharpe (MRS) agar (Oxoid) plus 2 μg/ml natamycin (Sigma-Aldrich) for the growing LAB, incubated at 30°C for 48 h; and acetic acid medium (1% glucose, 0.8% yeast extract, 0.5% bacteriological peptone, 15 g/liter agar, 0.5% ethanol, 0.3% acetic acid) plus 2 μg/ml of natamycin (Sigma-Aldrich) for the growing AAB incubated at 30°C for 5 days. The results obtained from three independent determinations were expressed as the means of the log CFU per gram. Yeast colonies (5 to 8 for each sampling point) were randomly isolated from the plate with the highest WLN dilution. These colonies were further purified by streaking and were then stored in 20% (vol/vol) glycerol. A 1-ml aliquot of the first 10-fold serial dilution was collected at each sampling and centrifuged at the maximum speed for 30 s.

3.2.3 Assessment of the yeast ecology by means of culture-dependent analysis

DNA extraction from single isolates was performed as described by Cocolin *et al.* [9] and normalized at 100 ng/liter. Isolates were grouped in relation to their restriction fragment length polymorphism (RFLP) profiles, which were obtained after enzymatic restriction of the amplified ITS-5.8S rRNA region, as previously described by Korabecná *et al.* [10]. The ITS-5.8S rDNA region of at least three representative isolates of each RFLP group was used for sequencing (GATC Biotech, Cologne, Germany). A repetitive extragenic palindromic PCR (REP-PCR) assay was performed on all the isolates previously identified as *S. cerevisiae* and *T. delbrueckii*, according to the procedure outlined in a previous study by Visintin *et al.* [8]. A starter culture from the REP-PCR profiles was compared with those of *S. cerevisiae* ID67 and *T. delbrueckii* ID103.

3.2.4 Chemical analysis of fermented cocoa beans

Fermented lyophilized cocoa bean pulp samples (0.20 g) were washed with 2 ml of pure hexane (Sigma-Aldrich, Milan, Italy) and vortexed for 5 min. The homogenate was centrifuged (6,000 × *g*, 4°C, 15 min), and the supernatant was removed. The washing process was repeated twice, and the precipitate was dried after the washings and resuspended with 10 ml of a 70:29.5:0.5 acetone–ultrapure water–formic acid solution (Sigma-Aldrich). The solution was vortexed, centrifuged, and clarified by filtration through 0.45-μm syringe filters (LLG-Labware, CA, USA) and then evaporated. The extract was resuspended with 5 ml of ultrapure water and passed through a C_{18} cartridge (Sep-Pack, USA). The column was washed with 5 ml of ultrapure water to recover the samples.

The high-performance liquid chromatography (HPLC) system (ThermoQuest Corporation, San Jose, CA, USA) was equipped with an isocratic pump (P1000), a multiple autosamplers (AS3000) fitted with a 20-μl loop, a UV detector (UV100) set at 210 nm, and a refractive index detector (Spectra system RI-150; Thermo Electro Corporation). The analyses of the sugars (glucose, fructose, and sucrose) were performed isocratically, at 0.6 ml · min^{-1} and 80°C, with a 300 by 7.8 mm inner diameter (i.d.) cation exchange column (Aminex HPX-87P) equipped with a cation Carbo-P Micro-Guard cartridge (Bio-Rad Laboratories, Hercules, CA, USA). The analyses of the organic acids (acetic, lactic, malic, succinic, oxalic, gluconic, tartaric, pyruvic, fumaric, and citric acid) were performed isocratically, at 0.8 ml · min^{-1} and 60°C, with a 300 by 7.8 mm i.d. cation exchange column (Aminex HPX-87H) equipped with a Cation H+ Micro-Guard cartridge (Bio-Rad Laboratories). The data treatments were carried out using the ChromQuest chromatography data system (ThermoQuest, Inc). Analytical-grade reagents were used as standards (Sigma-Aldrich, St. Louis, MO). All the samples of each biological replicate were analyzed in triplicate, and the identification of compounds was performed by comparing the retention time against the standard. The calibration curves of the standards were obtained by

injecting serial dilutions of glucose, sucrose, fructose, and acetic, lactic, malic, succinic, oxalic, gluconic, tartaric, pyruvic, fumaric, and citric acids, under the same conditions as the sample analyses. The concentrations of the compounds were calculated by plotting a linear curve of the areas obtained in each sample.

3.2.5 Volatile metabolites produced by the microbiota consortia

The dynamics of the volatile organic compounds (VOCs) of the fermented cocoa bean pulp were obtained under different previously lyophilized conditions using the headspace solid-phase microextraction (HS-SPME) technique, in which the fiber conditions and oven temperatures were set as previously described by Rodriguez-Campos *et al.* with some modifications [11]. Samples of each biological replicate were analyzed in triplicate. The analysis was conducted in a 20-ml vial filled with 2 ml of 20% NaCl and 0.1 g of the sample, and 10 µl of 5-nonanol in ultrapure water was added to each sample at a 50 mg/liter concentration as an internal standard for semi-quantification. The fibers with VOCs were injected into the gas chromatograph-quantitative mass spectrometer (GC-qQP2010 Plus; Shimadzu, USA), which was equipped with an autosampler (AOC-5000, PAL system; CombiPAL, Switzerland) and a DB-WAXETR capillary column (30 m by 0.25 mm, 0.25-µm film thickness; J&W Scientific, Inc., Folsom, CA). The injection mode was established at 260°C (1 min), and helium was used, at a constant flow rate of 1 ml/min, as the carrier gas. The detection was carried out by means of the electron impact mass spectrometer in total ion current mode, using ionization energy of 70 eV. The acquisition range was set at *m/z* 33 to 350 atomic mass units (amu). The peaks were identified by comparing the mass spectra of the peaks with the spectra of the MIST05 library and through a comparison of the retention indices (a matrix of a homologous series of C_8 to C_{24} was used) with an injected pure standard under the same sample conditions described above. Semiquantitative data (micrograms per

kilogram) were obtained by measuring the characteristic *m*/*z* peak area of each identified compound in relation to the added internal standard.

3.2.6 Statistical analyses

Statistical analyses were carried out using generalized linear mixed-effect model for a non-normally distributed data set. Mixed models were chosen because of their ability to capture both fixed (fermentation condition, inoculated with S, inoculated with ST, and non-inoculated; fermentation time, 0 to 120 h) and random effects (fermentation types, B and H) [12]. The *P* values were adjusted using Bonferroni's method and, when the mixed model revealed significant differences ($P < 0.05$), the Duncan honestly significant difference (HSD) test was applied. Mixed models were built and evaluated according to Crawley [13] using R version 3.3.2. The assessment of the mean difference between the box and heap fermentations over a specific fermentation period was subjected to a *t*-test, in which each fermentation condition was compared between fermentation methods (B and H). In addition, Spearman's correlation test was used to assess the correlations between the OTUs and to establish any changes in concentration over the fermentation period.

3.2.7 DNA extraction, library preparation, and sequencing

The total DNA was extracted from pellets of the cocoa matrices using a MasterPure complete DNA and RNA purification kit (Illumina, Inc., San Diego, CA), according to the manufacturer's instructions. Bacterial communities were studied by amplifying the V3 and V4 regions of 16S rRNA using the primers and under the conditions described by Klindworth *et al.* [14]. The yeast communities were studied by amplifying the ITS2 region using ITS3tagmix (5′-CTAGACTCGTCACCGATGAAGAACGCAG) and ITS-4ngs (5′-TTCCTSCGGCTTATTGATATGC) [15]. The PCR products were purified twice by means of an Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the

resulting products were tagged using a Nextera XT index kit (Illumina), according to the manufacturer's instructions. After the second clean-up step with the Agencourt AMPure kit, a 4 nM pool was obtained in which the weight of the library was measured by means of Qubit fluorometric quantitation (Thermo Fisher Scientific). A denatured 20 pM pool was obtained by mixing 5 µl of 0.2 N NaOH with 5 µl of the 4-nM pool. A final 10 pM library was combined with 10% PhiX. Sequencing was performed using a MiSeq instrument (Illumina) with V3 chemistry, according to the manufacturer's instructions, and 250-bp paired-end reads were generated.

3.2.8 Bioinformatics

The obtained paired-end reads were first assembled with the FLASH software [16], with default parameters. The joint reads were further quality filtered (Phred < Q20) using the QIIME 1.9.0 software [17]. Reads shorter than 250 bp were discarded using Prinseq. For the 16S data, the OTUs were picked at 99% of similarity threshold, and centroid sequences of each cluster were used to assign the taxonomy by mapping against the Greengenes 16S rRNA gene database, version 2013, as recently described [18]. The chloroplast and mitochondrial sequences were removed from the data set. For the ITS data set, 97% similarity was picked for the OTUs, by means of UCLUST clustering methods [19], and representative sequences of each cluster were used to assign the taxonomy using the UNITE rDNA ITS database, version 2012, by means of the RDP Classifier. Weighted and unweighted UniFrac distance matrices, as well as the OTUs table, were used to find differences between the fermentation processes (B and H) and under different conditions (inoculated and non-inoculated) in the Adonis and analysis of similarity (ANOSIM) statistical test in the R environment in order to avoid biases due to different sequencing depths. All of the samples of each data set were rarefied at the lowest number of reads after raw read quality filtering. QIIME was used to produce a filtered OTUs table

at 1% in at least 2 samples. The OTUs table in this chapter displays the highest taxonomy resolution reached when the taxonomy assignment was not able to reach the species level or when the genus or family name was displayed. The Kruskal-Wallis statistical package and Mann-Whitney tests were used to find significant differences in the microbial taxon abundance profiles and in the Shannon-Wiener diversity index (H′), according to the time, conditions, and methods ($P < 0.05$). Spearman's rank correlation coefficient was obtained as a measure of the association between the microbial OTUs that occurred in at least 2 samples and the chemical variables through the *psych* function and plotted through the *corrplot* package of R. The OTUs that occurred in at least 2 samples of the microbial communities were conglomerated, by means of hierarchical clustering analysis, using Ward's method, which was acquired through the *heatplot* function plotted by the *made4* package of R.

3.2.9 Accession numbers

The 16S and ITS rRNA gene sequences are available at the NCBI Sequence Read Archive under accession numbers SRP126069 and SRP126081, respectively.

3.3 RESULTS

3.3.1 Physical and microbiological changes that take place in box and heap cocoa fermentations

Temperature and pH were measured during the box and heap (B and H, respectively) fermentations at time zero and after 48, 96, and 120 h, as shown in Table 3.1. No significant difference ($P < 0.05$) between the considered conditions (inoculated and non-inoculated) was observed from the physical or microbiological analysis, while the temperature observed during the B and H

fermentations increased significantly from the initial values of 27°C to 43°C and 40°C, respectively, at the end of the fermentation ($P < 0.05$). The pH of the cocoa bean pulp was 3.5 at the beginning of the trial, and it increased to 4.2 and 4.7 at the end of the fermentation for the B and H fermentations, respectively ($P < 0.05$).

Table 3.1 Average changes in the physical and microbiological parameters during the inoculated and non-inoculated box and heap fermentation of cocoa bean pulp turned after 48 and 96 h

Parameter by fermentation		Inoculation[a]	Data by fermentation time [h][b]			
			0	**48**	**96**	**120**
BOX						
	°C	S	26.73 ± 0.60	36.20 ± 1.53	41.88 ± 1.78	43.70 ± 2.94
		ST	26.48 ± 0.34	35.10 ± 2.46	41.73 ± 2.06	42.78 ± 3.68
		C	26.73 ± 0.62	36.20 ± 1.53	41.88 ± 1.78	43.70 ± 2.94
		Avg	***26.58 ± 0.08***[c]	***35.80 ± 1.22***[b]	***42.09 ± 0.50***[a]	***43.33 ± 0.70***[a]
	pH	S	3.55 ± 0.03	3.88 ± 0.16	4.15 ± 0.11	3.96 ± 0.18
		ST	3.54 ± 0.01	4.00 ± 0.16	4.20 ± 0.15	4.18 ± 0.26
		C	3.55 ± 0.03	3.88 ± 0.17	4.27 ± 0.11	3.96 ± 0.18
		Avg	***3.57 ± 0.03***[c]	***4.08 ± 0.11***[b]	***4.31 ± 0.09***[a]	***4.15 ± 0.17***[ab]
Log CFU						
	Yeast	S	7.08 ± 0.05	7.19 ± 0.15	N.C	N.C
		ST	7.14 ± 0.01	7.05 ± 0.06	N.C	N.C
		C	7.19 ± 0.17	7.55 ± 0.27	N.C	N.C
		Avg	***7.14 ± 0.11***[a]	***7.26 ± 0.29***[a]	*N.C*	*N.C*
	LAB	S	5.38 ± 0.30	7.18 ± 0.02	5.11 ± 1.28	6.75 ± 0.17
		ST	6.13 ± 0.22	6.85 ± 0.27	4.33 ± 0.38	7.35 ± 0.25
		C	6.21 ± 0.74	6.88 ± 0.40	4.00 ± 0.00	5.49 ± 0.59
		Avg	***5.91 ± 0.61***[b]	***6.97 ± 0.31***[a]	***5.44 ± 2.85***[c]	***6.55 ± 0.94***[a]
	AAB	S	6.41 ± 0.11	7.01 ± 0.12	5.54 ± 1.78	5.63 ± 1.88
		ST	6.28 ± 0.21	6.96 ± 0.06	5.60 ± 1.84	7.34 ± 0.09
		C	6.28 ± 0.25	7.31 ± 0.26	4.00 ± 0.00	5.76 ± 0.49
		Avg	***6.32 ± 0.20***[a]	***7.09 ± 0.23***[a]	***5.05 ± 2.01***[b]	***6.69 ± 2.85***[a]
HEAP						
	°C	S	28.20 ± 1.15	38.17 ± 0.75	36.57 ± 1.80	40.07 ± 0.12
		ST	27.37 ± 0.32	39.00 ± 2.21	36.57 ± 0.90	39.38 ± 0.32
		C	26.27 ± 0.06	38.97 ± 0.32	39.37 ± 2.57	40.30 ± 0.56
		Avg	***27.28 ± 0.97***[c]	***38.71 ± 0.47***[b]	***38.40 ± 1.59***[b]	***40.07 ± 0.23***[a]
	pH	S	3.55 ± 0.01	4.24 ± 0.17	4.48 ± 0.79	4.90 ± 0.97
		ST	3.53 ± 0.01	4.32 ± 0.23	4.05 ± 0.40	4.52 ± 0.74
		C	3.50 ± 0.05	3.87 ± 0.08	4.24 ± 0.29	3.99 ± 0.33
		Avg	***3.54 ± 0.02***[b]	***4.28 ± 0.24***[a]	***4.26 ± 0.21***[a]	***4.71 ± 0.90***[a]
Log CFU						
	Yeast	S	7.16 ± 0.92	7.80 ± 0.15	7.13 ± 0.16	8.03 ± 0.29
		ST	6.76 ± 0.85	7.72 ± 0.15	7.24 ± 1.41	7.43 ± 0.07
		C	7.02 ± 0.71	6.62 ± 0.02	6.34 ± 0.04	7.24 ± 0.28
		Avg	***6.98 ± 0.20***[c]	***7.38 ± 0.66***[b]	***6.90 ± 0.48***[d]	***7.57 ± 0.41***[a]
	LAB	S	5.67 ± 0.25	7.28 ± 0.19	7.36 ± 0.04	7.69 ± 0.28
		ST	5.95 ± 0.29	7.07 ± 0.09	7.00 ± 0.01	7.50 ± 0.04
		C	5.72 ± 0.03	6.17 ± 0.21	7.40 ± 0.04	8.10 ± 0.13
		Avg	***5.78 ± 0.15***[d]	***6.84 ± 0.59***[c]	***7.25 ± 0.22***[b]	***7.76 ± 0.30***[a]

	AAB	S	6.20 ± 0.23	6.81 ± 0.14	8.33 ± 0.02	7.80 ± 0.18
		ST	5.70 ± 0.04	7.15 ± 0.28	8.08 ± 0.11	7.66 ± 0.01
		C	6.60 ± 0.04	5.65 ± 0.15	8.42 ± 0.02	8.56 ± 0.04
		Avg	***6.17 ± 0.45***[d]	***6.54 ± 0.79***[c]	***8.28 ± 0.18***[a]	***8.00 ± 0.49***[b]

[a] **S**, *S. cerevisiae*; **ST**, *S. cerevisiae* and *T. delbrueckii*; **C**, non-inoculated.

[b] Values are expressed as the mean ± SD from triplicate determinations. Different letters indicate statistical differences related to the fermentation period using the least significant difference test ($P < 0.05$). *P* values were adjusted using Bonferroni's method. NC, below the detection limit.

The yeast, LAB, and AAB population dynamics are reported in Table 3.1. The yeasts constituted the dominant population for the first 48 h in both processes (B and H), and they were already detected at high loads in the cocoa beans before the introduction of the starter strain inoculum, with an average value of 6.98 log CFU g^{-1} in the H fermentation and 7.14 log CFU g^{-1} in the B fermentation. On the other hand, the yeast population in the H fermentation remained at around 7 log CFU g^{-1}, even after 48 h, with the highest count recorded at the end of the process (7.57 log CFU g^{-1}). A significant difference between B and H fermentations was also observed in the LAB dynamics during the fermentation time, with a marked increase in the counts after 48 h in both fermentation processes (B, 5.91 to 6.55 log CFU g^{-1}; H, 5.78 to 7.76 log CFU g^{-1}), as shown in Table 3.1 ($P < 0.01$). High counts of AAB were observed at the beginning of the B and H fermentations (6.32 and 6.17 log CFU g^{-1}, respectively). However, this population showed a fluctuating trend during the B fermentation, whereas it increased over time during the H fermentation to a final count of 8.00 log CFU g^{-1} ($P < 0.01$). It should be noted that after 96 h, the AAB dominated the LAB and yeasts in both fermentation processes. Overall, higher counts were observed for the three considered microbial groups (yeasts, LAB, and AAB) in the H fermentations than in the B ones at 96 h, as shown in Table 3.1 ($P < 0.05$).

3.3.2 Identification of isolated yeast colonies and assessment of the dominance of the starter strains

In order to establish the yeast dynamics in the B and H fermentations, 104 yeast colonies were isolated from WLN agar plates. The ITS-restriction

fragment length polymorphism (ITS-RFLP) fingerprints identified *S. cerevisiae* and *T. delbrueckii* in 70% of the isolated colonies. Furthermore, REP-PCR fingerprints and a comparison with the starter profiles highlighted the presence of *S. cerevisiae* ID76 and *T. delbrueckii* ID103 in the cultivable mycobiota during the first 48 h of both the B and H fermentations. *S. cerevisiae* ID76 represented 68% of the isolates from the fermentations inoculated with *S. cerevisiae* (S) and 51% of the colonies isolated from the fermentations inoculated with *S. cerevisiae* and *T. delbrueckii* (ST). Finally, 38% of the colonies isolated from the ST fermentations were ascribed to a *T. delbrueckii* ID103 profile. Apart from the identification of the starter strains, *Hanseniaspora opuntiae* represented the most abundant autochthonous species, representing 31% of the colonies isolated from the non-inoculated fermentations (data not shown).

3.3.3 Dynamics of the non-volatile organic compounds during cocoa bean fermentation

The evolution of non-volatile compounds was determined during the B and H fermentation of the cocoa beans by means of high-performance liquid chromatography (HPLC), as shown in Figure 3.2. No significant differences were observed between the inoculated and non-inoculated fermentations through the analysis of the non-volatile compounds. At the beginning of the process, the B fermentations showed higher concentrations of glucose, fructose, and sucrose (24, 25, and 8 mg/g, respectively) than did the H fermentations (20, 23, and 10 mg/g, respectively), and significantly decreased levels of glucose, fructose, and sucrose during both fermentation processes (B and H) were observed over the fermentation period ($P < 0.05$).

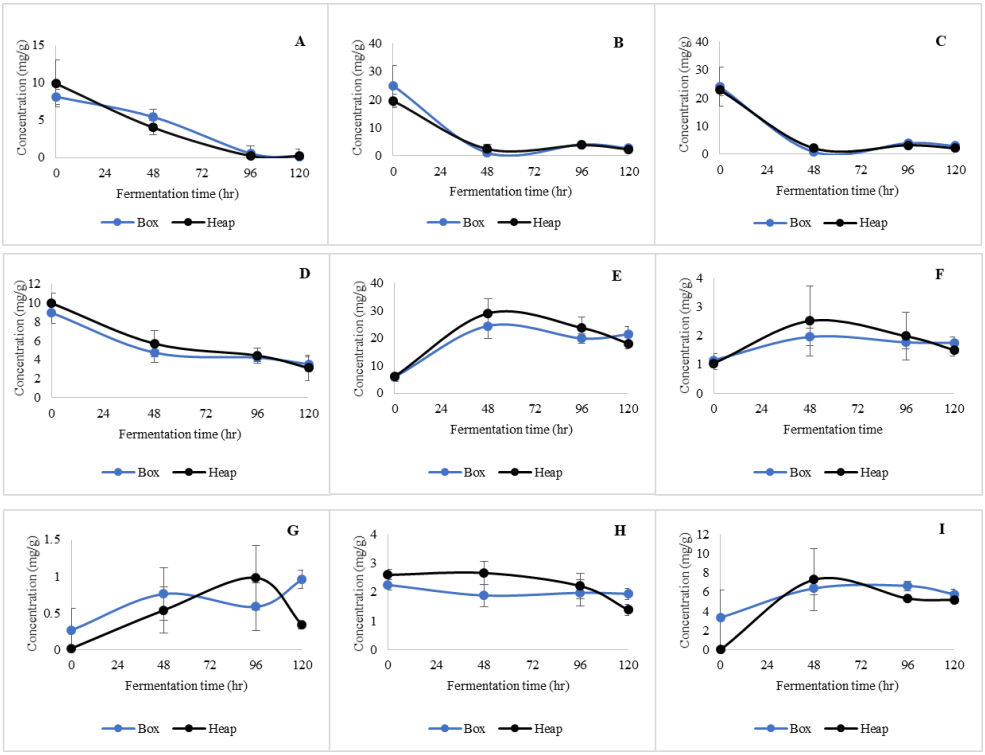

Figure 3.2 Dynamics of sugars and organic acid compounds in cocoa bean-pulp inoculated and non-inoculated during Box and Heap fermentations expressed as mg/g. Data are expressed as mean ± standard deviation (SD) values from triplicate determinations. **A)** Sucrose, **B)** Glucose, **C)** Fructose, **D)** Citric acid, **E)** Succinic acid, **F)** Malic acid, **G)** Acetic acid, **H)** Gluconic acid, and **I)** Lactic acid

As far as the overall content of organic acids is concerned, the highest concentration of organic acids in the cocoa bean pulp before the start of the fermentation was citric acid, and this was followed by succinic and gluconic acid in both fermentation processes (3.2). It should be pointed out that greater amounts of lactic and succinic acid were detected at 48 h, whereas the maximum production of acetic acid was observed at 96 h. Succinic acid was found to be the most abundant organic acid from 48 h to the end of both fermentation processes (B and H), with concentrations of up to 21 and 18 mg/g, respectively. The dynamics over time observed for the organic acids during both fermentation processes (B and H) were similar. A statistically significant decrease in the citric

and gluconic acid concentrations was observed during the B and H fermentations, and the lowest values were reached at the end of the fermentation ($P < 0.01$). On the other hand, an increase in the malic, succinic, lactic, and acetic acid concentrations was found during the fermentation period ($P < 0.01$) for both processes (B and H). No significant changes were observed for the oxalic, pyruvic, tartaric, or fumaric acids during B or H over the fermentation period (data not shown).

3.3.4 Volatilome during cocoa bean pulp fermentation

A total of 72 VOCs were identified by means of headspace solid-phase microextraction gas chromatography–quantitative mass spectrometry (HS-SPME/GC-qMS) on fermented cocoa bean pulp. No significant differences were observed between the inoculated and non-inoculated fermentations from the VOCs analysis at the end of the fermentation. At the beginning of the B and H fermentation processes, 2-pentanol, ethyl acetate, limonene, and 1,2-propanediol diacetate were found to be the most abundant VOCs, whereas acetic acid, limonene, 2-heptanol, phenylethyl alcohol, isopentyl alcohol, isovaleric acid, and benzeneacetaldehyde represented the most retrieved VOCs in the headspace at the end of both fermentations (Table 3.2). It should be pointed out that the total peak area of the VOCs at the end of the B fermentation was about twice as high as that of the H fermentation ($P < 0.01$).

Table 3.2 Total volatile compounds (µg/kg) detected at the beginning and the end of cocoa beans fermentations identified by GC-MS analysis produced from two different fermentation methods (Box and Heap) under different conditions (inoculated and non-inoculated).

	BOX																					
	At the begining (ug/kg)										At the end (ug/kg)									
Compound	**S**			**ST**			**C**			**MEAN**			**S**			**ST**			**C**			**MEAN**		
Ethyl acetate	1068.78	±	322.19	1531.8	±	375.5	798.99	±	34.81	1133.21	±	370.66	1203.40	±	860.7	453.63	±	116.81	793.86	±	249.34	816.96	±	375.42
Ethyl octanoate	4.60	±	0.62	0.00	±	0.00	7.23	±	2.14	3.94	±	3.66	83.99	±	15.94	60.79	±	10.02	56.21	±	14.91	66.99	±	14.89
Ethyl caproate	28.59	±	1.51	15.14	±	2.69	17.15	±	5.22	20.29	±	7.26	115.48	±	28.40	76.39	±	14.95	64.77	±	18.34	85.55	±	26.56
Methyl octanoate	15.00	±	1.44	8.91	±	1.12	14.73	±	4.40	12.88	±	3.44	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Ethyl benzoate	0.57	±	0.35	1.11	±	0.09	1.64	±	0.41	1.11	±	0.53	32.45	±	5.81	23.60	±	6.38	23.45	±	11.32	26.50	±	5.16
Ethyl-α-toluate	0.00	±	0.00	0.00	±	0.00	0.08	±	0.08	0.03	±	0.05	122.72	±	18.46	111.51	±	33.54	94.77	±	22.29	109.67	±	14.06
2-Pentanol, acetate	196.77	±	73.81	211.61	±	37.09	265.74	±	71.62	224.71	±	36.30	1002.98	±	105.5	438.19	±	162.67	851.84	±	205.09	764.34	±	292.39
Isopentyl alcohol, acetate	151.29	±	45.60	1.09	±	0.33	181.43	±	20.53	111.27	±	96.60	1198.90	±	212.6	657.66	±	115.32	1488.83	±	489.60	1115.13	±	421.87
Hexyl acetate	1.88	±	0.90	0.60	±	0.28	0.00	±	0.00	0.82	±	0.96	7.04	±	1.97	0.00	±	0.00	0.00	±	0.00	2.35	±	4.06
2,3-butanediol diacetate	27.00	±	10.41	24.82	±	4.08	21.62	±	5.55	24.48	±	2.71	7.29	±	3.74	9.15	±	1.18	13.06	±	4.54	9.83	±	2.94
1,2-propanediol, diacetate	913.95	±	123.51	673.62	±	143.1	726.96	±	94.10	771.51	±	126.21	309.60	±	114.5	452.64	±	119.67	340.33	±	208.67	367.52	±	75.30
β-phenylethyl acetate	4.22	±	1.39	9.87	±	2.15	7.71	±	0.80	7.27	±	2.85	307.16	±	51.11	302.99	±	102.25	490.95	±	355.36	367.04	±	107.33
Isoamyl benzoate	22.37	±	11.71	11.78	±	1.51	42.71	±	7.68	25.62	±	15.72	26.58	±	12.79	3.69	±	1.13	7.00	±	1.75	12.42	±	12.37
n-Hexyl butanoate	0.00	±	0.00	4.51	±	1.52	0.00	±	0.00	1.50	±	2.60	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Total ester	**2435.04**			**2494.9**			**2086.00**			**2338.65**			**4417.59**			**2590.25**			**4225.06**			**3744.30**		
Hexanal	266.27	±	60.76	200.16	±	54.09	73.54	±	13.29	179.99	±	97.93	39.19	±	9.77	29.55	±	7.85	27.66	±	6.69	32.13	±	6.19
Benzaldehyde	57.17	±	14.06	45.49	±	8.50	25.87	±	5.47	42.84	±	15.81	161.94	±	28.03	131.46	±	77.05	173.44	±	23.66	155.61	±	21.70
Benzeneacetaldehyde	770.20	±	253.72	505.67	±	123.3	401.44	±	54.96	559.10	±	190.09	892.26	±	114.9	1039.41	±	453.84	1069.12	±	140.50	1000.27	±	94.71
α-ethylidene-benzeneacetaldehyde	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	11.69	±	3.21	4.62	±	1.46	7.57	±	6.08	7.96	±	3.55
trans-2-Undecenal	0.00	±	0.00	0.00	±	0.00	0.61	±	0.30	0.20	±	0.35	0.00	±	0.00	4.26	±	0.91	3.28	±	0.11	2.51	±	2.23
Nonanal	19.33	±	4.87	12.48	±	2.78	42.13	±	19.95	24.65	±	15.52	23.97	±	5.83	25.35	±	4.08	38.12	±	13.75	29.15	±	7.80
2-methyl-butanal	132.28	±	25.38	103.14	±	17.09	68.96	±	4.79	101.46	±	31.69	125.80	±	37.94	113.27	±	62.65	68.52	±	23.69	102.53	±	30.11
Total aldehyde	**1245.25**			**866.95**			**612.56**			**908.25**			**1254.85**			**1347.93**			**1387.70**			**1330.16**		
2-Pentanol	6597.01	±	1488	2133.6	±	748.3	7985.97	±	603.0	5572.22	±	3057.7		±	281.9	431.28	±	229.69	1008.09	±	62.97	764.44	±	298.64
2-Heptanol	54.32	±	12.46	44.88	±	5.17	51.27	±	11.64	50.16	±	4.82	2291.74	±	769.5	1389.95	±	330.39	2015.58	±	453.58	1899.09	±	462.05
(Z)-3-Hexen-1-ol	5.76	±	1.41	3.30	±	1.22	5.69	±	2.16	4.92	±	1.40	4.04	±	1.04	2.98	±	0.76	2.60	±	1.66	3.20	±	0.75
1-Hexanol	41.50	±	3.00	35.43	±	7.45	31.26	±	10.64	36.06	±	5.15	37.22	±	8.75	32.05	±	5.65	32.00	±	17.09	33.76	±	3.00
1-Octen-3-ol	5.41	±	4.54	3.91	±	2.70	0.00	±	0.00	3.11	±	2.79	0.00	±	0.00	0.00	±	0.00	42.20	±	13.59	14.07	±	24.36
1-Heptanol	5.19	±	0.55	4.09	±	0.99	6.95	±	1.59	5.41	±	1.44	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
2-ethyl-1-hexanol	42.38	±	11.07	40.49	±	9.54	33.85	±	5.27	38.90	±	4.48	37.00	±	5.47	41.94	±	4.73	43.11	±	3.25	40.68	±	3.24
3-methyl-1-butanol	153.25	±	24.16	107.51	±	18.41	132.12	±	35.84	130.96	±	22.89	334.31	±	145.6	186.71	±	51.27	368.42	±	78.19	296.48	±	96.58
2-Hexanol	57.49	±	6.08	65.45	±	11.41	111.23	±	9.88	78.06	±	29.00	41.09	±	5.16	24.08	±	10.64	40.72	±	8.55	35.30	±	9.71
1-Pentanol	59.45	±	14.51	68.87	±	17.52	12.67	±	3.67	47.00	±	30.10	24.90	±	4.49	11.76	±	5.38	25.26	±	10.14	20.64	±	7.69
1-Octanol	9.08	±	0.99	4.86	±	1.46	15.46	±	5.50	9.80	±	5.34	32.87	±	13.79	18.50	±	4.38	25.15	±	1.39	25.51	±	7.19

Benzyl alcohol	5.56	±	1.08	4.27	±	0.96	4.25	±	1.18	4.69	±	0.75	8.04	±	1.32	10.33	±	3.39	7.82	±	0.47	8.73	±	1.39
Phenylethyl alcohol	61.26	±	6.12	45.88	±	7.14	38.19	±	7.21	48.45	±	11.74	936.34	±	120.7	1174.60	±	487.07	1864.90	±	373.95	1325.28	±	482.27
1-Heptadecanol	6.58	±	2.49	2.57	±	0.80	7.34	±	2.81	5.50	±	2.56	25.32	±	2.62	19.64	±	3.34	14.93	±	3.53	19.96	±	5.20
Isobutanol	213.39	±	65.42	17.18	±	7.62	10.90	±	2.96	80.49	±	115.14	17.87	±	5.35	11.76	±	3.16	26.28	±	8.29	18.64	±	7.29
1-Decanol	1.22	±	0.35	0.56	±	0.22	0.69	±	0.27	0.82	±	0.35	1.94	±	0.60	1.29	±	0.27	1.09	±	0.09	1.44	±	0.45
α-Methylbenzyl alcohol	43.63	±	13.03	24.44	±	10.75	98.38	±	12.54	55.48	±	38.37	55.88	±	4.79	58.64	±	16.30	52.33	±	11.33	55.62	±	3.16
1-Dodecanol	6.82	±	2.20	14.25	±	3.18	35.32	±	6.24	18.80	±	14.78	16.69	±	5.39	42.67	±	26.52	15.05	±	5.43	24.80	±	15.50
Ethanol	300.55	±	84.47	322.81	±	82.61	375.28	±	25.18	332.88	±	38.37	404.88	±	63.92	223.02	±	52.02	292.90	±	54.31	306.93	±	91.73
Decanal	4.75	±	1.51	4.37	±	0.83	13.72	±	3.70	7.62	±	5.29	5.44	±	1.53	7.58	±	3.05	7.09	±	1.63	6.70	±	1.12
Total alcohol	**7674.61**			**2948.8**			**8970.55**			**6531.32**			**5129.50**			**3688.78**			**5885.51**			**4901.26**		
2-Pentanone	277.43	±	181.9	128.56	±	18.55	1138.55	±	71.19	514.85	±	545.25	323.76	±	110.5	366.19	±	159.87	386.31	±	28.96	358.75	±	31.93
2-Heptanone	91.90	±	10.08	57.47	±	12.51	194.33	±	19.35	114.57	±	71.19	450.06	±	106.0	157.66	±	205.23	348.02	±	107.87	318.58	±	148.41
Acetoin	53.89	±	9.78	39.22	±	4.02	44.36	±	4.60	45.82	±	7.45	1129.92	±	279.0	1070.10	±	248.78	1000.23	±	170.89	1066.75	±	64.91
2-Nonanol	6.86	±	2.18	4.81	±	0.88	6.63	±	0.50	6.10	±	1.12	200.36	±	50.65	61.59	±	12.66	87.31	±	7.94	116.42	±	73.82
2-Undecanone	5.77	±	0.86	2.93	±	0.72	7.36	±	1.90	5.35	±	2.24	5.76	±	0.51	4.46	±	0.34	7.77	±	0.54	5.99	±	1.67
Acetophenone	161.17	±	59.28	155.68	±	43.9	751.86	±	115	356.24	±	342.63	205.89	±	47.02	208.12	±	66.78	186.19	±	36.61	200.07	±	12.07
2-Tetradecanone	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Dodecanal	2.11	±	0.43	0.00	±	0.00	1.03	±	0.07	1.05	±	1.05	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Total ketone	**599.14**			**388.68**			**2144.12**			**1043.98**			**2315.75**			**1868.11**			**2015.82**			**2066.56**		
β-trans-Ocimene	18.58	±	3.69	28.42	±	6.57	18.37	±	3.63	21.79	±	5.75	88.37	±	11.61	10.77	±	3.47	27.11	±	13.10	42.09	±	40.91
Sabinene	13.04	±	1.51	14.18	±	3.85	8.25	±	1.35	11.82	±	3.15	53.38	±	9.27	8.87	±	0.43	18.08	±	2.45	26.78	±	23.49
β-Myrcene	287.72	±	14.27	192.61	±	47.0	302.15	±	62.1	260.83	±	59.52	432.36	±	27.21	172.58	±	16.03	271.14	±	72.19	292.03	±	131.14
D-Limonene	1155.55	±	96.24	1027.2	±	109	790.11	±	134.	990.96	±	185.40	4896.50	±	929.	1149.67	±	104.92	2743.65	±	1387.2	2929.94	±	1880.3
cis-furan Linalool oxide	13.25	±	8.90	7.18	±	0.36	8.31	±	1.48	9.58	±	3.23	21.75	±	4.31	0.00	±	0.00	9.94	±	11.51	10.56	±	10.89
Limonene epoxide	92.45	±	61.70	75.00	±	14.7	51.17	±	5.92	72.87	±	20.73	5.80	±	2.99	0.00	±	0.00	0.00	±	0.00	1.93	±	3.35
α-Citral	4.52	±	3.08	5.15	±	1.07	1.72	±	0.22	3.80	±	1.83	4.43	±	1.11	16.76	±	3.73	21.67	±	1.60	14.29	±	8.88
β-Caryophyllene	2.17	±	0.22	1.27	±	0.43	1.53	±	0.56	1.66	±	0.47	2.95	±	0.53	2.72	±	1.05	5.08	±	1.56	3.59	±	1.30
α-Caryophyllene	11.51	±	1.35	7.34	±	0.97	10.48	±	1.47	9.78	±	2.17	17.96	±	2.91	15.02	±	4.68	27.36	±	6.20	20.11	±	6.44
cis- pyranoid-linalyol oxide	14.99	±	2.42	12.52	±	1.48	9.76	±	1.21	12.42	±	2.61	16.45	±	4.75	20.67	±	4.69	17.14	±	1.72	18.09	±	2.26
Carveol	4.99	±	3.72	3.92	±	0.69	3.32	±	0.78	4.08	±	0.85	0.37	±	0.28	0.17	±	0.05	0.48	±	0.05	0.34	±	0.16
cis-Geranylacetone	3.10	±	1.02	3.16	±	0.71	2.45	±	0.56	2.90	±	0.39	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
α-Limonene diepoxide	1.19	±	0.39	1.74	±	0.68	0.00	±	0.00	0.98	±	0.89	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Total monoterpene	**1623.07**			**1379.7**			**1207.63**			**1403.47**			**5540.32**			**1397.24**			**3141.66**			**3359.74**		
Acetic acid	96.10	±	35.24	107.97	±	16.28	109.77	±	35.4	104.61	±	7.43	5745.06	±	445.34	5079.09	±	1588	4670.67	±	2046	5164.94	±	542.32
Isobutyric acid	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	22.93	±	7.72	49.29	±	4.04	22.15	±	5.56	31.46	±	15.45
Isovaleric acid	8.07	±	4.20	8.03	±	0.79	6.73	±	1.47	7.61	±	0.76	897.84	±	166.4	1645.25	±	407.6	512.36	±	93.05	1018.48	±	576.00
Hexanoic acid	54.00	±	8.55	19.50	±	2.58	11.84	±	2.73	28.44	±	22.46	92.27	±	13.70	81.88	±	20.18	55.88	±	4.17	76.68	±	18.75
Heptanoic acid	5.56	±	1.36	0.00	±	0.00	3.30	±	2.27	2.95	±	2.80	13.48	±	2.68	12.12	±	2.16	8.54	±	1.09	11.38	±	2.56
Octanoic Acid	6.15	±	2.24	2.85	±	0.60	6.98	±	1.72	5.33	±	2.19	38.43	±	4.22	33.66	±	8.55	20.41	±	2.16	30.83	±	9.34
Total acid	**169.88**			**138.35**			**138.61**			**148.95**			**6810.02**			**6901.29**			**5290.01**			**6333.77**		
Phenol	2.47	±	0.49	2.47	±	0.46	2.54	±	0.47	2.49	±	0.04	6.33	±	0.88	9.99	±	2.72	7.27	±	0.81	7.87	±	1.90
o-Guaiacol	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	4.02	±	0.83	8.00	±	1.83	3.91	±	1.58	5.31	±	2.33
Total phenol	**2.47**			**2.47**			**2.54**			**2.49**			**10.36**			**17.99**			**11.18**			**13.18**		

	HEAP																							
	At the beginning (ug/kg)											At the end (ug/kg)												
Compound	**S**			**ST**			**C**			**MEAN**			**S**			**ST**			**C**			**MEAN**		
Ethyl acetate	856.78	±	51.3	707.26	±	177	350.01	±	31.1	638.02	±	260.39	765.55	±	203.9	568.61	±	142.4	53.58	±	23.8	462.58	±	367.64
Ethyl octanoate	4.04	±	0.25	3.90	±	1.11	22.73	±	5.53	10.22	±	10.83	53.90	±	14.47	99.55	±	29.77	18.90	±	8.35	57.45	±	40.44
Ethyl caproate	21.66	±	0.04	26.14	±	2.58	74.36	±	12.1	40.72	±	29.22	87.59	±	20.54	109.04	±	18.78	4.85	±	0.95	67.16	±	55.02
Methyl octanoate	12.35	±	0.51	17.47	±	1.71	0.00	±	0.00	9.94	±	8.98	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Ethyl benzoate	3.12	±	1.17	2.00	±	0.97	87.83	±	20.6	30.98	±	49.23	34.77	±	10.22	27.60	±	4.83	7.69	±	0.42	23.35	±	14.03
Ethyl-α-toluate	2.41	±	4.18	0.00	±	0.00	0.00	±	0.00	0.80	±	1.39	54.05	±	15.96	58.00	±	9.13	56.79	±	10.5	56.28	±	2.03
2-Pentanol, acetate	69.51	±	0.98	181.08	±	86.8	90.18	±	47.2	113.59	±	59.35	292.59	±	68.14	326.81	±	14.60	20.87	±	9.87	213.42	±	167.63
Isopentyl alcohol, acetate	79.60	±	5.01	112.76	±	12.6	14.78	±	1.15	69.05	±	49.84	415.53	±	75.82	506.51	±	4.47	2.66	±	1.15	308.23	±	268.51
Hexyl acetate	1.71	±	0.19	0.00	±	0.00	2.42	±	0.42	1.38	±	1.24	6.59	±	1.20	0.00	±	0.00	0.00	±	0.00	2.20	±	3.80
2,3-butanediol diacetate	14.32	±	1.84	17.70	±	3.03	207.11	±	50.4	79.71	±	110.34	196.31	±	52.93	3.49	±	0.77	106.24	±	13.5	102.02	±	96.48
1,2-propanediol, diacetate	753.88	±	62.3	929.04	±	173	143.75	±	23.1	608.89	±	412.23	364.46	±	92.43	219.04	±	15.36	203.37	±	24.4	262.29	±	88.83
β-phenylethyl acetate	23.82	±	5.57	5.92	±	0.39	7.42	±	0.45	12.38	±	9.93	138.62	±	39.83	85.37	±	8.42	66.61	±	7.93	96.87	±	37.36
Isoamyl benzoate	4.36	±	0.33	11.48	±	0.76	3.53	±	0.53	6.46	±	4.37	92.03	±	24.76	10.64	±	2.09	7.47	±	1.84	36.71	±	47.93
n-Hexyl butanoate	7.95	±	3.75	2.45	±	1.41	0.00	±	0.00	3.47	±	4.07	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Total ester	**1855.5**			**2017.19**			**1004.1**			**1625.6**			**2501.98**			**2014.6**			**549.04**			**1688.56**		
Hexanal	28.04	±	0.61	47.36	±	14.1	175.46	±	11.1	83.62	±	80.12	33.98	±	4.73	27.98	±	1.79	16.81	±	2.30	26.26	±	8.72
Benzaldehyde	48.98	±	7.60	24.50	±	2.31	48.14	±	5.64	40.54	±	13.90	122.13	±	39.53	69.30	±	11.00	67.10	±	14.4	86.18	±	31.16
Benzeneacetaldehyde	521.18	±	16.6	484.56	±	19.1	332.37	±	34.5	446.04	±	100.13	2027.33	±	635.2	665.86	±	96.47	666.89	±	79.9	1120.03	±	785.74
α-ethylidene-benzeneacetaldehyde	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.79	±	0.35	2.99	±	0.30	0.41	±	0.31	1.40	±	1.39
trans-2-Undecenal	0.00	±	0.00	1.52	±	0.61	0.00	±	0.00	0.51	±	0.88	4.36	±	1.06	5.69	±	1.05	2.59	±	0.68	4.21	±	1.55
Nonanal	11.50	±	0.81	18.81	±	5.83	33.26	±	12.8	21.19	±	11.07	20.45	±	2.59	15.31	±	6.28	14.33	±	3.88	16.70	±	3.29
2-methyl-butanal	83.51	±	5.65	83.30	±	17.7		±	3.00	77.14	±	10.85	239.67	±	55.66	85.34	±	1.57	75.02	±	3.49	133.34	±	92.23
Total aldehyde	**693.22**			**660.05**			**653.83**			**669.03**			**2448.72**			**872.47**			**843.15**			**1388.11**		
2-Pentanol	1501.2	±	43.5	5271.34	±	984	45.46	±	3.57	2272.7	±	2696.9	249.71	±	50.59	502.94	±	13.88	7.01	±	0.78	253.22	±	247.99
2-Heptanol	33.48	±	2.08	41.86	±	2.97	123.16	±	11.0	66.17	±	49.54	406.49	±	94.86	931.42	±	49.90	4.17	±	1.62	447.36	±	464.98
(Z)-3-Hexen-1-ol	3.92	±	0.30	4.68	±	0.29	7.64	±	0.87	5.41	±	1.96	3.61	±	0.83	1.08	±	0.46	0.43	±	0.12	1.71	±	1.68
1-Hexanol	22.80	±	1.96	25.49	±	2.97	83.75	±	7.73	44.02	±	34.44	29.78	±	6.14	25.55	±	0.74	10.92	±	0.74	22.08	±	9.90
1-Octen-3-ol	3.33	±	0.29	4.15	±	0.88	0.00	±	0.00	2.49	±	2.20	30.27	±	7.61	0.00	±	0.00	0.00	±	0.00	10.09	±	17.48
1-Heptanol	2.67	±	0.65	5.50	±	1.77	10.13	±	1.39	6.10	±	3.76	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
2-ethyl-1-hexanol	35.83	±	0.78	45.71	±	12.2	63.16	±	6.85	48.23	±	13.84	46.36	±	10.74	39.23	±	0.85	36.36	±	2.25	40.65	±	5.14
3-methyl-1-butanol	101.72	±	5.53	132.59	±	21.4	77.84	±	4.32	104.05	±	27.45	143.71	±	28.59	217.34	±	5.56	10.79	±	2.94	123.95	±	104.68
2-Hexanol	53.23	±	1.56	135.48	±	24.0	0.00	±	0.00	62.90	±	68.26	11.51	±	1.99	21.39	±	0.65	0.88	±	0.26	11.26	±	10.25
1-Pentanol	21.19	±	5.27	15.88	±	3.60	49.33	±	19.3	28.80	±	17.98	22.85	±	4.34	16.89	±	8.13	15.40	±	7.35	18.38	±	3.94
1-Octanol	5.31	±	0.21	10.36	±	1.83	31.23	±	3.43	15.64	±	13.74	15.27	±	3.42	19.46	±	1.90	13.06	±	0.68	15.93	±	3.25
Benzyl alcohol	4.51	±	0.24	4.27	±	0.28	4.77	±	0.39	4.52	±	0.25	9.05	±	2.69	8.63	±	1.25	5.94	±	1.05	7.87	±	1.69
Phenylethyl alcohol	90.79	±	7.39	36.44	±	2.34	46.80	±	5.10	58.01	±	28.86	741.14	±	206.3	653.88	±	104.1	462.46	±	77.3	619.16	±	142.54
1-Heptadecanol	7.27	±	4.58	12.12	±	7.76	7.70	±	1.55	9.03	±	2.68	10.32	±	2.93	24.23	±	2.09	11.59	±	3.72	15.38	±	7.69
Isobutanol	9.89	±	1.99	11.62	±	4.76	141.33	±	11.9	54.28	±	75.39	9.02	±	3.97	16.20	±	8.73	1.56	±	0.98	8.93	±	7.32
1-Decanol	0.98	±	0.28	1.20	±	0.44	0.93	±	0.27	1.04	±	0.15	1.39	±	0.36	1.14	±	0.11	0.82	±	0.10	1.12	±	0.29
α-Methylbenzyl alcohol	40.79	±	1.67	65.81	±	1.46	33.68	±	3.91	46.76	±	16.88	48.43	±	12.99	39.67	±	5.48	28.84	±	4.43	38.98	±	9.81
1-Dodecanol	4.04	±	1.52	2.85	±	1.24	34.40	±	9.89	13.76	±	17.88	8.26	±	1.94	44.12	±	19.63	27.45	±	12.6	26.61	±	17.94
Ethanol	267.50	±	8.34	367.30	±	90.9	159.91	±	1.18	264.90	±	103.72	455.21	±	84.87	444.01	±	22.29	25.09	±	3.94	308.10	±	245.16
Decanal	6.08	±	0.73	6.60	±	2.24	5.85	±	2.43	6.17	±	0.38	4.01	±	0.78	3.68	±	0.05	2.86	±	0.95	3.52	±	0.59

Total alcohol	**2216.6**			**6201.23**			**927.07**			**3114.9**			**2246.37**			**3010.8**			**665.65**			**1974.29**		
2-Pentanone	126.09	±	12.1	1161.33	±	310	60.63	±	6.57	449.35	±	617.46	166.32	±	38.38	260.31	±	7.60	196.38	±	13.5	207.67	±	48.00
2-Heptanone	66.55	±	3.88	153.32	±	14.8	67.85	±	18.7	95.91	±	49.72	323.49	±	67.91	421.29	±	37.61	7.23	±	1.53	250.67	±	216.42
Acetoin	34.49	±	3.82	45.68	±	6.72	119.51	±	11.7	66.56	±	46.19	201.35	±	38.59	309.03	±	1.90	228.34	±	13.5	246.24	±	56.03
2-Nonanol	4.70	±	0.21	4.83	±	0.27	5.22	±	0.64	4.92	±	0.27	25.97	±	5.93	53.15	±	5.89	67.45	±	8.75	48.85	±	21.07
2-Undecanone	5.48	±	0.10	5.67	±	0.22	8.86	±	1.26	6.67	±	1.90	5.28	±	1.11	0.00	±	0.00	7.91	±	0.86	4.40	±	4.03
Acetophenone	164.75	±	4.07	365.86	±	28.9	92.44	±	13.7	207.69	±	141.68	293.61	±	90.74	269.70	±	50.63	68.72	±	13.7	210.68	±	123.52
2-Tetradecanone	0.96	±	0.30	0.00	±	0.00	0.78	±	0.49	0.58	±	0.51	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Dodecanal	2.72	±	0.14	0.00	±	0.00	1.67	±	0.73	1.46	±	1.37	0.00	±	0.00	1.27	±	0.23	0.00	±	0.00	0.42	±	0.73
Total ketone	**405.74**			**1736.69**			**356.95**			**833.13**			**1016.02**			**1314.7**			**576.03**			**968.93**		
β-trans-Ocimene	23.20	±	0.75	9.07	±	2.79	18.06	±	1.27	16.77	±	7.15	83.70	±	19.28	42.64	±	4.94	2.18	±	0.55	42.84	±	40.76
Sabinene	15.37	±	1.01	6.49	±	0.46	16.11	±	2.03	12.66	±	5.36	49.82	±	10.00	19.58	±	2.74	1.12	±	0.46	23.51	±	24.59
β-Myrcene	323.51	±	8.79	236.09	±	17.3	222.68	±	39.2	260.76	±	54.75	514.78	±	103.4	269.25	±	50.15	182.06	±	15.2	322.03	±	172.52
D-Limonene	1326.0	±	48.9	794.55	±	39.4	2446.1	±	422	1522.2	±	843.07	5015.38	±	983.6	2024.9	±	336.2	254.40	±	34.6	2431.58	±	2406.4
cis-furan Linalool oxide	3.11	±	0.31	3.54	±	0.42	11.55	±	1.15	6.07	±	4.76	15.81	±	3.84	11.81	±	4.23	5.42	±	1.35	11.02	±	5.24
Limonene epoxide	44.49	±	5.03	34.74	±	7.80	319.79	±	31.4	133.01	±	161.83	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
α-Citral	5.27	±	0.22	11.55	±	4.37	4.54	±	1.30	7.12	±	3.85	13.89	±	3.39	16.99	±	1.87	10.75	±	0.98	13.88	±	3.12
β-Caryophyllene	2.08	±	0.06	2.36	±	0.49	6.59	±	0.80	3.68	±	2.53	2.68	±	0.73	3.46	±	2.10	6.86	±	0.39	4.33	±	2.23
α-Caryophyllene	11.05	±	0.20	10.76	±	5.32	25.83	±	3.57	15.88	±	8.62	19.61	±	4.87	20.69	±	2.24	47.99	±	4.81	29.43	±	16.08
cis- pyranoid-linalyol oxide	9.89	±	0.94	11.77	±	2.20	11.61	±	1.57	11.09	±	1.04	20.61	±	4.64	19.47	±	2.25	13.56	±	2.05	17.88	±	3.78
Carveol	2.06	±	0.22	1.34	±	0.29	34.35	±	2.43	12.58	±	18.85	0.00	±	0.00	0.00	±	0.00	0.11	±	0.11	0.04	±	0.07
cis-Geranylacetone	0.00	±	0.00	2.31	±	0.92	0.00	±	0.00	0.77	±	1.34	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
α-Limonene diepoxide	2.75	±	0.95	0.00	±	0.00	3.19	±	0.16	1.98	±	1.73	1.68	±	0.75	1.86	±	0.18	0.00	±	0.00	1.18	±	1.03
Total monoterpene	**1768.8**			**1124.56**			**3120.4**			**2004.6**			**5737.96**			**2430.7**			**524.45**			**2897.71**		
Acetic acid	72.02	±	12.4	80.97	±	45.3	67.33	±	17.8	73.44	±	6.93	710.32	±	205	739.73	±	85.68	997.40	±	175	815.82	±	157.94
Isobutyric acid	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	75.63	±	17.4	53.88	±	8.04	26.61	±	11.5	52.04	±	24.56
Isovaleric acid	6.64	±	1.75	6.12	±	1.15	12.34	±	1.37	8.37	±	3.45	1322.76	±	327	943.18	±	38.89	1245.9	±	216	1170.62	±	200.68
Hexanoic acid	69.31	±	14.5	23.74	±	7.61	39.61	±	15.0	44.22	±	23.13	71.06	±	15.3	44.06	±	8.74	46.12	±	13.0	53.75	±	15.03
Heptanoic acid	9.44	±	2.11	0.00	±	0.00	4.64	±	2.14	4.70	±	4.72	12.96	±	4.11	10.69	±	2.45	7.82	±	3.00	10.49	±	2.58
Octanoic Acid	5.21	±	2.88	7.68	±	1.59	6.83	±	3.87	6.58	±	1.25	17.99	±	11.7	31.76	±	6.52	19.08	±	6.20	22.94	±	7.65
Total acid	**162.63**			**118.52**			**130.76**			**137.31**			**2210.74**			**1823.3**			**2342.9**			**2125.66**		
Phenol	2.53	±	0.15	3.98	±	0.44	1.81	±	0.13	2.77	±	1.10	58.06	±	14.9	42.73	±	5.43	14.94		2.27	38.58	±	21.86
o-Guaiacol	0.12	±	0.21	0.62	±	0.09	0.00	±	0.00	0.25	±	0.33	95.16	±	27.5	72.70	±	10.47	12.80	±	2.38	60.22	±	42.57
Total phenol	**2.66**			**4.59**			**1.81**			**3.02**	**±**	**1.43**	**153.22**			**115.43**			**27.74**			**98.80**	**±**	**64.37**

Values are expressed as mean ± SD from triplicate determinations. **Abbreviations: S**, *S. cerevisiae*; **ST**, *S. cerevisiae* and *T. delbrueckii*; **C**, non-inoculated.

3.3.5 Mycobiota of cocoa beans during fermentation

A total of 1,304,936 raw reads (2 × 250 bp) were obtained, and 1,217,061 reads passed the filters applied through QIIME, with an average value of 31,975 ± 22,635 reads/sample and a mean sequence length of 411 bp. The rarefaction analysis and the estimated sample coverage (ESC) were satisfactory for all of the samples with an ESC average of 97%, while the alpha diversity indicated a higher level of complexity in the B fermentations than in the H fermentations ($P < 0.05$). Overall, 18 fungal OTUs were identified during the fermentations, as shown in Table 3.3. A statistically significant difference between conditions was found, with a higher relative abundance of *Hanseniaspora opuntiae* in the non-inoculated fermentation (46.23%) than in those inoculated with *S. cerevisiae* and *T. delbrueckii* (ST; 25.60%) ($P < 0.05$). In addition, a significantly higher presence of *T. delbrueckii* was observed in the fermentations inoculated with the mixed yeast culture (ST, 22.23%) than in the fermentations inoculated only with *S. cerevisiae* (S) and in the non-inoculated fermentation (0.03 and 0.11%, respectively, $P < 0.01$, Table 3.3).

The inoculated cocoa beans (S and ST) in both fermentation processes (B and H) showed a dominance of *H. opuntiae*, *Candida jaroonii*, *S. cerevisiae*, *T. delbrueckii*, and *Pichia pijperi* at time zero (Table 3.3). In addition, *H. opuntiae*, *P. pijperi*, and *C. jaroonii* were the most predominant in the non-inoculated B fermentations at the beginning of the process, while *H. opuntiae*, *P. pijperi*, and *Botryosphaeria* spp. reached the highest incidence in the non-inoculated H fermentations. However, *H. opuntiae*, *S. cerevisiae*, *P. pijperi*, and *Kluyveromyces marxianus* were the most abundant at the end of both fermentations (B and H). The mycobiota dynamics were similar overtime for the inoculated and non-inoculated B and H fermentations. *S. cerevisiae* significantly increased over time in both processes, while *H. opuntiae* significantly decreased, as shown in Table 3.3 ($P < 0.01$).

Table 3.3 Incidence of the fungal taxonomic groups by amplicon sequencing expressed as relative abundances

	Relative abundance																							
	Box				Heap				Box				Heap				Box				Heap			
	0	48	96	120	0	48	96	120	0	48	96	120	0	48	120		0	48	96	120	0	48	96	120
Taxonomic group	***S. cerevisiae***								***S. cerevisiae + T. delbruecki***								**Non-inoculated**							
Botryosphaeria	1.33	0.43	0.46	0.21	0.59	1.55	0.13	0.13	0.53	0.44	0.91	3.51	0.56	0.32	0.59		0.66	0.20	1.08	0.16	11.07	0.00	0.47	0.12
Candida	3.33	1.35	1.21	1.27	1.03	0.46	0.31	0.74	1.89	0.97	0.77	5.56	0.53	0.34	0.72		2.83	1.48	2.59	0.49	0.89	0.24	1.39	1.73
Candida butyri	2.23	0.27	0.42	0.50	0.16	0.03	0.07	0.09	0.77	0.69	0.34	0.40	0.15	0.04	0.07		0.70	0.21	1.16	0.09	0.07	0.00	0.07	0.03
Candida diversa	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	5.50	0.00	0.00	0.00		0.00	1.00	0.96	0.00	0.00	0.00	0.00	0.00
Candida inconspicua	0.00	0.58	2.63	7.82	0.06	0.03	0.25	0.32	0.00	0.74	1.51	12.97	0.00	0.04	0.94		0.03	2.61	0.96	13.47	0.00	0.87	1.62	1.43
Candida jaroonii	13.07	4.40	3.63	1.24	4.01	1.65	0.59	1.00	8.06	3.80	2.29	1.24	3.41	1.55	1.96		4.34	2.18	3.04	0.86	1.89	0.24	1.27	0.80
Candida quercitrusa	2.27	1.03	0.78	0.32	1.31	0.94	0.32	0.81	1.40	0.72	1.17	0.49	1.27	0.99	1.34		2.43	1.08	1.04	0.16	0.47	0.09	1.46	0.89
Ceratocystis	3.08	5.98	2.93	1.42	1.43	0.99	0.50	0.50	2.46	2.91	2.91	3.41	1.27	1.56	2.32		4.19	2.45	9.32	4.81	2.07	0.87	1.53	0.80
Hanseniaspora opuntiae	38.33	40.98	39.72	28.21	49.76	39.10	48.67	64.37	36.78	37.25	15.62	0.38	43.73	29.51	11.70		54.21	48.11	29.13	21.78	69.22	72.35	35.05	44.81
Issatchenkia	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.27	0.25	0.00	0.37		0.00	0.41	0.37	0.00	0.00	0.00	2.46	1.18
Kluyveromyces marxianus	0.00	0.28	0.18	8.91	0.06	0.13	11.18	1.79	0.00	0.42	0.38	15.76	0.00	0.13	2.80		0.01	4.61	1.03	43.54	0.09	7.76	7.23	1.98
Lasiodiplodia theobromae	0.83	0.61	2.07	1.33	0.49	0.49	0.09	0.07	3.30	0.91	1.22	0.90	0.80	0.09	0.25		4.94	0.61	0.92	0.27	0.10	0.16	0.07	0.16
Penicillium	0.62	0.39	1.09	0.16	0.19	0.13	0.00	0.06	0.12	0.16	0.31	0.25	0.10	0.19	0.27		0.55	0.12	0.24	0.10	0.15	0.02	0.52	0.21
Pichia	0.56	0.09	0.05	0.16	0.12	0.12	0.01	0.78	0.06	0.09	0.15	0.60	0.07	0.01	0.49		0.37	0.41	0.55	0.19	0.19	0.00	1.21	1.28
Pichia pijperi	10.90	10.23	12.75	15.36	10.21	14.55	6.79	8.42	7.67	9.57	9.05	18.01	13.62	18.35	17.13		11.32	10.91	14.24	8.38	10.37	6.29	29.42	24.14
Saccharomyces cerevisiae	12.95	28.62	28.30	30.22	26.08	37.67	30.29	19.11	7.73	21.50	31.97	15.98	7.67	21.57	33.96		0.28	19.95	27.12	3.35	0.37	10.28	11.92	17.96
Saccharomycopsis	0.72	0.35	0.32	0.15	0.13	0.15	0.07	0.13	1.12	0.30	0.32	0.03	0.27	0.10	0.32		0.82	0.49	1.21	0.22	0.52	0.13	0.32	0.34
Torulaspora delbrueckii	0.06	0.01	0.04	0.01	0.03	0.00	0.01	0.04	25.35	16.32	28.18	8.48	23.02	20.45	22.50		0.02	0.07	0.27	0.03	0.12	0.09	0.13	0.09

Only OTUs with an incidence above 1% in at least 2 samples are shown. Values are expressed as the mean from duplicate determinations. The abundance of OTUs in the 2 biological replicates of each sampling time were averaged. Samples are labeled according to fermentation period (0, 48, 96 and 120 h), fermentation method (Box and Heap), and condition (inoculated with S, ST and non-inoculated

3.3.6 Bacterial community of the fermented cocoa beans

The total number of paired sequences obtained from the fermented cocoa beans reached 4,159,213 raw reads. After merging, a total of 2,655,230 reads passed the filters applied through QIIME, with an average value of 63,220 ± 45,781 reads/sample and a mean sequence length of 445 bp. The rarefaction analysis and Good's coverage, expressed as a percentage (91%), also indicated satisfactory coverage of all the samples. Alpha diversity only indicated a higher level of complexity over the fermentation period ($P < 0.05$). No significant difference was observed when the different conditions (inoculated with S or ST and non-inoculated) were compared or between processes (B and H). The taxonomic classification of bacterial communities includes the family, genus, and species levels.

Overall, the most abundant OTUs detected at 48 h in both the inoculated and non-inoculated B fermentations were *Acetobacter pasteurianus*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* (Table 3.4). It should be noted that *A. pasteurianus* and *L. fermentum* remained the two most abundant OTUs at the end of the box fermentation under both conditions (inoculated or non-inoculated), and these were followed by *Bacillus* species. As far as the inoculated H fermentations are concerned, *A. pasteurianus*, *L. fermentum*, and *Acetobacteraceae* were the most abundant OTUs detected at 48 h, and *A. pasteurianus* and *L. fermentum* remained the dominant OTUs over the entire fermentation period (Table 3.4). Instead, the non-inoculated H fermentations were characterized by high relative abundances of *L. fermentum*, *A. pasteurianus*, and *L. plantarum* at 48 h, while *L. fermentum*, *Bacillus* spp., and *Klebsiella* spp. took over and dominated at the end of the process. Regarding bacterial dynamics, we observed an increased in the relative abundances under different conditions for *L. fermentum*, *L. plantarum*, *A. pasteurianus*, *Bacillus* spp., *Acetobacteraceae*, and *Lactobacillaceae* over the

fermentation period, while *Erwinia* spp., *Gluconobacter* spp., *Trabulsiella* spp., and *Enterobacteriaceae* decreased over time ($P < 0.01$), as shown in Table 3.4

Table 3.4 Classification at family/genus level of occurrence of the bacterial taxonomic groups achieved by means of amplicon sequencing expressed as relative abundances

	Relative abundance																							
	Box				Heap				Box				Heap				Box				Heap			
	0	48	96	120	0	48	96	120	0	48	96	120	0	48	96	120	0	48	96	120	0	48	96	120
Taxonomic group	***S. cerevisiae***								***S. cerevisiae + T. delbrueckii***								**Non-inocualted**							
Acetobacter pasteurianus	3.16	13.81	62.90	52.83	1.05	40.27	76.99	69.22	3.02	42.67	63.42	86.96	0.96	57.24	79.19	24.74	2.40	18.22	42.67	64.24	4.70	28.52	36.67	45.93
Acetobacteraceae	0.19	1.73	1.01	1.29	0.00	16.87	6.23	5.08	0.29	4.70	0.86	1.05	0.10	11.79	4.41	0.86	0.10	0.58	3.45	2.40	0.38	5.42	1.44	0.10
Acinetobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.00	0.00	0.05	0.00	0.00	0.00	0.00	4.99	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00
Acinetobacter guillouiae	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.86	0.10	0.00	0.10	0.00	0.00	0.00	0.00	1.25	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.14
Acinetobacter rhizosphaerae	0.00	0.00	0.00	0.00	0.00	0.00	0.19	1.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.25	0.00	0.00	0.00	0.00	0.05	0.00	0.19	0.24
Bacillus	0.00	0.00	0.00	16.35	0.00	0.00	0.00	0.77	0.00	0.00	11.94	0.29	0.00	0.00	0.00	3.36	0.00	0.10	0.00	4.41	0.05	0.00	0.96	8.96
Dyella	1.68	0.58	0.10	0.14	0.77	0.10	0.00	0.00	0.43	0.38	0.10	0.00	0.19	0.19	0.00	0.00	1.25	0.29	0.58	0.19	0.96	1.10	0.53	0.14
Enterobacteriaceaee	3.26	1.34	1.20	0.29	0.00	0.00	0.00	0.10	0.96	0.58	0.10	0.00	0.19	0.00	0.00	0.10	1.82	1.53	0.58	0.19	2.21	1.53	0.62	0.34
Erwinia	4.94	1.68	1.10	0.53	0.19	0.19	0.00	0.00	1.63	0.72	0.19	0.00	0.29	0.10	0.00	0.00	3.45	1.53	0.77	0.58	2.30	1.82	1.05	0.34
Gluconobacter	3.36	1.25	0.38	0.05	1.63	1.34	0.38	0.19	4.07	1.15	0.05	0.10	2.97	1.25	0.38	0.10	2.40	0.67	0.58	0.00	2.88	1.34	0.10	0.10
Klebsiella	0.34	0.43	0.19	0.24	0.00	0.00	0.19	0.19	1.10	0.19	0.10	0.00	0.00	0.00	0.10	0.48	1.25	1.15	0.48	0.10	0.38	0.34	0.34	3.93
Lactobacillaceae	0.05	2.06	3.88	1.20	0.00	0.38	0.00	0.10	0.05	1.68	0.96	0.00	0.00	0.10	0.19	0.10	0.10	5.37	2.21	1.25	0.19	0.62	1.10	0.34
Lactobacillus plantarum group	1.44	15.58	12.27	4.31	0.00	2.11	0.38	0.19	0.91	10.74	1.92	0.67	0.00	1.15	0.19	1.63	0.58	28.57	14.09	5.47	0.29	3.74	6.62	1.49
Lactobacillus fermentum	0.19	31.59	3.40	8.63	0.00	10.07	7.19	5.75	0.14	6.14	10.12	1.44	0.00	9.20	6.90	12.18	0.10	3.55	0.58	7.00	0.05	30.87	13.71	16.73
Lysinibacillus	0.00	0.00	0.00	0.58	0.00	0.00	0.00	0.67	0.00	0.00	1.63	0.00	0.00	0.00	0.00	0.96	0.00	0.00	0.00	0.67	0.00	0.00	0.19	0.05
Trabulsiella	5.13	3.12	2.49	1.53	0.38	0.29	0.00	0.00	2.64	0.81	0.53	0.19	0.58	0.10	0.00	0.10	5.75	1.73	1.53	0.86	2.97	4.12	0.96	0.38

Only OTUs with an incidence above 1% in at least 2 samples are shown. Values are expressed as the mean from duplicate determinations. The abundance of OTUs in the 2 biological replicates of each sampling time were averaged. Samples are labeled according to fermentation period (0, 48, 96 and 120 h), fermentation method (Box and Heap), and condition (inoculated with S, ST and non-inoculated).

3.3.7 OTUs co-occurrence and/or co-exclusion during cocoa bean fermentation

When the relative abundance of the bacterial and yeast populations was plotted, considering the OTUs of all the conditions (inoculated with S, inoculated with ST, and non-inoculated) of each fermentation method (B and H) together, it was possible to observe microbial co-occurrence or co-exclusion dynamics between the two different communities, as shown in Figure 3.3.

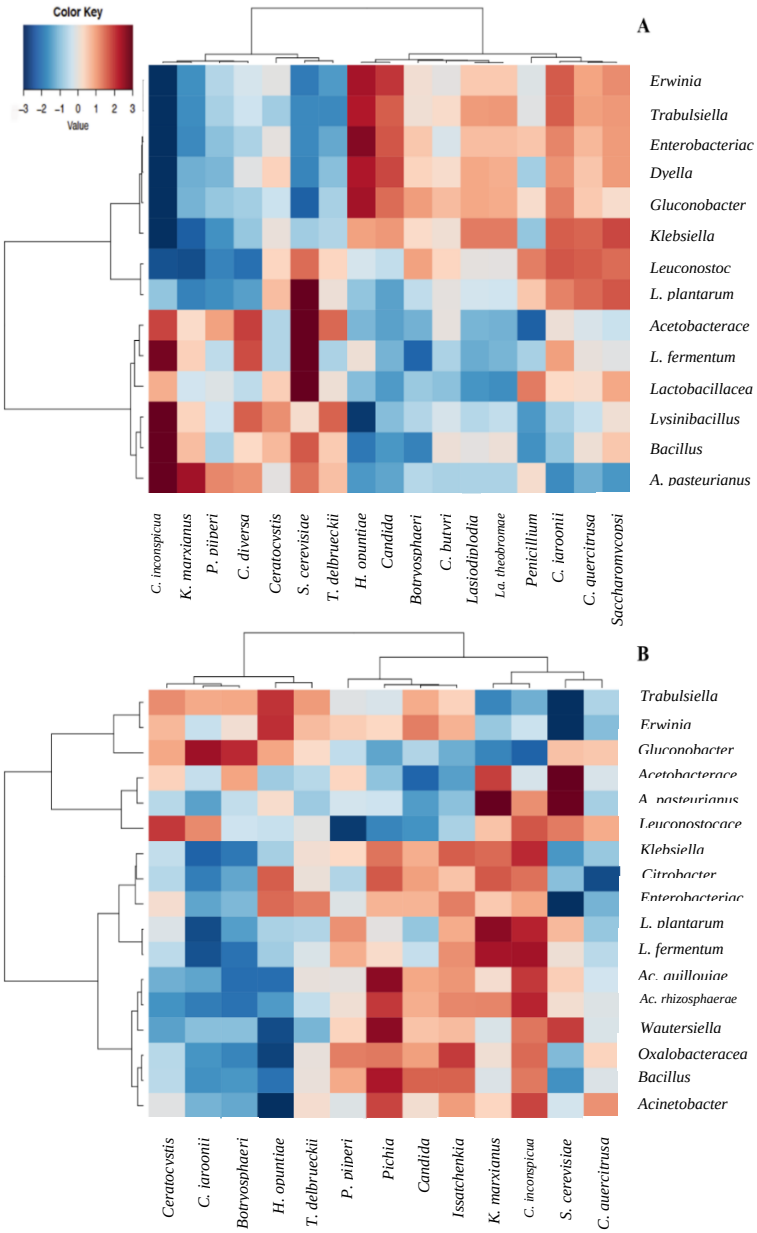

Figure 3.3 Spearman's correlation between microbial OTUs observed with an incidence above 1% in at least 2 samples. Figures are labeled according to fermentation method used, box (A) and heap (B). Rows and columns are clustered by Ward's linkage hierarchical clustering. The intensity of the colors represents the degree of correlation between fungal and bacterial OTUs, as measured by the Spearman's correlation, where blue represents a negative degree of correlation and red a positive degree of correlation. **Abbreviations:** L: *Lactobacillus*; AC: *Acinetobacter*; C: *Candida*; H: *Hanseniaspora*; P: *Pichia*; S: *Saccharomyces*; T: *Torulaspora*

Overall, *L. plantarum*, *A. pasteurianus*, and *Enterobacteriaceae* family were negatively associated with the main yeast OTUs (*S. cerevisiae*, *K. marxianus*, *Candida inconspicua*, and *P. pijperi*) in B fermentation, while, *S. cerevisiae* was positively correlated with *Acetobacteraceae* and *Lactobacillaceae*, whereas *A. pasteurianus* was positively correlated with *K. marxianus* and *C. inconspicua* and negatively correlated with *C. jaroonii* and *H. opuntiae* ($P < 0.05$). However, *H. opuntiae* was positively associated with the presence of the *Enterobacteriaceae* family, as well as with *Gluconobacter* spp. ($P < 0.05$). It is worth noting that *H. opuntiae* and *C. jaroonii* were found to be positively associated with the minor OTUs *Citrobacter* and *Erwinia* ($P < 0.05$, Figure 3.3A).

The *L. fermentum* in the H fermentation showed a positive correlation with *K. marxianus* and *C. inconspicua* and a negative correlation with *C. jaroonii* (Figure 3.3B, $P < 0.05$). *S. cerevisiae* was positively correlated with *Acetobacteraceae* and with *A. pasteurianus* (Figure 3.3B).

3.3.8 Correlation between sugar and organic acid compounds and microbiota populations detected by means of HPLC

Significantly different correlations were observed between the changes found in the concentration of sugars, organic acids, and microbes in the B and H fermentations, as shown in Figure 3.4 ($P < 0.05$). Overall, the most abundant microbial species in the fermented cocoa beans in the B fermentation, that is, *H. opuntiae*, *A. pasteurianus*, *K. marxianus*, *L. plantarum*, and *S. cerevisiae*, were statistically positively associated with intermediate metabolites, such as the citric, lactic, and succinic acids ($P < 0.05$), while *Bacillus* spp., *L. plantarum*, *A. pasteurianus*, and *L. fermentum* were statistically negatively correlated with the energy/carbon substrates ($P < 0.05$). In addition, sucrose was positively correlated with the presence of *H. opuntiae* ($P < 0.05$) and negatively correlated with *A. pasteurianus* and *Bacillus* spp. ($P < 0.05$). Citric acid was negatively

correlated with *Bacillus* spp. and *S. cerevisiae* but positively correlated with *H. opuntiae*, *Gluconobacter* spp., and *Erwinia* spp. ($P < 0.05$). *L. fermentum* was negatively correlated with fructose, glucose, gluconic acid, and pyruvic acid ($P < 0.05$). Finally, succinic acid was positively associated with *A. pasteurianus*, *C. inconspicua*, and *K. marxianus*, and lactic acid was positively related to *L. plantarum* and *S. cerevisiae*, as shown in Figure 3.4A ($P < 0.05$).

Few statistically significant correlations were found in the H fermentation (Figure 3.4B). In short, *A. pasteurianus* was found to be negatively associated with sucrose, while *Gluconobacter* spp. were positively related with sucrose ($P < 0.05$). In addition, *A. pasteurianus*, *K. marxianus*, *L. plantarum*, and *L. fermentum* were positively associated with succinic and lactic acid, as shown in Figure 3.4B ($P < 0.05$).

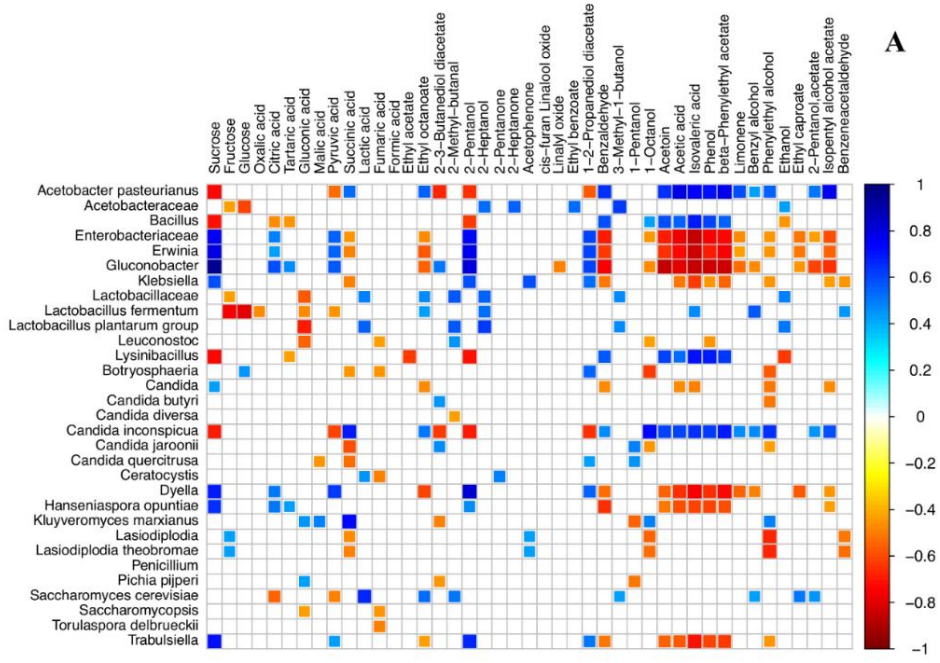

Figure 3.4 Correlation plot showing Spearman's correlation between the microbial OTUs and metabolites observed with an incidence above 1% in at least 2 samples. The samples are labeled according to the box (A) and heap (B) fermentation methods. Only significant associations between the OTUs and metabolites are shown ($P < 0.05$). The intensity of the colors represents the degree of correlation between the bacterial communities and metabolites, as measured by Spearman's correlation, where the blue color represents a positive degree of correlation and red a negative correlation between the sugars, organic acids, and OTUs

3.3.9 Correlation between the microbiota and volatilome profile

Significantly different associations were observed between the secondary metabolites and the main OTUs in the B and H fermentations, as shown in Figure 3.4 ($P < 0.05$). The main bacterial and fungal taxa in the B fermentations (Figure 3.4A), that is, *S. cerevisiae*, *H. opuntiae*, *L. plantarum*, *A. pasteurianus*, *K. marxianus*, *C. inconspicua*, and *L. fermentum*, were statistically correlated with the key aroma and fermentative markers, while the minor OTU bacteria (*Enterobacteriaceae* family, *Trabulsiella* spp., *Erwinia* spp., and *Gluconobacter* spp.) and *H. opuntiae* were statistically negatively correlated with the acids and phenols. In short, positive correlations were found between *S. cerevisiae* and

ethyl octanoate, 2-methylbutanal, and 3-methylbutanol, between *H. opuntiae* and 2-pentanol ($P < 0.05$), between *L. plantarum* and 2-heptanol, 2-methylbutanal, 3-methyl-1-butanol, and ethanol, and between *L. fermentum* and ethyl octanoate, 2-heptanol, benzyl alcohol, and isovaleric acid ($P < 0.05$). In addition, *A. pasteurianus*, *C. inconspicua*, and *Bacillus* spp. were also positively correlated with acetoin, acetic acid, isovaleric acid, phenol, limonene, benzyl alcohol, and phenylethyl alcohol ($P < 0.05$), while these compounds were positively correlated with *H. opuntiae* and the minor bacterial OTUs, as shown in Figure 3.4A ($P < 0.05$).

In general, in H fermentation some of the most abundant microbes (*A. pasteurianus*, *T. delbrueckii*, *S. cerevisiae*, and *K. marxianus*) and *Acetobacteraceae* family showed several significantly positive correlations with VOCs. *A. pasteurianus* was positively correlated with ethyl octanoate, 2-heptanol, 2-hepanone, *cis*-furan-linalool oxide, benzaldehyde, acetoin, β-phenylethylacetate, 3-methyl-1-butanol, limonene, 2-pentanol acetate, phenylethyl alcohol, ethanol, and isopentyl alcohol ($P < 0.05$, Figure 3.4B). *T. delbrueckii* was positively associated with 2-heptanone ($P < 0.05$). *S. cerevisiae* was positively correlated with 3-methyl-1-butanol and ethanol ($P < 0.05$). Finally, *Acetobacteraceae* was positively correlated with ethyl octanoate, 2-heptanol, 2-heptanone, *cis*-furan-linalool oxide, 3-methyl-1-butanol, acetoin, limonene, phenylethyl alcohol, ethanol, and isopentyl alcohol, while *K. marxianus* was positively correlated with benzaldehyde, acetoin, acetic acid, benzyl alcohol, and β-phenylethylacetate ($P < 0.05$, Figure 3.4B).

3.4 DISCUSSION

In this chapter, the changes that have taken place in the physicochemical composition, microbial counts, and microbiota diversity of cocoa beans in two different fermentation processes, that is, in boxes (B) and heaps (H), inoculated or non-inoculated with yeasts as a starter culture, have been investigated. The

ability of the survival and growth of selected starter strains, in this case, *S. cerevisiae* ID67 and *T. delbrueckii* ID103 during cocoa fermentation is one of the most important features to ensure their effect during this process. These starter strains have shown the ability to coexist with autochthonous microbial communities in fermented cocoa beans. However, the yeast cultures used in the present chapter did not significantly modify the microbiological dynamics, physicochemical parameters, or metabolites produced at the end of the fermentation, whereas the same starter strains influenced the fermentative process and the quality of the end products in at least one cocoa hybrid variety [8]. It is important to note that the initial yeast load in a previous study was lower than those observed in our study, and this might explain the discrepancies on the impact of the same yeast culture during cocoa fermentation [8]. The different fermentation practices, the cocoa variety, and the use of different starter cultures on-site during cocoa bean fermentation play important roles in the success of the starter culture used during fermentation and might also explain the discrepancies found between studies [4,5,7,8,20–24]. Our results confirmed that the performance of starter cultures on cocoa fermentation might change from geographic origin. Moreover, the effectiveness of the cultures depends on the complexity of the microbial consortia. This, in turn is influenced directly by the fermentation method used, each of which is characterized by its own microenvironment and is affected by oxygen availability, local agriculture practices, temperature, amount of cocoa mass used, etc.

During fermentation, cocoa beans constitute an ecological niche for a wide range of microbes. The advances made in studying the dynamics of cocoa microbial communities have shown that the composition of these communities follows predictable patterns that report a rapid decline in yeast counts after 48 h, when the sugars are depleted, a rise in temperature, and an increase in LAB and AAB [25–29]. The great impact on the microbial dynamics and succession during cocoa fermentation have been explained by considering the use of different cultivar varieties, fermentation methods, environmental conditions, and

harvesting and post-harvesting methods, as well as external factors, such as cross-contamination (equipment, operators, insect interactions, and microbial populations from previous fermentations) [24,26].

The use of molecular biology tools and the improvement of culturing techniques have facilitated the detection of new yeast, LAB, and AAB species. A restricted microbial population that includes *H. opuntiae, A. pasteurianus,* and *L. fermentum* has already been reported for fermented cocoa beans and has also been detected in our study [7,23,30]. However, some discrepancies can be observed among the most abundant microbial species in fermented cocoa beans, which may vary considerably from country to country. Through the application of amplicon-based sequencing in this chapter, we were able to detect unusual yeasts, such as *C. jaroonii*, *Lasiodiplodia theobromae*, and *Botryosphaeria*, during cocoa fermentation, none of which had previously been detected. Noteworthy, there is a lack of information available regarding the incidence of minor microbial groups [31]. In spite of the great advances made in microbial ecology, through next-generation sequencing, microbial species-level identification and strain-level differentiation still represent a challenge that needs to be addressed in the future to achieve an accurate identification.

This chapter, in an attempt to gain more knowledge about the range of potential interactions between microbial communities, describes a possible co-occurrence and co-exclusion. Our results showed a modulation of the LAB due to the presence of the yeast culture, in agreement with previous observations [32]. It should be noted that in this chapter shows that these associations depended on the type of fermentation process, and the correlation data set was used to explore the possible microbial dynamics, interactions, and metabolism. This information can offer information about the kinetics of substrate consumption and aroma production by the microbiota present in fermented cocoa beans. However, it is important to note that the correlations depend on the number of samples in which a type II error reflects the failure to reject a null hypothesis that is not true.

Therefore, further research is needed to assess the interactions between microbial communities and metabolites produce during cocoa fermentation.

The dynamics of the non-volatile compounds have shown successful competition for nutrients by the microbial populations within the cocoa fermentations. The ability of the fungal and bacterial communities to reduce sugars that were observed in this chapter has been studied in detail and is supported by previous studies [33,34]. As far as the organic acid dynamics are concerned, citric acid showed the highest concentration at the beginning of both fermentation and then decreased over time. This utilization of citrate has been attributed to bacteria, which metabolize it into acetic acid, carbon dioxide, and lactic acid [35]. However, not only bacteria can utilize citrate as an energy source: some isolates within *Candida krusei* have also been reported to assimilate citrate during cocoa fermentation [36]. However, *C. krusei* was not detected in our study during cocoa fermentation; while, the most abundant yeasts found in this study, *H. opuntiae* and *S. cerevisiae*, have never shown the capability to assimilate citrate *in vitro* [37]. Therefore, it is hypothesized that the observed citrate assimilation in our study was due to LAB, such as the highly abundant *L. fermentum*, as also supported elsewhere [38]. The high concentrations of succinic acid from 48 h to the end of the fermentation are likely related to the metabolic activity of the LAB, since these bacteria have shown the capability to produce succinic acid from citrate fermentation or convert fumaric and malic acids to succinic acid [39,40]. The reduction in pH in the pulp caused by LAB producing lactic acid favors the growth of AAB species, such as *A. pasteurianus*, which are capable of producing acetic and malic acids [39,41].

Biochemical reactions play key roles in the formation of VOCs in fermented cocoa beans [32,42]. Here, we observed that the dynamics of VOCs during fermentation changed in the concentration, as did their composition. According to Koné *et al.* [43], *P. kudriavzevii* and *S. cerevisiae* are the most important producers and contributors of cocoa aroma compounds, and these are followed by *Wickerhamomyces anomalus*, *Geotrichum* spp., and *Pichia*

galeiformis. One also notes that desirable cocoa aroma compounds, such as 2-heptanol, ethyl acetate, and 2-phenylethanol, were found in both fermentation processes, as previously identified by Ramos *et al.* [5]. The principal producers of alcohol, ester, and acid compounds have been linked to yeasts, such as *S. cerevisiae*, *Candida* spp., and other yeast species that have not been identified in this chapter on fermented cocoa beans [43–45]. Apart from the production of VOCs by fungi, AAB is known to oxidize alcohols, such as ethanol, isoamyl alcohol, and 2-phenylethanol, to produce acids and acetaldehydes [46,47].

In this chapter, we observed that the main bacterial group found increased the concentration of succinic, acetic, and lactic acids, acetoin, alcohols, esters, and acetaldehydes. Overall, the biochemical contribution to food ecosystems might change according to the complexity of the microbial consortia [48]. Therefore, further research is needed to understand the role of other compounds, such as free amino acids, oligopeptides, and polyphenols, in the development of microbes and aroma compounds [2,32].

3.5 ACKNOWLEDGMENTS

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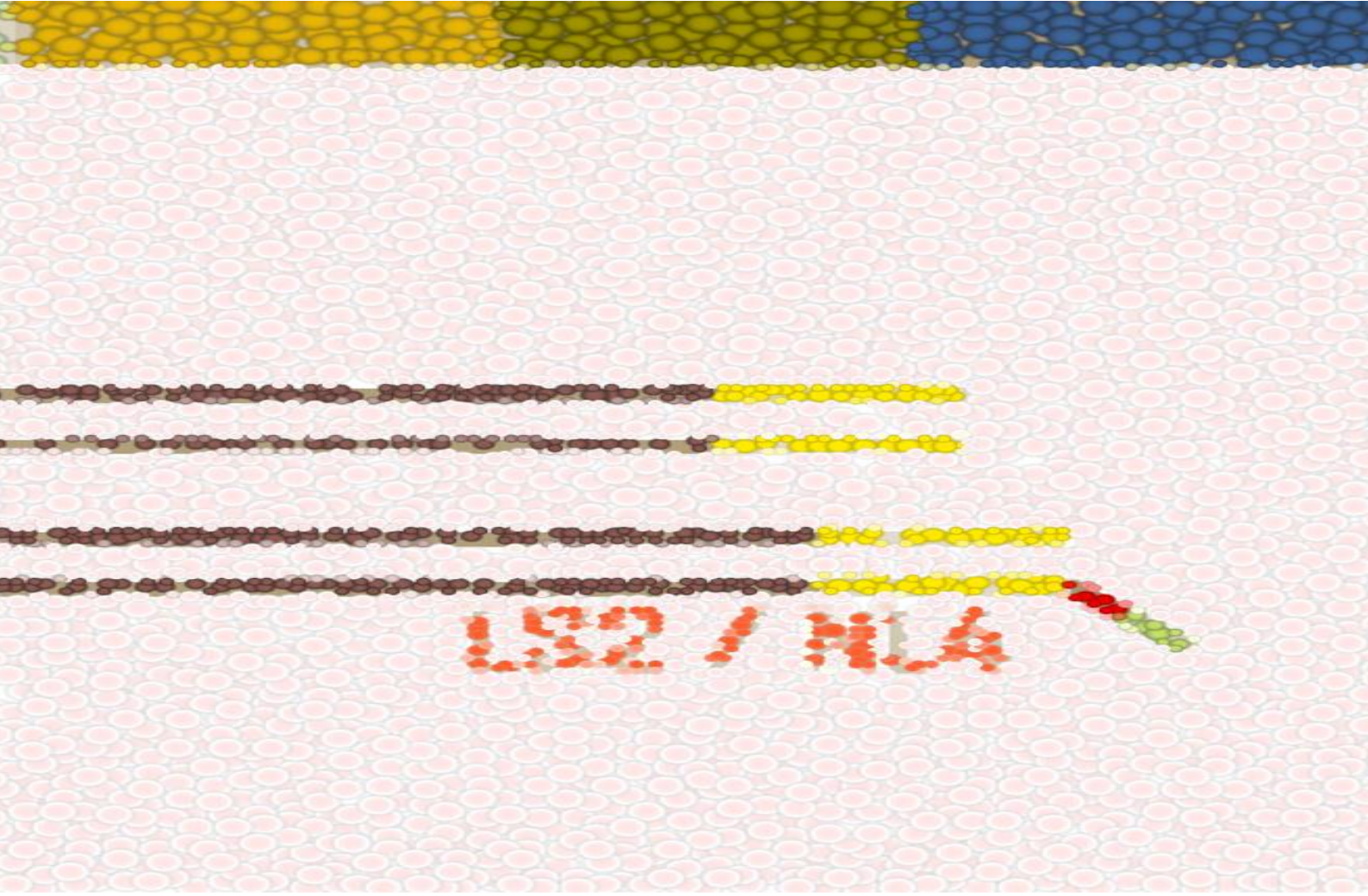
METATAXONOMIC COMPARISON



BETWEEN ITS2 AND 26S rRNA

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ABSTRACT

Next-generation sequencing has been used to strengthen our knowledge about taxonomic diversity and ecology of fungi within food ecosystems. However, primer amplification and identification bias could edge our understanding of the fungal ecology. The aim of this chapter is to compare the performance of two primer pairs over two nuclear ribosomal RNA regions of the fungal kingdom, namely the ITS2 and 26S regions. Fermented cocoa beans were employed as biological material, and the fungal ecology during fermentation was studied using amplicon-based sequencing tools, making use of a manually curated 26S database constructed, and validated with SILVA's database. To explore potential biases introduced by PCR amplification of fungal communities, a mock community of known composition was prepared and tested. The relative abundances observed for ITS2 suggest that species with longer amplification fragments are underestimated and concurrently species that render shorter amplification fragments are overestimated. However, this correlation between amplicon length and relative abundance is not valid for all the species analyzed. Variability in the amplificon lengths contributed to the preferential amplification phenomenon. DNA extracted from twenty fermented cocoa bean samples were used to assess the performance of the two target regions. Overall, the metataxonomic data set recovered similar taxonomic composition and provided consistent results in OTUs richness among biological samples. However, 26S region provided a higher alpha diversity index and greater fungal rRNA taxonomic depth and robustness results compared with ITS2. Based on the results of this chapter we suggest the use of the 26S region for targeting fungi. Furthermore, this chapter showed the efficacy of the manually curated reference database optimized for annotation of mycobiota by using the 26S as a targeting gene.

4.1 INTRODUCTION

Fungi are eukaryotic microorganisms that belong to one of the most diverse kingdoms on Earth [1]. They play an important role in the safety, quality, and stability of all foodstuff to some degree, whether they are required during processing or whether they have a negative impact during shelf life. Therefore, tracking fungal communities on food systems has been a concern in food research. To date, most recent studies on the microbial diversity of fermented food such as vegetables, seafood, beverages, cheese, olives, and spontaneously fermented American cool ship ale fermentations have employed amplicon sequencing approaches [2–6].

Illumina sequencing platform has been currently providing a sensitive description of the microbial dynamics within food ecosystems. Some of the advantages of using this technology are that it yields greater sequencing coverage and increased sample throughput at a lower cost *per sample* compared to other platforms [7,8]. The sensitivity of this approach relies on the high coverage and accurate taxonomic resolution of short amplicon length [8]. Recent advances in the microbial diversity using next-generation sequencing technologies (NGS) have underlined the importance of the reliability of PCR primers targeting a specific genetic marker [9]. In spite of the importance of the amplification of shorter fragments amplified by PCR in NGS, recent studies described a more reliable community of fungi using shorter Internal Transcribed Spacer amplicons (ITS) of the nuclear ribosomal RNA (rRNA) [9,10].

The ITS region is considered the universal barcode for identification of fungi. This spacer is composed of the ITS1 and ITS2 regions, separated by the 5.8S gene. These two regions (ITS1-2) are characterized by high evolutionary rates and are edged by highly conserved regions with suitable target sites for universal primers [11]. However, the complete ITS region located between the 18S and 28S genes in the nuclear ribosomal RNA is considered too long for 454

sequencing or other NGS [12]. Therefore, various primers are used to amplify parts of the ITS region. In this chapter, we selected the primer ITS3ngs that targets a site in the ITS2 and the degenerate reverse primer ITS4 which targets the ITS-flanking site in the ribosomal large subunit (LSU) encoding regions [13] based on their ability to amplify fungal species through *in silico* analysis [12,14]. Besides the use of ITS as a target region for fungi identification, the nuclear rRNA large subunit (LSU/28S/26S) and small subunit (SSU/18S) genes have also been often used to address fungal diversity [15–21]. To bring an overall perspective, most yeasts have been identified from sequence divergence in the D1/D2 domain of LSU rRNA [22]. Despite the great resolution to recognized yeast species through 26S rRNA sequencing reactions, little is known about the potential uses and bias that can be introduced when using this target region in NGS. In this context, it is necessary for ecological studies to compare different targeting regions to describe the most accurate and reliable ecological populations in a food system. Given the nature of current challenges, the selection of a suitable genetic marker for the identification of fungi will help researchers to clear current issues insight into the selection of primer sets.

The main focus of this chapter is to address sequencing target regions and primer biases on one of the dominating taxonomic groups of fungi in the Dikarya, Ascomycota, which represents 53% of the described species of true Fungi [23]. This phylum is important in the food industry and serves as a source for biomass production, but also includes known human and plant pathogens [24,25]. The present research focused on the assessment of two different targeting sites for amplicon-based Illumina NGS studies. We tested the 26S primer set, delivering high coverage and accurate taxonomic assignment of short (~ 400 bp) fungal amplicon *versus* the performance with the ITS2 region. This research intends to bring new insights into the field of taxonomic assignment, validation, and resolution of uncertainties on using amplicon-sequencing approaches for fungi identification by using mock samples as well as fermented samples. Attention

was paid for monitoring fungi in mock communities and biological samples, where taxonomic assurance of the technique, and mapping and monitoring fungi dynamics are investigated for food applications.

4.2 MATERIALS AND METHODS

4.2.1 Primer selection and in silico analysis

Primer pairs targeting the ITS2 region [14], and the D1 domain of 26S rRNA gene [26], were selected and reported in Table 4.1. For the amplification of the D1 domain of the 26S, we modified the LS2-MF primer sequence position from reverse to forward, corresponding to nucleotide position 266 of *Saccharomyces cerevisiae* 26S gene as described by Cocolin *et al.* (2000) and a reverse primer NL4 [27]. The Illumina overhang adapter sequences were added to locus-specific sequences. The D1 region from the 26S gene was amplified *in silico* to compare primer specificity and taxonomic coverage of both LS2-MF and NL4 by using Primer Prospector [28] against the constructed 26S databases and SILVA's database.

Table 4.1 Primers used for Illumina MiSeq sequencing

Primer	Feature	Primer sequence	TR	AL	Reference
ITS3tagmix1	Fwd	5'-CTAGACTCGTCACCGATGAAGAACGCAG-3'	ITS2	385	Tedersoo *et al.* 2015
ITS4ngs	Rev	5'- TTCCTSCGCTTATTGATATGC-3'			Tedersoo *et al.* 2015
LS2-MF	Fwd	5'-GAGTCGAGTTGTTTGGGAAT-3'	LSU D1	369	This study
NL-4	Rev	5'-GGTCCGTGTTTCAAGACGG-3'			Jespersen *et al.* 2005

Abbreviations: TR, Target region; AL, Amplicon length

4.2.2 Mock community preparation, DNA extraction, and PCR amplification

Strains of yeast and filamentous fungi listed in Table 4.2 (DISAFA collection, Torino) were used and cultured on Malt Extract Agar (Oxoid, Milan, Italy) plus 25 mg l^{-1} streptomycin (Sigma, Milan, Italy) incubated at 28°C for 72 h for yeast and 7 to 10 days for fungi. DNA extraction from yeasts was carried out from a loopful of grown culture while 250 mg of mycelium was scraped from the plate for filamentous fungi. DNA extraction was carried out as described by Cocolin *et al.* (2000). The DNA from each strain was quantified by using the Qubit dsDNA assay kit (Thermo Fisher Scientific, Milan, Italy) and standardized at 5 ng/µl. A pool (Mock-DNA) containing each of the standardized strain DNA was then obtained and subject to amplification of the ITS2 and the 26S regions. PCR was carried out for the two target regions using a PCR mixture prepared with 12.5 µl of the 2X Kapa HiFi HotStart ReadyMix Taq (Roche, Milan, Italy), 1 µM each primer, 2.5 µl of DNA template, and PCR-grade water. Each PCR were subject to the following amplification conditions: thirty cycles of 30 s of denaturation (95 °C), 30 s of primer annealing (55 °C), and 30 s of primer elongation (72 °C), followed by a final elongation step (72 °C) of 10 min.

The amplification of each fungal strain was carried out by using the same couple of primers, each amplicon was then purified using the Agencourt AMPure XP beads (Beckman Coulter Genomics) and quantified using Qubit dsDNA assay kit. Based on the amplicon size of DNA assessed by using a Biorad experion workstation (Biorad, Milan, Italy), amplicons concentration was determined. Amplicons were diluted at 20 mM and aliquots of 10 µl were pooled together to construct a Mock-Amp. In total, two independent Mock-DNA and Mock-Amp were obtained by two independent DNA extraction, quantification and pooling procedure.

Table 4.2 Fungal strains used for sequencing analysis and respective amplicon length

Fungal species	Size bp	
	26S	ITS2
Alternaria alternata	454	414
Aspergillus flavus	455	430
Aspergillus fumigatus	459	439
Candida sake	467	393
Fusarium oxysporum	462	405
Fusarium verticillioides	458	415
Galactomyces geotrichum	502	324
Hanseniaspora opuntiae	435	484
Hanseniaspora osmophila	462	520
Kluyveromyces marxianus	427	521
Penicillium brevicompactum	456	428
Penicillium glabrum	458	426
Pichia kudriavzevii	472	431
Pichia membranifaciens	466	398
Plectosphaerella cucumerina	457	441
Saccharomyces cerevisiae	460	496
Saccharomycodes ludwigii	452	470
Schizosaccharomyces pombe	488	562
Starmerella bacillaris	389	361
Torulaspora delbrueckii	461	518

4.2.3 DNA extraction and PCR amplification of fermented cocoa beans

A total of twenty fermented cocoa beans samples were collected and DNA extracted as following the original study [29]. Samples were collected during a fermentation period of 0, 48, 96 and 120 h. Detailed information of samples is reported in Mota-Gutierrez *et al*. (2018). Briefly, total DNA was extracted from the pellet of the cocoa matrix by using the MasterPure Complete DNA and RNA Purification kit (Illumina Inc, San Diego, CA) following the

manufacturer's instructions. DNA was quantified by using the Qubit dsDNA assay kit (Thermo Fisher Scientific), standardized at 5 ng/µl and subject to amplification of the two target regions using primers and procedure as described above.

4.2.4 Library preparation and sequencing

Sequencing was performed for the two target regions and the three target samples (Mock-DNA, Mock-Amp, and fermented cocoa samples). After the first purification step following the Illumina sample preparation procedure, the library was combined with the sequencing adapters and dual indices using the Nextera XT Index Kit (Illumina, San Diego, USA), obtaining the multiplexed paired-end libraries. Individual libraries concentration in nM was calculated based on the size of amplicons by using a Biorad Experion workstation (Biorad) and diluted to 4 nM, denaturated with 0.2 N NaOH and spiked with 20% (v/v) of PhiX. The combination of pool library and PhiX were diluted to 12 pM and paired-end sequencing was performed on the MiSeq platform, using MiSeq Reagent Kit V3 (2 x 250bp) (Illumina, San Diego, USA), following the standard Illumina sequencing protocol.

4.2.5 Constructed 26S rRNA sequence database

The construction database of the fungal rRNA gene sequence of the 26S gene was used to select primers, which amplify the D1 region of broad fungal taxa. The sequences were downloaded from the nucleotide database of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/nucleotide/; accessed March 07, 2018). The database was constructed using the large subunit rRNA gene sequences, 23 381 sequences were downloaded using the query word "26S rRNA". The final constructed database consisted of 4 phyla, 27 classes, 172 families, and 442 fungal strains. Incomplete sequences or sequences with absent taxonomies were removed. Duplicate sequences and sequences that clustered together at 99% of

similarity were discarded by using Prinseq and USEARCH, respectively [30]. A taxonomy file, matching exactly seven taxonomic levels (root, subphylum, class, order, family, genus and species) was generated from the corresponding taxonomy strings to be compatible with the implementation in the NGS analysis pipeline QIIME. Both files were manually curated for accuracy and consistency. Sequences obtained from the constructed 26S database from biological and mock samples were compared using the SILVA database. All sequences identified by the D1 domain of 26S rRNA sequence analysis from biological samples and mock communities were compared with the constructed 26S database**.**

4.2.6 Bioinformatics

Paired-end reads (2x250 bp) were first merged using the FLASH software [31], with default parameters. Joint reads were further quality filtered (Phred < Q20) using the QIIME 1.9.0 software [7]. Chimeras were then removed with the adopted USEARCH version 8.1 software. Lastly, OTUs were picked at 99% of similarity by means of UCLUST clustering methods [32] and representative sequences of each cluster were used to assign taxonomy. For the 26S data, each cluster was used to assign taxonomy using the Constructed 26S rRNA gene database and SILVA, while for the ITS dataset the UNITE rRNA ITS database version 2012, by means of the RDP Classifier was used. Sequences were double-checked using the BlastN search tool (http://www.ncbi.nlm.nih.gov/blast/) to confirm the taxonomy assignment. The cocoa samples datasets (ITS and 26S) were rarefied at 10,018 reads after raw read quality filtering and clustering, OTUs tables were obtained and filtered for OTUs occurring at 1% of the relative abundance in at least 2 samples. While for mock community reads from the two target regions were rarefied at 17,313 reads.

4.2.7 Statistical analyses

Statistics and plotting were carried out in the R environment (www.r-project.org). Alpha diversity indices were calculated using the diversity function of the vegan package [33]. OTUs table was used to find differences between target regions by Anosim statistical test in R environment. A two-sided permutation test with 999 permutations was performed to compare the OTUs distribution and alpha diversity between the two datasets. Pairwise Kruskal-Wallis Wilcoxon test or one way- ANOVA coupled with the Duncan honestly significant difference (HSD) test were used as appropriate to determine significant differences in alpha diversity or OTUs abundance from mock communities and biological samples. Statistical analysis was acquired through the function *aov* through the *stats* package and principal component analysis was plotted using the function *dudi.pca* through the *made4* package using R version 3.3.2

4.2.8 Accession numbers

The ITS and 26S rRNA gene sequences are available at the Sequence Read Archive of the National Center for Biotechnology Information (NCBI), under the SRA accession number SRP126081 (fermented cocoa samples ITS) and SRP150401 (fermented cocoa samples 26S and mock sequences data).

4.3 RESULTS

4.3.1 In silico performance of 26S primers

We performed an *in silico* analysis of the 26S primer set against our constructed database and SILVA using Primer Prospector. LS2-MF primer showed the lowest weighted score indicating higher coverage across both database sequences and a lower number of mismatches if compared with NL4

(data not shown). Comparing the taxonomic coverage of LS2-MF and NL4 against *Zygomycota*, *Glomeromycota*, *Ascomycota* and *Basidiomycota* sequence, LS2-MF showed the best performance with coverage higher than 80% for all the phyla except for *Glomeromycota*, while NL4 account for the 20% of the coverage against our constructed database (Figure 4.1). Regarding the performance of the primer sets against the SILVA's database, the score of the primers was higher compared with our database (data not shown).

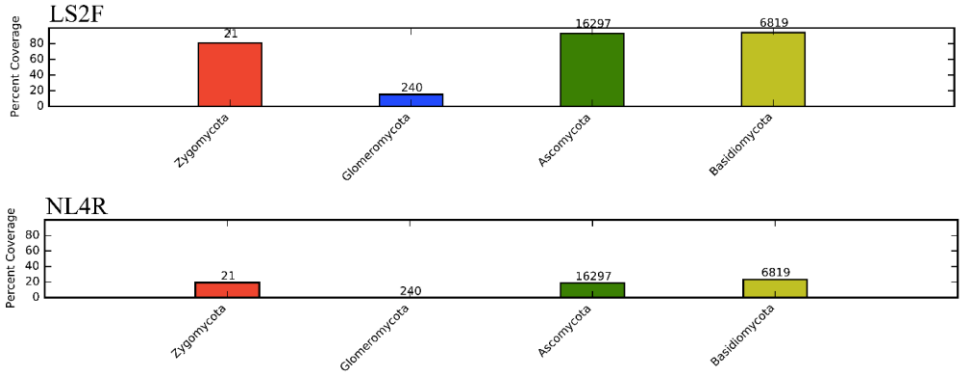

Figure 4.1 Taxonomic coverage of 26S primer sets

4.3.2 Performance of primers by mock community analysis

A mock community containing twenty fungal species (Table 4.2) was prepared to validate the performance of the two target regions. The possible effect of the bias introduced by PCR (Mock-DNA) and that of sequencing (Mock-Amp) was then evaluated. Amplicon length of the single species showed little variation when the 26S gene was amplified (461 ± 30 bp) while for ITS2 we observed greater dispersion in size (445 ± 55 bp, Table 4.2). A significant difference in mycobiota composition (Anosim statistical test, $P < 0.05$) by using the two target

regions or mock communities (DNA or Amp) was observed by Principal Component Analysis (Figure 4.2).

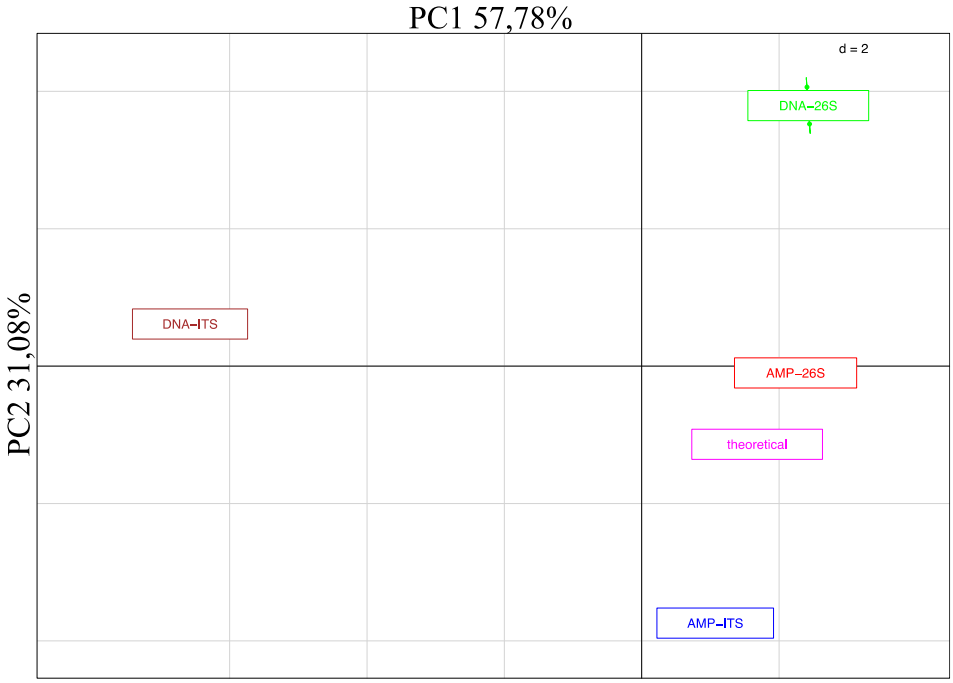

Figure 4.2 Principal component analysis (PCA) based on mock mycobiota composition

In both samples (Mock-DNA and Mock-Amp), the target region ITS2 showed similar abundances with respect to the theoretical value for two fungal species, namely *Torulaspora delbrueckii* and *Plectosphaerella cucumerina* (Table 4.3). Similarly, with the 26S target gene, for six species, abundances retrieved from both Mock-DNA and Mock-Amp samples were comparable to the theoretical values (*Aspergillus fumigatus, Pichia membranifaciens, Pichia kudriavzevii, Penicillium glabrum, Penicillium brevicompactum,* and *Starmerella bacillaris*). Furthermore, for the 26S region, the species *Alternaria alternata, Aspergillus flavus*, and *Fusarium oxysporum* rendered different abundances in the Mock-DNA and the Mock-Amp, but in both samples, the values were comparable to the theoretical.

For 18 out of the 20 fungal species tested, the ITS2 region resulted in underestimation or overestimation with respect to the theoretical value in the Mock-DNA, Mock-Amp or both ($P < 0.05$). Four species were significantly overestimated [*A. fumigatus* (439bp), *Fusarium verticillioides* (415bp), *Kluyveromyces marxianus* (521bp) and *P. brevicompactum* (428bp)] while other 4 were significantly underestimated in both samples [*Galactomyces geotrichum* (324bp), *Hanseniaspora opuntiae* (484bp), *Schizosaccharomyces pombe* (562bp) and *Starmerella bacillaris* (361bp)]. Interestingly, *G. geotrichum* and *S. pombe* were not detected in any of the two samples. Nine more species resulted to be significantly different from the theoretical value (either higher or lower) in the Mock-DNA or Mock-Amp sample only (Table 4.3).

Table 4.3 Relative abundance (%) of the fungal species identified in mock communities amplified using two different target regions. The expected concentration is referred to as theoretical

OTU	Theoretical	26S		Theoretical	ITS2	
		DNA	**AMP**		**DNA**	**AMP**
Alternaria alternata	5 ^{ab}	3.62 ± 0.34 ^{b}	5.71 ± 0.47 ^{a}	5 ^{a}	7.67 ± 0.94 ^{b}	6.63 ± 0.09 ^{ab}
Aspergillus flavus	5 ^{ab}	5.78 ± 0.46 ^{a}	4.03 ± 0.07 ^{b}	5 ^{a}	9.39 ± 0.17 ^{b}	7.19 ± 0.26 ^{a}
Aspergillus fumigatus	5 ^{a}	4.82 ± 1.34 ^{a}	3.71 ± 0.09 ^{a}	5 ^{a}	8.41 ± 0.20 ^{b}	6.14 ± 0.01 ^{c}
Candida sake	5 ^{a}	0.24 ± 0.20 ^{b}	0.06 ± 0.02 ^{b}	5 ^{a}	4.57 ± 0.38 ^{a}	6.19 ± 0.03 ^{b}
Fusarium oxysporum	5 ^{ab}	3.99 ± 0.25 ^{b}	5.08 ± 0.22 ^{a}	5 ^{a}	8.11 ± 0.00 ^{b}	6.33 ± 0.04 ^{a}
Fusarium verticillioides	5 ^{a}	6.27 ± 0.04 ^{c}	4.63 ± 0.02 ^{b}	5 ^{a}	10.44 ± 0.12 ^{c}	6.60 ± 0.27 ^{b}
Galactomyces geotrichum	5 ^{a}	13.39 ± 0.61 ^{c}	6.94 ± 0.11 ^{b}	5 ^{b}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Hanseniaspora opuntiae	5 ^{b}	2.94 ± 0.16 ^{a}	3.46 ± 0.33 ^{a}	5 ^{c}	0.70 ± 0.07 ^{a}	3.67 ± 0.01 ^{b}
Hanseniaspora osmophila	5 ^{a}	10.63 ± 0.56 ^{b}	4.73 ± 0.13 ^{a}	5 ^{b}	1.63 ± 0.34 ^{a}	4.78 ± 0.08 ^{b}
Kluyveromyces marxianus	5 ^{b}	3.68 ± 0.08 ^{a}	3.33 ± 0.16 ^{a}	5 ^{a}	8.55 ± 0.33 ^{b}	6.71 ± 0.08 ^{b}
Penicillium brevicompactum	5 ^{a}	6.00 ± 0.92 ^{a}	3.55 ± 0.29 ^{a}	5 ^{a}	14.39 ± 0.09 ^{c}	9.04 ± 0.08 ^{b}
Penicillium glabrum	5 ^{a}	4.86 ± 0.19 ^{a}	4.88 ± 0.22 ^{a}	5 ^{b}	7.08 ± 0.11 ^{a}	5.69 ± 0.07 ^{b}
Pichia kudriavzevii	5 ^{a}	7.24 ± 1.89 ^{a}	10.76 ± 0.45 ^{a}	5 ^{b}	0.12 ± 0.14 ^{a}	2.11 ± 0.16 ^{b}
Pichia membranifaciens	5 ^{a}	6.63 ± 1.83 ^{a}	4.82 ± 0.28 ^{a}	5 ^{b}	2.58 ± 0.39 ^{a}	3.57 ± 0.13 ^{b}
Plectosphaerella cucumerina	5 ^{b}	1.11 ± 0.13 ^{a}	4.33 ± 0.02 ^{b}	5 ^{b}	3.51 ± 0.62 ^{a}	4.45 ± 0.26 ^{b}
Saccharomyces cerevisiae	5 ^{b}	1.86 ± 0.09 ^{a}	4.20 ± 0.49 ^{b}	5 ^{a}	4.74 ± 0.03 ^{a}	5.44 ± 0.10 ^{b}
Saccharomycodes ludwigii	5 ^{b}	3.51 ± 0.00 ^{a}	4.49 ± 0.00 ^{b}	5 ^{b}	0.68 ± 0.03 ^{a}	4.66 ± 0.03 ^{b}
Schizosaccharomyces pombe	5 ^{b}	5.00 ± 0.11 ^{b}	3.71 ± 0.19 ^{a}	5 ^{b}	0.01 ± 0.00 ^{a}	0.00 ± 0.00 ^{c}
Starmerella bacillaris	5 ^{a}	4.21 ± 1.68 ^{a}	5.91 ± 0.11 ^{a}	5 ^{b}	0.16 ± 0.07 ^{a}	0.19 ± 0.10 ^{b}
Torulaspora delbrueckii	5^{c}	2.46 ± 0.02 ^{a}	3.90 ± 0.17 ^{b}	5 ^{a}	5.38 ± 0.11 ^{a}	5.30 ± 0.52 ^{a}

Values are expressed as the mean from duplicate determinations (%). Different letters indicate statistical difference related to relative abundances of mock communities using least significant difference test ($P < 0.05$). *P*-values were adjusted using Bonferroni's method. Different color showed no difference (grey), underestimation (light green) or overestimation (light blue) between mock samples and theoretical data

When the 26S region was targeted, 11 out of the 20 species were either underestimated or overestimated in the Mock-DNA, Mock-Amp or both. More specifically, 4 species were underestimated in both types of samples [*Candida sake* (467bp), *H. opuntiae* (435 bp), *K. marxianus* (427 bp), *T. delbrueckii* (461 bp)] and only one was overestimated [*G. geotrichum* (502bp)]. Five more species were significantly different from the theoretical value (either higher or lower) in the Mock-DNA or Mock-Amp sample only (Table 4.2 and 4.3). One also notes that we did not observe a clear correlation between amplicon size and over or underestimation.

In the Mock-Amp samples, correct relative quantification was obtained for 13 out of the 20 species targeting the 26S region and for 10 out of 20 species with the ITS2 region. In the Mock-DNA samples, correct relative quantification was obtained for 10 out of 20 species in the 26S region and 4 out of 20 species targeting the ITS2 (Table 4.3).

Remarkably, *G. geotrichum* and *S. pombe* were only detected when the 26S region was targeted while *H. opuntiae* was the only species that was consistently underestimated, independently from the target region or sample. Overall, 26S sequencing data aligned better to theoretical abundance values for the fungal species tested than did the ITS sequencing data.

4.3.3 Mycobiota in biological samples

The sequencing of twenty fermented cocoa beans samples collected during a previous experiment, after amplification with the primers ITS2 and 26S showed a mean sequence length of 412 and 390 bp, respectively and an estimated sample coverage of 97.73 and 95.87%, respectively. The 26S target region revealed greater OTUs richness compared to the ITS2 region as shown in Figure 4.3 ($P < 0.05$).

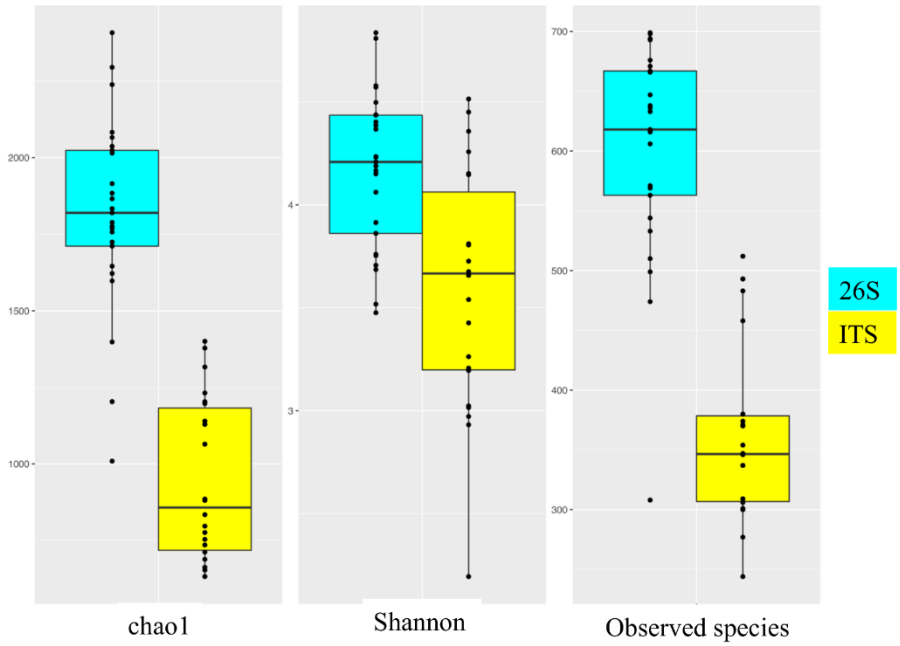

Figure 4.3 Boxplots describe α-diversity measures (Chao1, Shannon index and number of observed species) of fermented cocoa bean samples. Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively

Overall, 20 and 37 fungal OTUs were identified during the fermentation using the primer set ITS2 and 26S, respectively. In addition, we observed differences in length distribution across the two target genes. Histogram of reads length of 26S showed that the higher reads proportion were around 380 bp while for ITS2 we observed a varied distribution of the length of the reads around 370 bp, 400 bp, 420 bp, and 450 bp. Eleven OTUs, namely *Candida jaroonii*, *Candida tallmaniae*, *Fusarium* spp., *Hanseniaspora* spp., *H. opuntiae*, *Hanseniaspora uvarum*, *K. marxianus*, *S. cerevisiae*, *Saccharomycopsis crataegens*, *T. delbrueckii*, and *Pichia pijperi* were detected by both targeting regions (Figure 4.4).

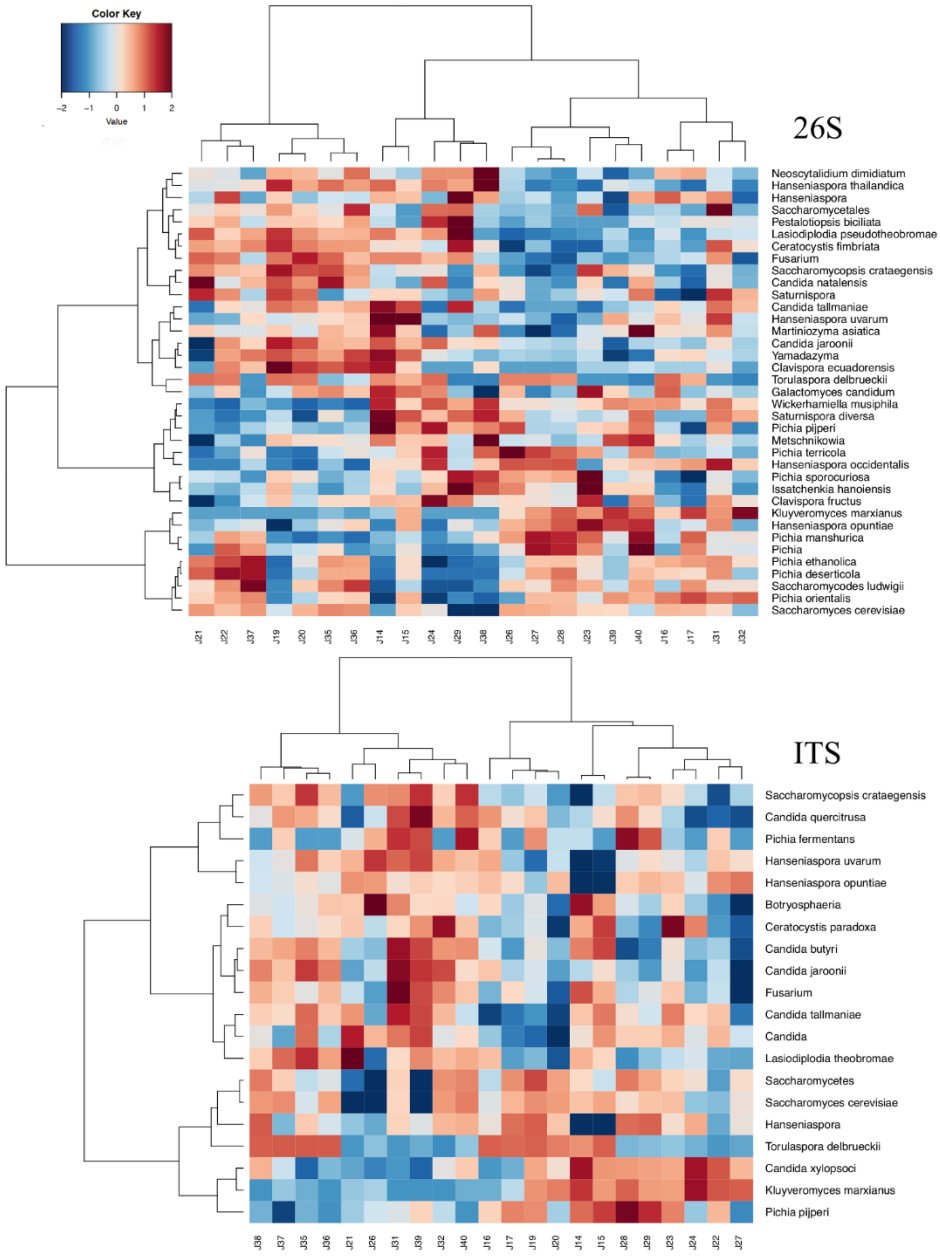

Figure 4.4 Distribution of OTUs in fermented cocoa bean samples in the amplicon datasets divided into 26S (upper figure) and ITS (lower figure). Only OTUs with an incidence above 1% in at least 2 samples are shown

The relative abundance of several fungal species was significantly different according to the type of amplicon used ($P < 0.05$, Figure 4.5), in which significantly higher relative abundance was found for *S. cerevisiae*, *P. pijperi*, and *H. uvarum* using ITS2 target gene, while *Hanseniaspora* spp. showed higher abundance when using the 26S ($P < 0.05$, Figure 4.5).

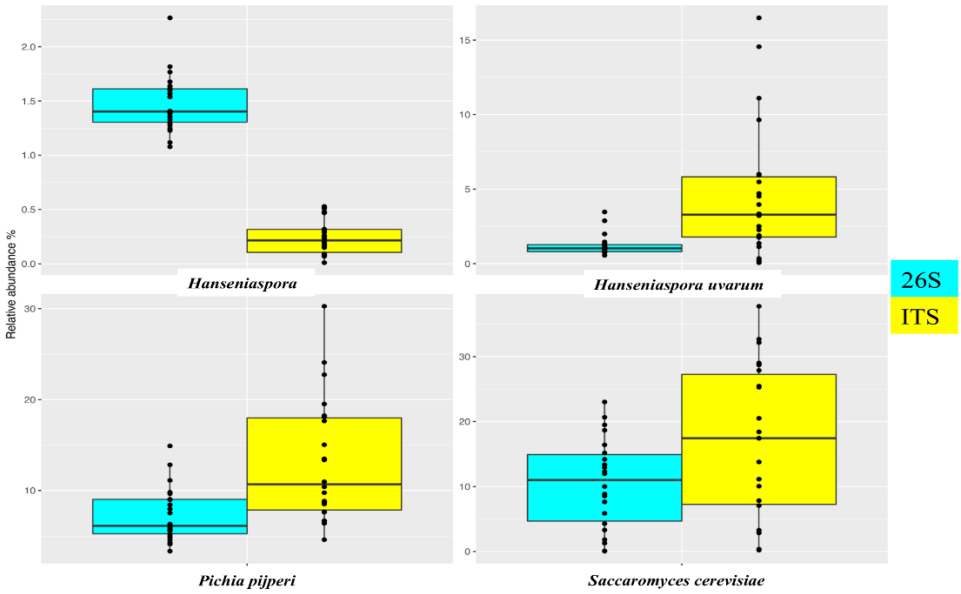

Figure 4.5 Boxplots describe statistically different species detected in fermented cocoa bean samples analyzed with two different target genes. Individual points and brackets represent the relative abundance and the theoretical standard error range, respectively

4.3.4 Performance of the newly constructed 26S database against SILVA

To validate the new 26S database, biological samples and mock communities identified by the D1 domain of 26S rRNA sequence analysis were compared with SILVA's database [34]. A significant difference in the mycobiota composition of the two databases was observed in mock communities (Anosim

statistical test, $P < 0.05$). In detail, the constructed 26S database assigned successfully the twenty fungal species from the mock DNA, while SILVA's database assigned only nine (*A. alternata, Hanseniaspora osmophila, K. marxianus, P. kudriavzevii, P. membranifaciens, Saccharomycodes ludwigii, S. pombe, S. bacillaris*, *T. delbrueckii*, Table 4.4).

Table 4.4 Relative abundance of mock communities identified by two different fungal databases

	Constructed	**SILVA**	**Constructed**	**SILVA**
Fungal species	DNA		AMP	
Torulaspora delbrueckii	2,46 ± 0,02	2,28 ± 0,16	3,90 ± 0,17	3,85 ± 0,01
Starmerella bacillaris	4,21 ± 1,68	3,44 ± 0,94	5,91 ± 0,11	5,93 ± 0,01
Schizosaccharomyces pombe	5,00 ± 0,11	4,85 ± 0,16	3,71 ± 0,19	3,57 ± 0,12
Saccharomycodes ludwigii	3,51 ± 0,00	2,99 ± 0,30	4,49 ± 0,00	4,15 ± 0,11
Saccharomyces cerevisiae	1,86 ± 0,09	0,00 ± 0,00	4,20 ± 0,49	0,00 ± 0,00
Rizoctonia solani	0,00 ± 0,00	0,00 ± 0,00	4,21 ± 0,23	0,00 ± 0,00
Plectosphaerella cucumerina	1,11 ± 0,13	0,00 ± 0,00	4,33 ± 0,02	0,00 ± 0,00
Pichia membranifaciens	6,63 ± 1,83	6,71 ± 1,89	4,82 ± 0,28	4,83 ± 0,30
Pichia kudriavzevii	7,24 ± 1,89	3,62 ± 1,69	10,76 ± 0,45	6,20 ± 0,55
Penicillium glabrum	4,86 ± 0,19	0,00 ± 0,00	4,88 ± 0,22	0,00 ± 0,00
Penicillium brevicompactum	6,00 ± 0,92	0,00 ± 0,00	3,55 ± 0,29	0,00 ± 0,00
Kluyveromyces marxianus	3,68 ± 0,08	3,23 ± 0,22	3,33 ± 0,16	3,32 ± 0,04
Hanseniaspora osmophila	10,63 ± 0,56	10,50 ± 0,47	4,73 ± 0,13	4,70 ± 0,06
Hanseniaspora opuntiae	2,94 ± 0,16	0,00 ± 0,00	3,46 ± 0,33	0,00 ± 0,00
Galactomyces geotrichum	13,39 ± 0,61	0,00 ± 0,00	6,94 ± 0,11	0,00 ± 0,00
Fusarium verticillioides	6,27 ± 0,04	0,00 ± 0,00	4,63 ± 0,02	0,00 ± 0,00
Fusarium oxysporum	3,99 ± 0,25	0,00 ± 0,00	5,08 ± 0,22	0,00 ± 0,00
Candida sake	0,24 ± 0,20	0,00 ± 0,00	0,06 ± 0,02	0,00 ± 0,00
Aspergillus fumigatus	4,82 ± 1,34	0,00 ± 0,00	3,71 ± 0,09	0,00 ± 0,00
Aspergillus flavus	5,78 ± 0,46	0,00 ± 0,00	4,03 ± 0,07	0,00 ± 0,00
Alternaria alternata	3,62 ± 0,34	3,45 ± 0,18	5,71 ± 0,47	5,57 ± 0,21

Values are expressed as the mean from duplicate determinations (%)

Interestingly, *S. cerevisiae* was not detected by using the SILVA's database from the mock communities. In contrast, no significant differences in the OTUs distributions and the alpha diversity calculations were observed between the two datasets from biological samples. In detail, the constructed 26S database assigned 37 fungal species, described above, while SILVA's database assigned 35 (data are not shown). However, we observed that *S. cerevisiae* and *H. uvarum* were not detected by using SILVA's database.

4.4 DISCUSSION

New tools and molecular techniques have been used to detect microbial ecology in the past decades. Recently, the interest in the use of amplicon sequencing to identify taxonomically relevant taxa in food has increased. However, this approach has potential biases as previously described [35,36] where primer selection is considered one of the most important sources of biases [15–19,21,37–39]. The 26S region (D1 domain) of the rRNA encoding gene and the ITS2 region have been proposed as good candidates for identifying fungal species when using NGS technologies due to the high taxonomic resolution [14]. In this chapter, we performed a comparative evaluation of two regions as amplicon sequencing targets for the identification of fungi and it also describes the mycobiota community in food matrices.

Recently the 26S region has been studied using the Roche 454 technology [20]. However, this platform has been shown to result in high sequencing errors due to A and T rich homopolymers [40], while Illumina does not present this sequencing error [41]. Our results and a previous study revealed that the 26S gene is a reliable target site for both NGS technologies (Roche 454 and Illumina) for eukaryotic species [20]. In order to evaluate the effect of the target gene used we compared the sequencing results of both DNA samples and mock communities. In this chapter, different relative abundances were obtained for both mock

communities and biological samples and these differences were based on the PCR target used. We observed in such cases that the ITS2 target region led to underestimations of species with longer fragments (*S. pombe,* and *H. opuntiae*), while an overestimation of shorter fragments occurred (*F. vercillioides, A. fumigatus,* and *P. brevicompactum*). However, it should be pointed out that this correlation between amplicon length and estimation is not valid for all the species analyzed. Apart from the amplification length, other parameters, such as sampling errors, different primers alignment efficiencies during PCR amplification, the performance of degenerate primers used during PCR amplification influence the relative abundance calculations of taxa within samples [10,42].

Besides the underestimation of abundances observed when using NGS, "identification bias" is also a common error to amplicon-based analyses, where minor groups are poorly represented [23]. The lack of updated reliable public reference data set and the discrepancies to refer to fungal species have been recently demonstrated for the ITS sequences [23]. This is also in accordance with our results, suggesting that our new database for the 26S, validated by the widely use SILVA, proved to be a curated and rich database to be used. Differences between the two databases regarding the taxonomic classification of sequences were obtained. The newly constructed 26S database delivered a more precise taxonomic assignment of the sequences. This could be due to the fact that the SILVA's database comprised also non-microbial sequences, incomplete sequences, and sequences with unassigned taxonomy. In contrast, each taxonomy in our database was double-checked to get the higher taxonomic resolution, obtaining clearly more robustness results in terms of taxonomic assignment from the biological samples. Special attention must be paid on the miss-identification of fungal strain on the currently available database. This present issue is pointed out in this chapter, in which *S. cerevisiae* and *H. uvarum* were misidentified from fermented samples, using SILVA's database.

Given the intricate nature of PCR, the amplification of biological samples has been problematic [42]. Our results exhibit a high proportion of fungal coverage (98 - 96%) by both primer pair sets, which suggest that fungi account for roughly the complete eukaryote rRNA in fermented cocoa beans on average. The results also highlight the lower biodiversity of fungal communities for ITS2 compared with the 26S region in fermented cocoa beans, which contradicts previous studies where ITS region has been used in NGS studies as the universal primer set for fungi [4,14,39]. Such discrepancies between outcomes of different studies may arise on account of the biased quantification of relative abundance of taxa due to the uneven length of ITS fragments [12], the preferential amplification of rRNA genes for certain taxa by PCR, sequencing bias due to unequal amplification of the target gene or due to inaccurate taxonomic classification of reference databases [43]. Despite these challenges, the greater recovery trends in the community composition in the 26S target region observed here, have been supported from previous studies, where higher discrimination power of species identification in early diverging lineages of LSU compared with ITS was reported [44].

This chapter suggests that greater biodiversity in biological samples were obtained when targeting the 26s target region compared with the universal primer ITS. However, it should be noted that the present chapter shows the performance of a new pair of primers targeting the 26S region for fungal strains. Therefore, the novelty of these primer sets is also our limitation, that can be successfully overcome through future research focusing on the use of small fragments of the LSU region to target fungal species, that could support our observations. The combination of both target genes, where species identification can be performed applying ITS and phylogenetic analysis with 26S, is highly recommended and the use of both will depend on the purpose of taxa investigation [44,45]. From a molecular microbial ecology perspective, in terms of classification of marker-gene sequences, there is evidently a need for more extensive testing of primers

targeting different genes and *loci*, to support and identify all fungal species in NGS studies. Clearly, the benefits of identifying fermented microbial diversity may bring important advancements to the food industry, such as discrimination of starter culture to improve food quality or to accelerate processes. This chapter provides new insight into the selection of better primers and taxonomical assignment to study fungal ecology, which should enable food research to gain a better view of the microbial diversity present in a range of fermentations avoiding biases. One also notes, the limited availability of updated databases to assess ecological populations.

4.5 ACKNOWLEDGMENT

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EXPLORING THE FUNTIONAL

CAPABILITIES OF THE COCOBIOTA

In preparation Mota-Gutierrez, J., Ferrocino, I., Giordano M., Suarez-Quiroz, M.L., Gonzalez-Ríos, O., Cocolin L. Bacteria and yeast during cocoa fermentation: Exploring its functional capabilities and aroma and flavor formation of aroma

FERMENTED COCOA MICROBIOME APRIL 2020

ABSTRACT

A desirable chocolate aroma is considered a marker for high-quality. However, the flavor characteristic depends on the genetics of the cocoa variety and post-harvesting methods. This chapter aimed to identify the dynamic changes in microbial community structure and the volatilome profile, microbial associations as well as bacterial metabolic functions that could contribute to the aroma generation in two different Mexican cocoa varieties (*Criollo* and *Forastero*) during the five days of spontaneous fermentation. Microbial richness analysis indicated a higher level of complexity across fermentation time in the fungal and bacterial communities ($P < 0.05$). Remarkably, the beta diversity analysis of the 16S rRNA gene sequence data showed a clear separation between the cocoa varieties, indicating a higher level of complexity in *Criollo* varieties compared to *Forastero* ($P < 0.05$). In general, we observed that the fermentation was driven by *Hanseniaspora opuntiae, Saccharomyces cerevisiae, Acetobacter pasteurianus, Lactobacillus cacaonum,* and *Lactobacillus plantarum* group. A significant difference between the relative abundance of minor microbial species during the fermentation of the two different cocoa varieties was observed ($P < 0.05$). The predicted metagenomes confirmed differences in the correlations between the cocoa varieties and indicated that minor bacteria species play an important role in the biosynthesis of unsaturated fatty acids, and protein metabolism. From the metabolic production point of view, seventy volatile compounds (VOCs) were detected using gas chromatography. A significant difference in the concentration of VOCs between the cocoa varieties during fermentation was found, mainly in *Criollo* at the beginning of the fermentation. However, the quantitative descriptive analysis performed using a trained panel showed no differences between cocoa varieties, while the roasting process had a positive influence on the sensorial profile of the cocoa beans. In addition, the sensorial perception of fermented cocoa beans changes over fermentation time as

a function of the microbial metabolic activity and development. These results allowed us to advance the functions encoded at the level of DNA explaining how succession in microbial associations regulates the aroma development of fermented cocoa beans and the fermentation process.

5.1 INTRODUCTION

The chocolate industry is growing over the last decades, according to the Food Agriculture Organization, the trends of the annual cocoa production in the world between 2000 and 2017 has increased and the main cocoa producers are located in West Africa and South Asia [1]. However, this cocoa production has only increased one type of cocoa varieties (*Forastero*). On the market, cocoa is classified into two groups based on their flavor properties, fine or bulk cocoa [2].

Fine or flavor cocoa is characterize by their nutty cocoa flavor, dry or molasses and floral notes and is originally produced from *Criollo, Trinitario* and *Nacional* varieties [3–5]. These cocoa varieties are mainly produced in Venezuela, Central America, Mexico, Madagascar, Sri Lanka, Samoa, and Ecuador, while the bulk cocoa is mainly produced from *Forastero* and hybrids in the Lower Amazon and West Africa. One of the main differences between fine and bulk cocoa is the levels of sweetness, floral and caramel notes from which the level of these notes on the bulk cocoa is lower compared to the fine [6,7]. Cocoa can also be classified base on the physical aspects of the fruit and their niche of the cocoa market. *Criollo* varieties are characterized by the white or light brown appearance of the seed and are mainly used to produce high-quality chocolate, while purple seeds are observed when cutting open *Forastero* varieties and are mainly used to produce cocoa powder, cocoa mass, and chocolate [6,8].

The transformation of the characteristic mouthwatering cocoa aroma is developed after fermentation, drying and roasting processes [9]. Each of these cocoa processes plays an important role in the flavor development of the product [10]. The fermentation step includes the establishment of restricted and minor microbial populations that interact indirectly as they compete for common resources. A successful fermentation should ensure the development of the desirable flavor fingerprint after microbial behavior [11]. In general, microbes play a key role in the host defense, including their action against pathogen

invasion, nutrient processing and energy balancing through the metabolic process. However, the biochemistry behind is still not well understood at the molecular level.

Since colonization with non-pathogenic microbiota is essential to ensure a successful fermentation and flavor development; it is important to understand how the composition of the cocoa microbial ecosystem is established and their mechanism of action. Recently, amplicon sequencing and metagenomic analysis are providing interesting insights into the composition of the cocoa microbial communities and their effect on the cocoa mass [11–17]. However, not all microbial ecosystems from different types of cocoa varieties have been elucidated. This chapter aims to assess the link between the microbial composition of different fermented cocoa varieties (*Criollo* and *Forastero*) and the aromatic profile developed. This information will provide new insights into the discrimination of microbial communities in different cocoa varieties and to explore the metabolic pathways activated during cocoa fermentation. The combination of these different approaches may contribute to the development of new alternatives for cocoa processing based on biochemical and functional values.

5.2 MATERIALS AND METHODS

5.2.1 Fermentative, drying and roasting procedures

Criollo and *Forastero* varieties were obtained from Jardín Clonal Serranas, located in Comalcalco, Tabasco, Mexico at the end of the main-crop of 2018 (December 2017 - February 2018). The average ambient temperature and relative humidity were around 25°C and 76% in the field, respectively during fermentation. Cocoa samples were harvested by traditional methods including, opening pods and beans with unclean machetes and adhering pulp and beans were taken by hand. Spontaneous fermentation was carried out with 80 kg of cocoa

beans placed in wooden boxes of 200 kg capacity, for 5 days at ambient temperature covered with jute sacks. All trials were performed in duplicate (n=4) from an on-site fermentation, following local agricultural practices: cocoa beans were manually turned once per day, to obtain uniform fermentation and after 120 h the fermentation was stopped. During fermentation, 1.5 kg of cocoa beans were taken at 0, 24, 48, 72 and 120 h and placed in sterile bags in iced and transported to the laboratory. Samples were taken before turning cocoa beans for analyses. Every sample of cocoa beans was dried in a convection oven at 70°C for 8 h [18]. The beans were de-shelled manually and roasted in a laboratory-scale using a coffee-roaster (Solocafe, Coatepec, Veracruz, Mexico) for 14 min at 95 °C for sensorial analysis [19].

5.2.2 Physical and textural characteristics of cocoa pods

During the post-harvest 80 kg of cocoa pods were cut-open and five random pods were taken to measure physical properties such as (length, width, and thickness) and textural characteristics.

5.2.3 Evolution of temperature and pH during cocoa fermentation

Temperature and pH values were measured at the same sampling time during fermentation by an average of nine random zones of the cocoa bean-pulp mass using a pH-thermometer (Hanna, Romania).

5.2.4 Culture-dependent microbiological analysis

Beans samples (20 g) were aseptically scooped from the fermenting mass at 0, 48, 96 and 120 h and mixed with 180 ml of 0.1% ringer's solution and vigorously shaken for 3 min to give a uniform homogenate. Decimal dilutions in quarter-strength ringer's solution were prepared from which aliquots (0.1 ml) were spread-inoculated in duplicate over the surface of plates of agar media for the isolation and enumeration of specific organisms. The preparation of the media

used are described, Malt Extract Agar (MEA, yeasts; Dibico, DF, Mexico) plus 2 µg/mL of chloramphenicol (Sigma-Aldrich) for counting total yeasts, incubated for 3/5 days at 30°C, de Man-Rogosa-Sharpe agar (MRS, LAB; Dibico, DF, Mexico) plus 50 µg/mL natamycin (Oxoid, Basingstoke, Hampshire, UK) for growing LAB, incubated at 30°C for 48 h, and Acetic Acid Medium agar (AAM, AAB; Oxoid) plus 50 µg/mL natamycin for growing acetic acid bacteria (AAB) incubated at 30°C for 3/5 days [11]. Results were expressed as means of Log colony forming units (CFU) g^{-1} from two independent determinations.

5.2.5 Metabolite target analysis of fermented cocoa beans

The dynamics of the volatilome profile of two different fermented cocoa beans-pulp varieties previously lyophilized were obtained using headspace solid-phase micro-extraction technique (HS- SPME), setting fiber conditions and oven temperatures as previously described by Mota-Gutierrez *et al.* [20]. Samples were analyzed by triplicates of each biological replicate. The analysis was conducted in a 20 ml vial filled with 2 ml of 20% NaCl and 0.1 g of the sample and adding to each sample 10 µl of 5-nonanol in ultrapure water at 10 mg/L concentration as an internal standard for the semi-quantification. The fiber with the sample was injected into the Gas Chromatography-qMass Spectrometry (GC-qQP2010 Plus, Shimadzu, USA), equipped with an auto-sampler (AOC-5000, PAL System, CombiPAL, Switzerland) and a DB-WAXETR capillary column (30m × 0.25 mm, 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA). The injection mode was established at 260 °C (1 min) using helium as carrier gas at a constant flow rate of 1 ml/min. The detection was carried out by the electron impact mass spectrometer in total ion current mode, using ionization energy of 70 eV. The acquisition range was settled as *m/z* 33-350 amu. The identification of the peaks was calculated by comparing the mass spectra of the peaks with the spectra of the MIST05 library and through comparison of the retention indices (a matrix of a homologous series of C8-C24 was used) with a pure standard injected following the same sample conditions, described above. Semi-quantitative data (µg/kg)

were obtained by measuring the characteristic *m*/*z* peak area of each identified compound with the added internal standard.

5.2.6 Quality assessment of dried cocoa beans

Fermented, dried cocoa beans were evaluated for appearance and quality, making use of the cut test. A total of 400 beans were cut lengthwise (100 beans for each fermentation box) through the middle to expose both halves of the cotyledons in full daylight and categorized in one of the following categories: fully brown, brown/purple, purple, slaty and white.

5.2.7 Fermentation index

This index was determined according to the method of Ching *et al.* [21]. Ground fermented and dried cocoa beans (0.5 g) was added to a mixture of methanol/HCl (97:3) and homogenized. The mixture was left at 4°C for 16 h and filtered using Eaton-Dikeman filter paper. The filtrate was collected and the ratio of the absorbance at 460 nm and 530 nm was determined using a UV-visible spectrophotometer (Shimadzu, Japan).

5.2.8 Fermented and roasted cocoa nibs sensory analysis

The sensory tests were conducted to characterize the changes of the fermentation properties of cocoa beans by means of quantitative descriptive analysis (QDA), using a trained panel. Five Mexican judges (undergraduate students between the ages 20 -22 y; two females, three males) passed the screening criteria (good health, non-smoker, ability to work well on a panel, interest in participating, no allergic reactions to consumption of cocoa and its derivatives, and passed the olfactory and taste tests). Panelists were trained in sessions of 3 h per week for 22 weeks. In detail, a taste detection test was carried out to familiarize panelist with characteristic taste descriptors (five attributes), using solutions with concentrations of 10 $g.L^{-1}$ sucrose (sweet), 0.27 $g.L^{-1}$ of caffeine (bitter), 0.4 $g.L^{-1}$ citric acid (acidic), 2 $g.L^{-1}$ sodium chloride (salty) and

0.5 $g.L^{-1}$ potassium alum (astringent), when panelists were correct in 100% of the test, it was considered acceptable. Finally, the panelists were trained to memorize twenty-five odors descriptors for cocoa beans using an aroma kit as odor standards (Le Nez du cafe).

The trained panel worked in a sensory laboratory with adequate lighting and a temperature of 25°C. Prior to the evaluation, 5 g of fermented cocoa beans from different fermentation boxes (n=4) at different sampling points (0, 24, 48, 72 and 120 h) were dried, peeled, crush and placed in centrifuge tubes covered with aluminum. A total of 200 containers for odor and taste testing were prepared. The sealed containers were left for 15 min at a temperature of 40°C to promote the accumulation of odors. All panelist was asked to wear eye masks to avoid visual difference between samples. Fermented cocoa beans were presented individually in random order and assessed by the trained panel over two sessions (two replicates).

Odor testing was performed first, each panelist inhaled the odors and cast their judgment in which they were asked to mark the stimulus perceived. When required, the panelist used roasted coffee beans to desaturate their olfactory system. Finally, panelists were asked to evaluate the samples for the five predefined attributes of taste. For this test, the panelists were asked to rinse their mouth out with water between samples. The sensory perception was determined by the total percentage perception of the trained panel carried out twice for each type of test.

5.2.9 High-throughput sequencing workflow

The total DNA from raw and fermented cocoa samples in triplicate at each sampling point was collected and 2 ml of the first 10-fold serial dilution was directly centrifugated. The total DNA from fermented cocoa beans was extracted using a MasterPure Complete DNA & RNA Purification kit (Illumina Inc, San Diego, CA), according to the manufacturer's instructions. Samples were

amplified for bacterial communities targeting the V3 and V4 regions of 16S rRNA using the primers and under the conditions described by Klintword *et al.* [22], while the fungi communities were amplified using LS2-MF (5'-GAGTCGAGTTGTTTGGGAAT-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') primers targeting the 26S region [20]. Sequencing was performed following the Illumina sample preparation procedure. Briefly, the PCR products were purified twice using the Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and the DNA quality was checked on the NanoDrop 2000c instrument (Thermo Scientific, USA) and quantified on a Qubit 2.0 fluorometer (Invitrogen, USA). The resulting products were fragmented and tagged with sequencing adapters by using a Nextera XT index kit (Illumina). Amplicons were mixed at 4 nM pool and measured on Qubit Fluorometric Quantitation 2.0 (Thermo Fisher Scientific). In addition, the quality and size distribution of the libraries were assessed by using high-sensitivity DNA chips and DNA reagents on a Bioanalyzer 2100 (Aligent, USA). This pool was then denaturated to 20 pM pool obtained by mixing 5µl of NaOH 0.2N with 5 µl of the 4 nM pool. Finally, a 12 pM library was combined with 10% PhiX. Pool samples were load to the MiSeq instrument (Illumina) with V3 chemistry, and 250-bp paired-end reads were generated.

5.2.10 Bioinformatics and metagenome prediction

Raw illumina fastq files were first demultiplexed, assembled, filtered and analyzed using QIIME 1.9.0 software [23]. In deep, the obtained paired-end reads were assembled using the FLASH software [24], with default parameters and the truncated reads receiving a quality score of < Q20 were obtained. Any reads shorter than 250 bp were discarded using Prinseq. Primer sequences were trimmed from the ends of each sequence, and operational taxonomic units (OTUs) were assigned by means of UCLUST, with a threshold of 99% and 97% pairwise identity for the 16S and 26S rRNA data, respectively. The taxonomic assignation of the centroid sequences of each cluster was assessed by mapping

against the Greengenes 16S rRNA gene database, version 2013 for the 16S data [25] and our constructed 26S database. The chloroplast and mitochondria sequences were removed from the dataset. QIIME was also used to calculate the abundance-weighted and unweighted UniFrac distance matrices, as well as the OTUs table, was used to determine differences between the microbial communities from different cocoa varieties (*Criollo* and *Forastero*) and under different box fermentations using the Adonis and Anosim statistical tests in the R environment. All the samples of each dataset were rarefied at the lowest number of reads after the raw read quality filtering. QIIME was used to produce a filtered OTUs table at above 1% in at least 2 samples. The OTUs table displays the highest taxonomy resolution reached, when the taxonomy assignment was not able to reach the species level, or when the genus or family name was displayed. The generalized linear mixed model (glmm) test was used to find significant differences in the microbial taxa abundance profiles and the Shannon-Wiener diversity index *H',* according to time, variety and number of boxes ($P < 0.05$). Spearman's rank correlation coefficient was obtained as a measure of the association between the microbial OTUs that occurred in at least 2 samples using the *psych* function and plotted through the *corrplot* package of R. The OTUs that occurred in at least 2 samples of the microbial communities were conglomerated by means of hierarchical clustering analysis using Ward's method, which was acquired through the *heatmap.2* function plotted by the *gplots* package of R.

The prediction of functional profiles in the microbial communities of fermented cocoa beans was performed by the PICRUSt analysis (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, http://picrust.github.io/picrust/). For this analysis, the OTUs at 97% of identity were picked using the pick_closed_reference_otus.py script of QIIME 1.9.0 [23] against the Greengenes database [26]. The metagenome was predicted from normalized data for 16S rRNA gene copy numbers. KEGG orthologs were identified from the inferred metagenomes and the table obtained was rarefied at

the lowest number of sequence/sample. KEGG orthologs were then collapsed at level 3 of hierarchy and the resulting table was imported in the GAGE *Bioconductor* package in R to identify biological pathways overrepresented or underrepresented between the different cocoa varieties over fermentation time (www.r-project.org). Validation of the accuracy of metagenome prediction was calculated using the Nearest Sequenced Taxon Indexes (NSTI), this algorithm calculated how closely related the microbes in a given sample and the microbes with sequenced representatives; NSTI with lower values indicated a closer means relationship.

5.2.11 Statistical analyses

Statistical analyses were carried out using glmm for non-normally distributed data set. Mixed models were chosen because of their ability to capture both fixed (cocoa varieties: *Criollo* and *Forastero* and fermentation time: 0-120 h) and random effects. The number of boxes (n = 4) was treated as random factors for all the analysis except for the statistical analysis of the sensory profile data, in which the number of subjects (n =5) were treated as random effects and set a binomial family [27]. The *P*-values were adjusted using Bonferroni's method and when the mixed model revealed significant differences ($P < 0.05$), the Duncan honestly significant difference (HSD) test was applied. Mixed models were built and evaluated according to Crawley [28] using R version 3.3.2. The assessment of the mean difference between the cocoa varieties over a specific fermentation period was subjected to glmm. In addition, Spearman's correlation test was used to assess the correlations between the OTUs and to establish any changes in concentration over the fermentation period.

5.3 RESULTS

5.3.1 Cocoa pod properties

The physical and textural characteristics of both cocoa varieties are described in Table 5.1. In general, a similar average of pod length, width and weight are observed for both varieties, while the number of beans per pod and external thickness was different between cocoa varieties (Table 5.1).

Table 5.1 Physical and textural properties of two different cocoa varieties

	Criollo	***Forastero***
Physical properties		
Pod length (cm)	20.10	22.00
Pod diameter (cm)	30.30	30.00
Pod weight (g)	546.50	547.50
External thickness (cm)	2.50	1.50
Number of beans per pod	31.00	39.00
Weight of 100 beans	166.58	140.22
Number of seeds per 100 g	62.00	71.50
Textural characteristics		
Color	Yellow	Green/yellow
Shape	Angoleta type	Angoleta type
Roughness	Little	Little

5.3.2 Physical and microbiological dynamics through cocoa fermentation

No significant difference between cocoa varieties was observed through physical (temperature and pH) and microbiological analysis. However, the average global temperature and pH of all boxes significantly increased from initial values of 28°C to 36°C and 3.71 to 4.24, respectively as shown in Table 5.2 ($P < 0.05$).

The analysis of the culture-dependent method showed that yeast, LAB and AAB populations changed over the fermentation time (Table 5.2). Overall, at the beginning of the fermentation all counts (yeast, LAB, and AAB) were at the same order of magnitude, while at the end of this process, AAB population reached the highest load between the three microbial groups. Yeast and LAB populations reached the highest load at 48 h and decreased rapidly ($P < 0.05$, Table 5.2), whereas AAB reached the highest load at 72 h and decreased rapidly at the end of the fermentation ($P < 0.05$, Table 5.2).

Table 5.2 Average changes in physical and microbiological parameters during spontaneous box fermentation of cocoa bean-pulp turned every 24h

		Fermentation time (h)				
		0	24	**48**	**72**	**120**
°C						
	A	**28.37 ± 0.06 [d]**	**28.68 ± 0.25 [d]**	**33. 85± 1.06 [c]**	**39.09 ± 0.73 [a]**	**36.36 ± 0.76 [b]**
pH						
	A	**3.71 ± 0.13 [a]**	**3.83 ± 0.11 [a]**	**3.63 ± 0.44 [a]**	**3.89 ± 0.14 [a]**	**4.24 ± 0.61 [a]**
Yeast (Log CFU)						
	C1	4.55	5.42	4.81	1.70	1.40
	C2	4.54	5.71	5.84	4.42	2.06
	F1	4.31	5.35	6.68	1.70	1.70
	F2	5.21	4.50	6.30	1.70	1.65
	A	**4.65 ± 0.39 [a]**	**5.24 ± 0.52 [a]**	**5.91 ± 0.81 [a]**	**2.38 ± 1.36 [b]**	**1.70± 0.27 [b]**
LAB (Log CFU)						
	C1	4.99	5.64	5.65	5.04	3.70
	C2	4.99	6.08	6.06	4.55	3.40
	F1	4.46	5.90	5.66	4.13	3.70
	F2	4.65	6.00	5.86	4.90	3.40
	A	**4.77 ± 0.26 [b]**	**5.91 ± 0.19 [a]**	**5.81 ± 0.20 [a]**	**4.66 ± 0.41 [b]**	**3.55 ± 1.74 [c]**
AAB (Log CFU)						
	C1	4.26	5.64	5.82	5.66	4.40
	C2	4.40	4.00	5.62	5.68	5.49
	F1	4.40	5.19	4.88	6.10	4.90
	F2	4.00	5.00	4.90	5.96	4.54
	A	**4.27 ± 0.19 [b]**	**4.96 ± 0.70 [ab]**	**5.31 ± 0.49 [ab]**	**5.85 ± 0.22 [a]**	**4.83 ± 0.49 [ab]**

Values are expressed as the mean ± SD from duplicates determinations. Superscripts indicate statistical difference related to the fermentation time using the least significant difference test ($P < 0.05$). *P*-values were adjusted using Bonferroni's method. **Abbreviations; C1:** Box 1 *Criollo* variety, **C2:** Box 2 *Criollo* variety; **F1:** Box 1 *Forastero* variety, **F2:** Box 2 *Forastero* variety.

5.3.3 Fermented cocoa metabolite products

The head-space solid phase micro-extraction gas chromatography-qmass spectrometry (HS-SPME-GC/MS) method identified 70 VOCs in both cocoa fermented varieties. At the beginning of the fermentation 2-pentanol, ethanol, benzeneacetaldehyde, and ethyl acetate were the most abundant VOCs detected, while after 24, and 48 h ethyl acetate and ethanol remained the most detected VOCs in both cocoa varieties. Notably, after 72 h to the end of the fermentation, acetic acid and ethyl acetate took over and dominated. Overall, ethyl acetate was the most abundant VOCs observed regardless of the fermentation time and the cocoa variety. The dynamics across time observed for VOCs during both fermentations were similar. In general, we observed a decline in the total headspace concentration of alcohols as cocoa fermentation time increased, while an increase of acids and esters was observed regardless of the type of cocoa varieties (data not shown).

Interestingly, a significant difference between the concentration of VOCs of the two different fermented cocoa varieties (*Criollo* and *Forastero*) was observed at specific fermentation time. In detail, the concentration of 2-methylbutanal, 3-methylbutanal, 2-methyl-2-butenal, nonanal, decanal, benzaldehyde, benzeneacetaldehyde, D-limonene, isopropyl dodecanoate, benzyl alcohol, n-tetradecanol, nonanoic acid, decanoic acid, and methyl jasmonate were significantly higher in *Criollo* than in *Forastero* at the beginning of the fermentation, while significantly higher concentrations of 1,2 propanediol diacetate in *Forastero* was observed over the whole fermentation process as shown in Figure 5.1.

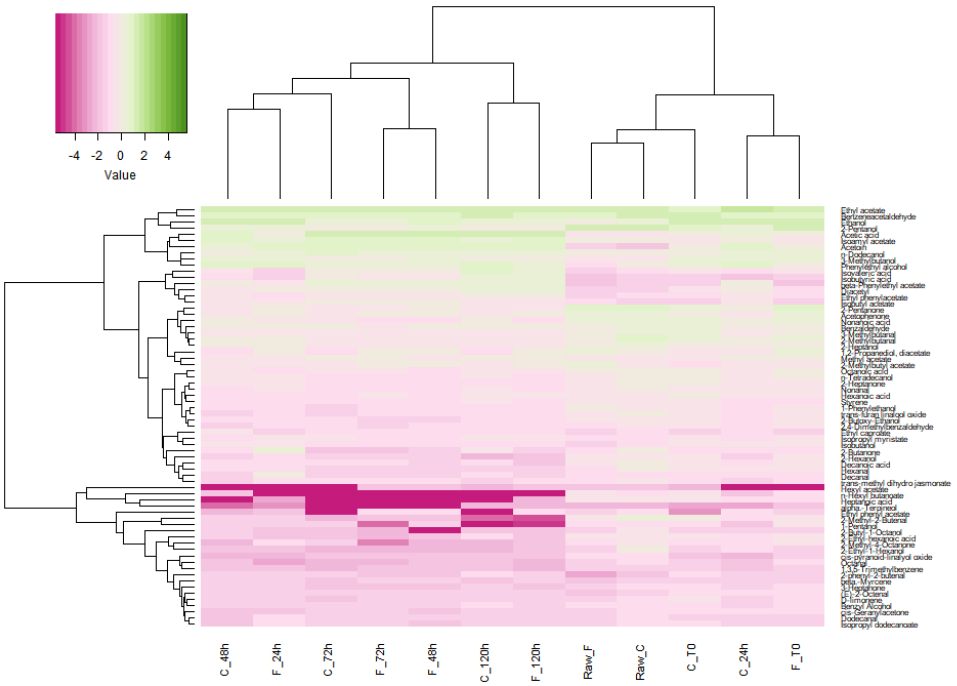


Figure 5.1 Volatile compounds detected during cocoa beans fermentations identified by GC-MS analysis produced from two different cocoa varieties during spontaneous fermentation. Only VOCs observed above 1% are shown. **Abbreviations:** *Criollo* (C) and *Forastero* (F)

5.3.4 Quality assessment of fermented cocoa beans

A significant difference between the percentage of bean surface color (purple and white) of the two different cocoa varieties (*Criollo* and *Forastero*) was observed ($P < 0.05$, Table 5.3). The cut test showed that fermented *Criollo* varieties obtained the highest percentage of white color at the end of the fermentation (ranging from 53–76%), and the lowest of purple (ranging from 0–3%), while *Forastero* varieties showed the highest percentage of brown (ranging from 92–88%) and lowest in purple (0%) as shown in Table 5.3. In addition, a significant difference between fermentation boxes was also observed during the evolution of the fermentation index (Table 5.3, $P < 0.05$). Overall, *Criollo* beans from the first box showed greater values than 1 after 48 h, which indicates that

the beans were fully fermented since then, while the same variety but in a different box reached values greater than 1 after 72 h. In contrast, *Forastero* beans from both boxes showed fully fermentation after 120 h (Table 5.3).

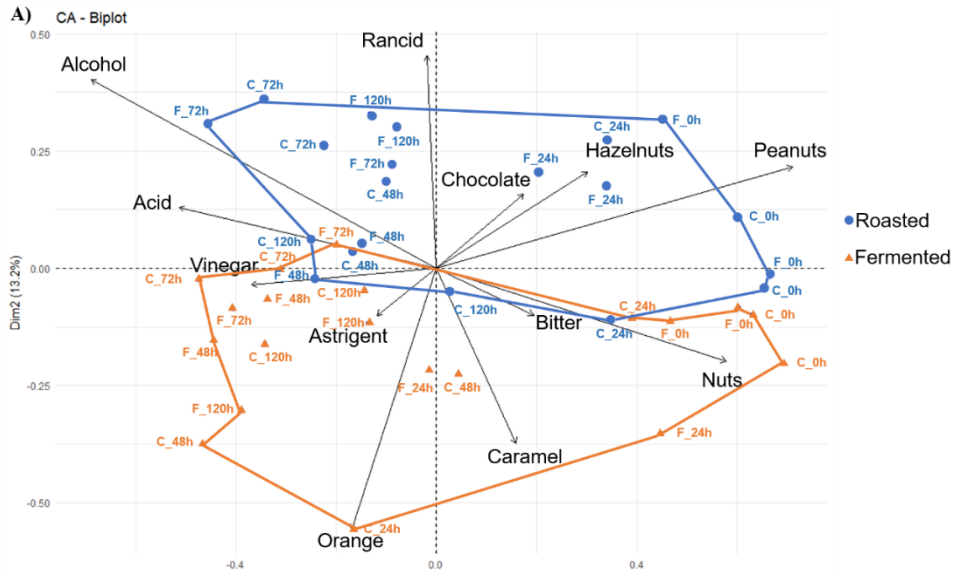
Table 5.3 Effect of fermentation of dried cocoa beans surface color and fermentation index

		Fermentation time (h)				
		0	**24**	**48**	**72**	**120**
FERMENTATION INDEX						
	C1[a]	0.76	0.78	1.31	1.98	2.00
	C2[b]	0.73	0.82	0.91	1.39	2.36
	F1[c]	0.41	0.41	0.58	0.82	1.13
	F2[c]	0.40	0.40	0.54	0.80	1.12
CUT TEST						
Slaty						
	C1	5.00	0.00	0.00	0.00	0.00
	C2	0.00	0.00	0.00	0.00	0.00
	F1	0.00	0.00	0.00	0.00	4.00
	F2	0.00	0.00	0.00	0.00	0.00
Purple						
	C1[b]	26.00	22.00	15.00	11.00	3.00
	C2[b]	24.00	19.00	23.00	18.00	0.00
	F1[a]	97.00	90.00	90.00	91.00	0.00
	F2[a]	90.00	89.00	84.00	84.00	0.00
Purple/brown						
	C1	0.00	0.00	5.00	2.00	2.00
	C2	0.00	0.00	0.00	0.00	0.00
	F1	0.00	0.00	5.00	5.00	0.00
	F2	0.00	0.00	4.00	7.00	0.00
Brown						
	C1	0.00	0.00	0.00	11.00	39.00
	C2	0.00	0.00	0.00	16.00	22.00
	F1	0.00	0.00	0.00	4.00	92.00
	F2	0.00	0.00	0.00	0.00	88.00
White						
	C1[a]	65.00	65.00	73.00	68.00	53.00
	C2[a]	69.00	70.00	68.00	63.00	76.00
	F1[b]	2.00	9.00	4.00	0.00	3.00
	F2[b]	8.00	11.00	10.00	7.00	10.00

Values are expressed as mean from 100 seeds for the Cut test. Superscripts indicate statistical difference using the least significant difference test ($P < 0.05$). *P*-values were adjusted using Bonferroni's method.
Abbreviations; C1: Box 1 *Criollo* variety, **C2:** Box 2 *Criollo* variety; **F1:** Box 1 *Forastero* variety, **F2:** Box 2 *Forastero* variety.

5.3.5 Sensory profile and sensorial evolution of fermented and roasted cocoa beans

A quantitative descriptive analysis (QDA) was performed using a trained panel and the contingency table obtained was subjected to correspondence analysis (CA) to visualize the relationship between the different cocoa processes and the aroma and flavor cited (Figure 5.2). The results clearly showed that the sensorial perception of fermented cocoa beans changes over fermentation time and cocoa processing, while no influence was observed between cocoa varieties (Figure 5.2A). In detail, dimension 1 explains the difference between the cocoa processing, in which roasted cocoa beans were described with a peanut, hazelnut, chocolate, rancid, alcohol and acid notes, while the fermented cocoa beans were described with a nut, caramel, bitter, orange and vinegar attributes (Figure 5.2A). Interestingly, the cocoa samples at 48 and 120 h were not clustered regardless of the cocoa process (Figure 5.2B).



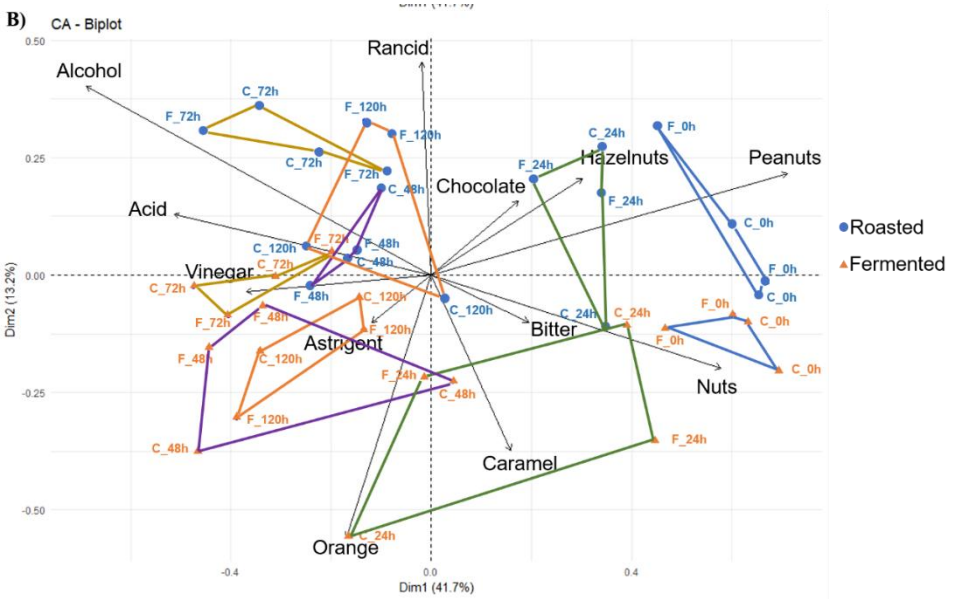


Figure 5.2 Correspondence analysis factor (CA) map showing the flavor profile of fermented and roasted cocoa beans from two different cocoa varieties over time. The cocoa beans were analyzed by a trained panel, and descriptors that were more perceived are displayed. The CA loading and scores for dimension 1 and 2 are represented based on the type of cocoa process, including roasting (blue circles) and fermentation (orange triangles). **Abbreviations:** *Criollo* (C) and *Forastero* (F).

The evolution of the flavor and aroma description judged by the sensory panel were well discriminated concerning the cocoa process and time ($P < 0.05$, Figure 5.3). Concerning taste notes, when cocoa beans were more fermented, the "bitterness" note was perceived as being less dominant, while "acid" was more dominant in both cocoa processing (Figure 5.3, $P < 0.05$). In detail, the moat attributes used to describe the sensory profile of fermented and roasted cocoa beans by the sensory panel were tamarind, apple, pineapple, vinegar, peanut, hazelnut, and chocolate. Besides these aroma attributes, honey was also used as an attribute to describe the sensory profile of fermented cocoa beans, while orange was used to describe the profile of roasted cocoa beans. However, the perception of the "vinegar", "alcohol", "peanut", "honey", and "pineapple" notes changed significantly over time on both processes (Figure 5.3, $P < 0.05$). One

notes, that the attributes “nut”, “rancid” and “tamarind” also changed significantly over time in fermented cocoa beans, while “apple” and “orange” for roasted samples ($P < 0.05$, Figure 5.3). In detail, the fermented cocoa beans at time 0 were perceived as the most dominant for the “peanut” note, while the increase in fermentation time induced an increase in the “alcohol”, “vinegar”, and “tamarind” notes (Figure 5.3A, $P < 0.05$). In addition, the perception of the “vinegar” and “tamarind” notes remained the most dominant attributes perceived by the panelist at the end of the fermentation, followed by “pineapple” and “rancid” (between 72 and 120 h). Interestingly, only the perception of the “rancid” note showed a significant difference at 72 h between varieties, in which this note was more described by the *Criollo* variety compared with *Forastero* (Figure 5.3A, $P < 0.05$).

In contrast, we observed that a "pineapple" note was characterized by not fermented roasted cocoa beans. An increase in fermentation time in roasted cocoa beans induced an increase in the “vinegar” and “alcohol” attributes (Figure 5.3B, $P < 0.05$). Interestingly, only the "honey" attribute showed a significant difference after 24 h between varieties. In detail, this note was more dominant in *Forastero* varieties compared to *Criollo* samples (Figure 5.3B, $P < 0.05$).

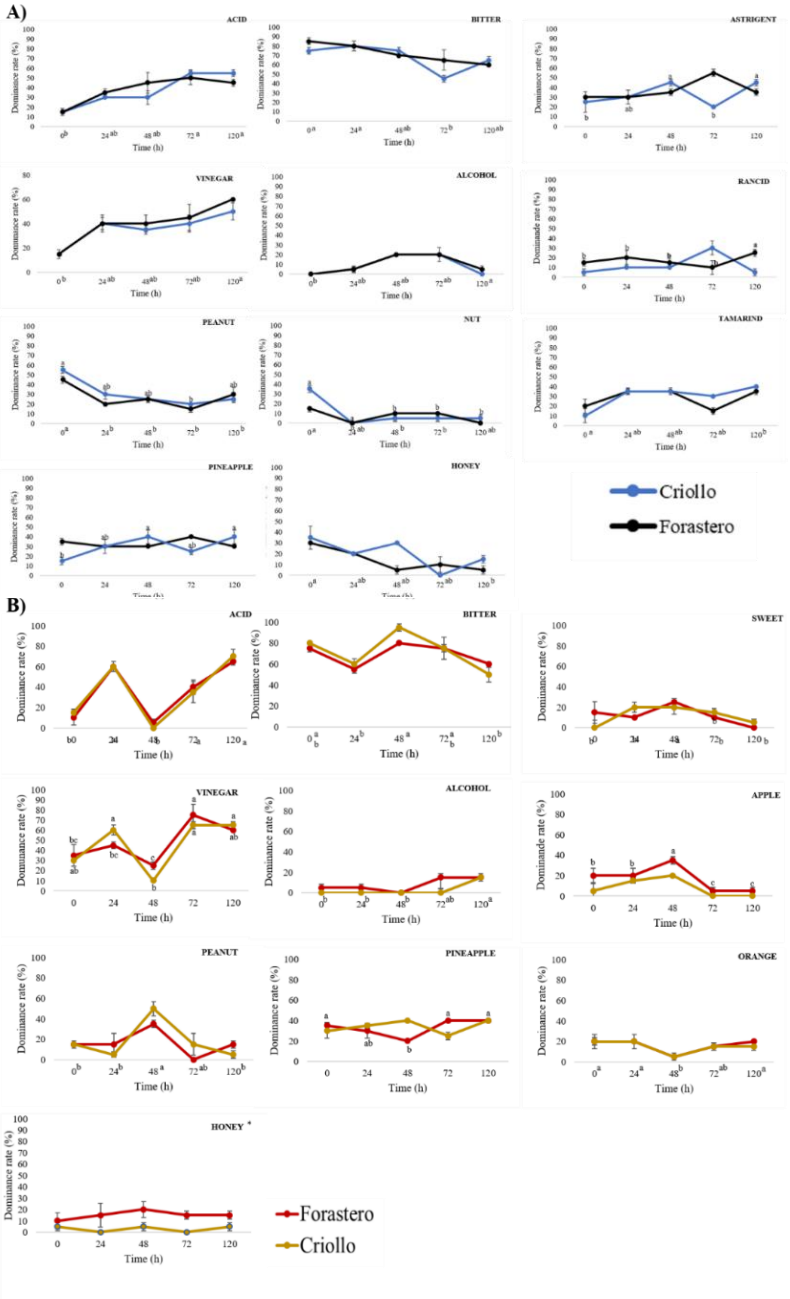


Figure 5.3B The evolution of the dominance rate of roasted cocoa beans from two different cocoa varieties for eleven attributes over time. Data are expressed as percentage ± SD values from 10 determinations for each fermentation time. Superscripts and asterisks indicate statistical differences using the least significant difference test ($P < 0.05$). *P*-values were adjusted using Bonferroni's method

5.3.6 Fungi diversity in fermented cocoa beans

The total number of raw read of the fungal community obtained from fermented cocoa beans was 895,971 and only 829,150 reads passed the filters applied through QIIME, with an average value of 37,688 ± 26,639 reads/sample, and a mean sequence length of 411 bp. The average good's coverage expressed as a percentage (98%) and the rarefaction analysis indicated a satisfactory population size. In addition, the species richness analysis indicated a higher level of diversity only across fermentation time ($P < 0.05$). Overall, 30 fungal OTUs were identified during the fermentations. In general, we observed two separate groups, the first group include the two most abundant yeast species, *Hanseniaspora opuntiae* and *Saccharomyces cerevisiae* and the rest the less abundant fungal group during cocoa fermentation regardless the cocoa variety (Figure 5.4). In addition, a significant increment of *S. cerevisiae* over fermentation time and a decrease of *H. opuntiae* was observed (Figure 5.4, $P < 0.05$).

A significant difference between the relative abundance of minor mycobiota species in the two different varieties studied were observed (Figure 5.4, $P < 0.05$). In detail, a significantly higher abundance of *Wickerhamomyces anomalus* and *Pichia myanmaensis* was characterized in *Criollo* varieties at time zero, while *Starmerella bacillaris, Hanseniaspora thailandica, Martiniozyma asiatica, Pichia orientalis, Rhizopus oryzae,* and *Saturnispora diversa* were more abundant in *Forastero* (Figure 5.4, $P < 0.05$). After 24 h, *Saccharomyces* species (*S. cerevisiae, Saccharomyces cariocanus*) characterized the *Criollo* variety, while the *Hanseniaspora (Hanseniaspora occidentalis, H. opuntiae, H. thailandica), Pichia (Pichia pijperi* and *Pichia terricola)* and *Martiniozyma* genus *(M. asiatica)* characterized the *Forastero* (Figure 5.4, $P < 0.05$). Interestingly, after 48 hours to the end of the fermentation significantly higher relative abundance of the minor mycobiota group (*H. thailandica, M. asiatica, R.*

oryzae, *Cyphellophora europaea*, *Torulaspora delbrueckii* and *Galactomyces candidum*) in *Forastero* varieties was observed compared with *Criollo*, while only at the end of the fermentation higher relative abundance of *P. myanmaensis* and *W. anomalus* in *Criollo* varieties were observed (Figure 5.4, $P < 0.05$). One also notes that a significantly higher abundance of *M. asiatica* was observed during the whole fermentation time in *Forastero* varieties.

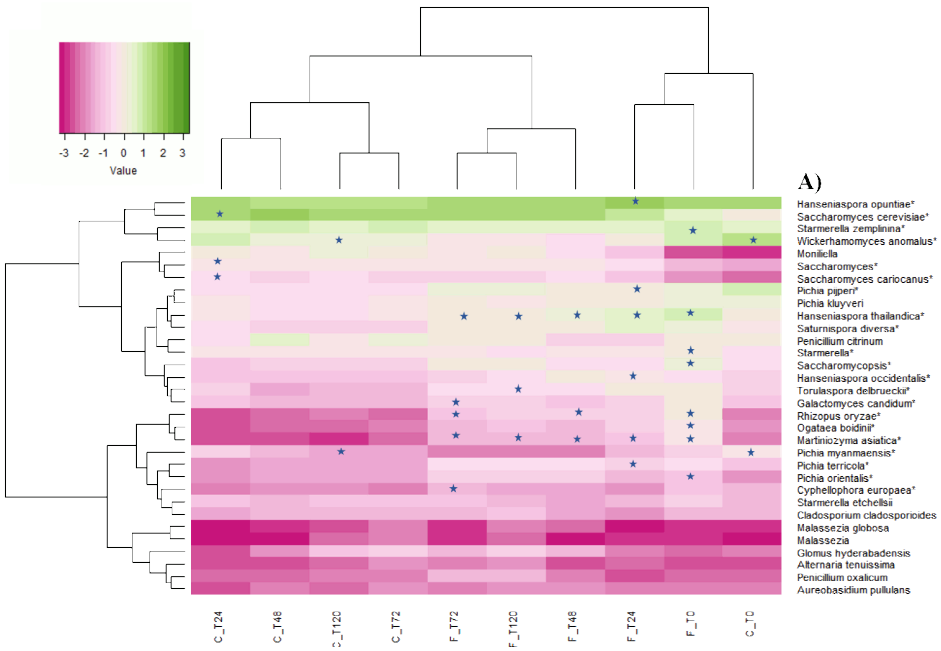


Figure 5.4 Spearman's correlation between the relative abundance of **fungal OTUs** observed with above 1% in at least 2 samples. Rows and columns are clustered by Ward linkage hierarchical clustering. The intensity of the colors represents the degree of correlation between fungal OTUs as measured by the Spearman's correlation with pink (negative degree of correlation) and green (positive degree of correlation). **Abbreviations:** *Criollo* (C) and *Forastero* (F)

5.3.7 Bacterial diversity in fermented cocoa beans

A total of 2,655,230 raw reads (2x250 bp) were obtained and 1,834,144 reads passed the filters applied through QIIME, with an average value of 43,670 ± 32,993 reads/sample, and a mean sequence length of 440 bp. The average estimated sample coverage was 97% indicating a satisfactory population size,

also supported by the rarefaction analysis. In addition, the diversity of the bacterial community analysis (α-diversity) indicated a higher level of complexity in *Criollo* compared to *Forastero* fermentations and a significant difference across fermentation time (Figure 5.4B, $P < 0.05$). In detail, at time 0 a significantly higher relative abundance of *Swaminathania* in *Forastero* was observed, while *Lactobacillus plantarum* group was characterized by *Criollo* varieties (Figure 5.5, $P < 0.05$). After 48 h, a significantly higher relative abundance of *Swaminathania* and *Lactobacillus cacaonum* were observed in *Forastero* varieties, while higher relative abundances of *Acetobacter pasteurianus, Curtobacterium, Lactobacillus brantae, Lactobacillus brevis,* and *Lactobacillus manihotivorans* were observed for *Criollo* (Figure 5.5, $P < 0.05$).

Overall, 27 bacterial OTUs were identified during the fermentations. The classification at a family level such as *Leuconostocaceae* refers to all the possible OTUs at different genus level excluding *Leuconostoc pseudomesenteroides* correctly identified**.** We observed two separate microbial communities of fermented cocoa beans, the first group include the four most abundant bacterial species, *A. pasteurianus, L. reuteri, L. cacaonum,* and *L. plantarum* group and the rest the less abundant bacterial group during cocoa fermentation regardless the cocoa variety (Figure 5.4B). In detail, both cocoa varieties at time 0 showed the dominance of *A. pasteurianus* and the unknown species of the *Leuconostocaceae* family, while after 24, 48, and 72 h we observed that *A. pasteurianus* remained the most abundant OTUs detected followed by *L. cacaonum* and *L. plantarum* group. Notably, at the end of the fermentation, *A. pasteurianus* and *L. reuteri* took over and dominated. Microbial dynamics of both cocoa varieties were similar over fermentation time, *A. pasteurianus* and *L. reuteri* significantly increased over fermentation time regardless of the cocoa varieties, while *L. plantarum* group, the unknown species of the *Leuconostocaceae* family and the minor bacterial group decreased ($P < 0.01$).

Interestingly, *L. cacaonum* increased and reach the highest abundance after 72 h and then decreased after 120 h as shown in Figure 5.5 ($P < 0.01$).

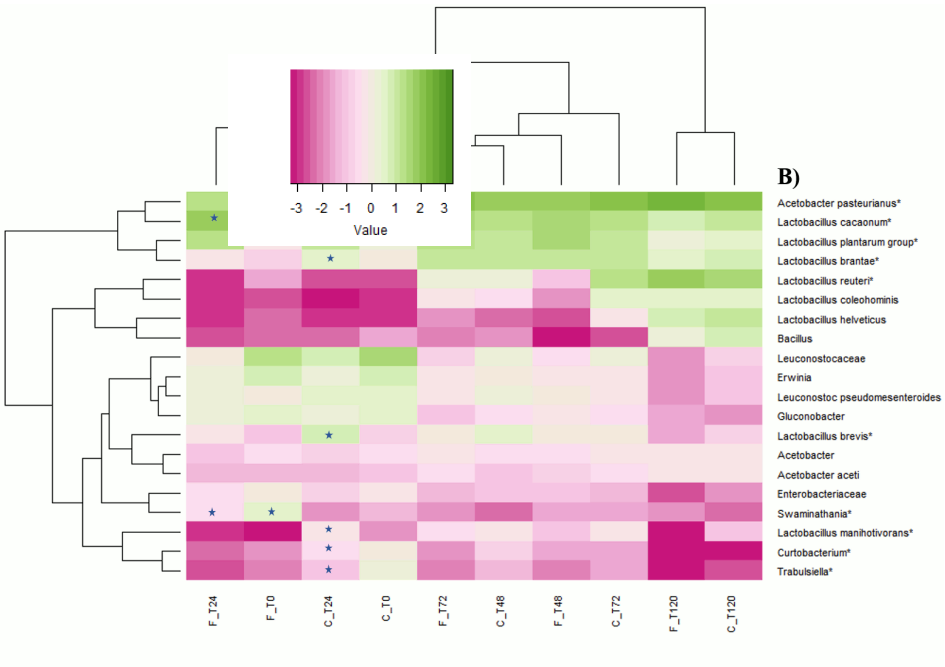


Figure 5.5 Spearman's correlation between the relative abundance of the **microbial OTUs** observed with above 1% in at least 2 samples. Rows and columns are clustered by Ward linkage hierarchical clustering. The intensity of the colors represents the degree of correlation between bacterial OTUs as measured by the Spearman's correlation with pink (negative degree of correlation) and green (positive degree of correlation). **Abbreviations:** *Criollo* (C) and *Forastero* (F)

5.3.8 Significant co-occurrence and/or co-exclusion relationships among the fermented cocoa microbial communities

A pairwise correlation matrix adjusted for multiple comparisons of the relative abundances of bacterial and fungal populations was used to visualize the microbial co-occurrence and/or co-exclusion dynamics between the two fermented cocoa varieties as shown in Figure 5.6. In general, we observed a co-occurrence relationship between the most abundant bacterial species (*A. pasteurianus*, *L. plantarum*, *L. reuteri*, and *L. brantae)* and *S. cerevisiae*, and a

co-exclusion relationship between the most abundant bacterial species and *P. myanmaensis* and *W. anomalus* in both cocoa varieties (Figure 5.6, $P < 0.05$). Besides the correlation of the most abundant bacterial species, the minor bacterial group (*Enterobacteriaceae, Erwinia,* and *Gluconobacter)* was positively associated with *Galactomyces candidum, H. opuntiae, P. kluyveri, P. myanmaensis, S. diversa,* and *W. anomalus,* while a negative association between the minor bacterial group describe above and the minor fungi group (*Glomus, Malassezia, Moniliella and Penicillium* species) was observed (Figure 5.6, $P < 0.05$). Interestingly, we observed a positive association between *Swaminathania* species and *H. opuntiae, P. kluyveri, P. myanmaensis, S. diversa, T. delbrueckii,* and *W. anomalus,* while the same bacteria species have a negative association with *Moniliella* and *S. cerevisiae* in both cocoa varieties (Figure 5.6, $P < 0.05$).

Concerning the difference between the correlation plots of the two cocoa varieties, we observed that more significant correlations were calculated in *Forastero* varieties compared with *Criollo,* in which different or more associations between the minor fungi group (*H. occidentalis, Martiniozyma asiatica, Ogataea, Pichia orientalis, P. pijperi, Saccharomyces cariocanus, Saccharomycopsis, Starmerella,* and *Torulaspora delbrueckii*) are observed (Figure 5.6B, $P < 0.05$).

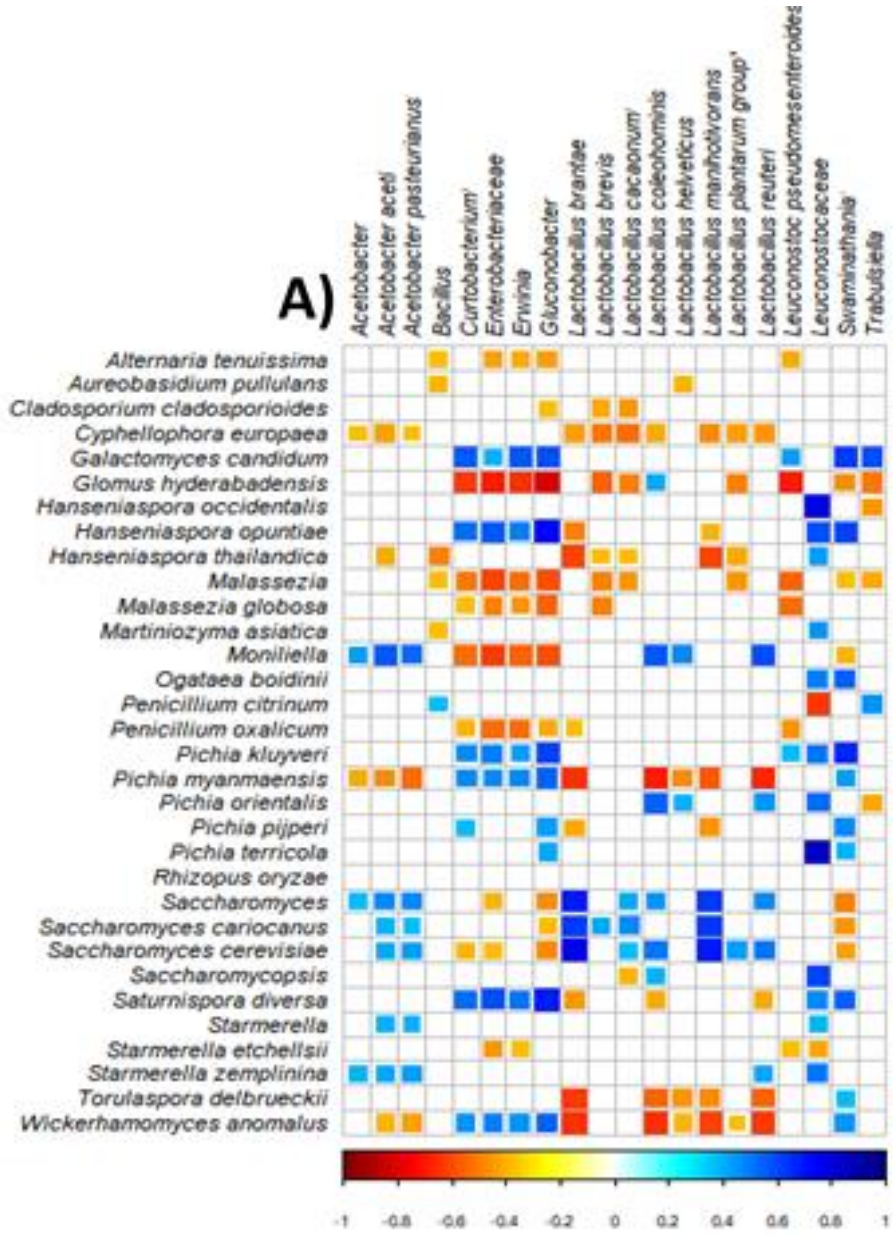


Figure 5.6 Correlation plot showing Spearman's correlation between microbial OTUs observed with an incidence above 1% in at least 2 samples. Figure are label according to the type of cocoa variety **A)** *Criollo* and **B)** *Forastero*. Significance associations between OTUs are only shown ($P < 0.05$). The intensity of the colors represents the degree of correlation between fungal and bacterial OTUs as measured by the Spearman's correlation, where the color blue represents a positive degree of correlation and red a negative correlation between microbial communities

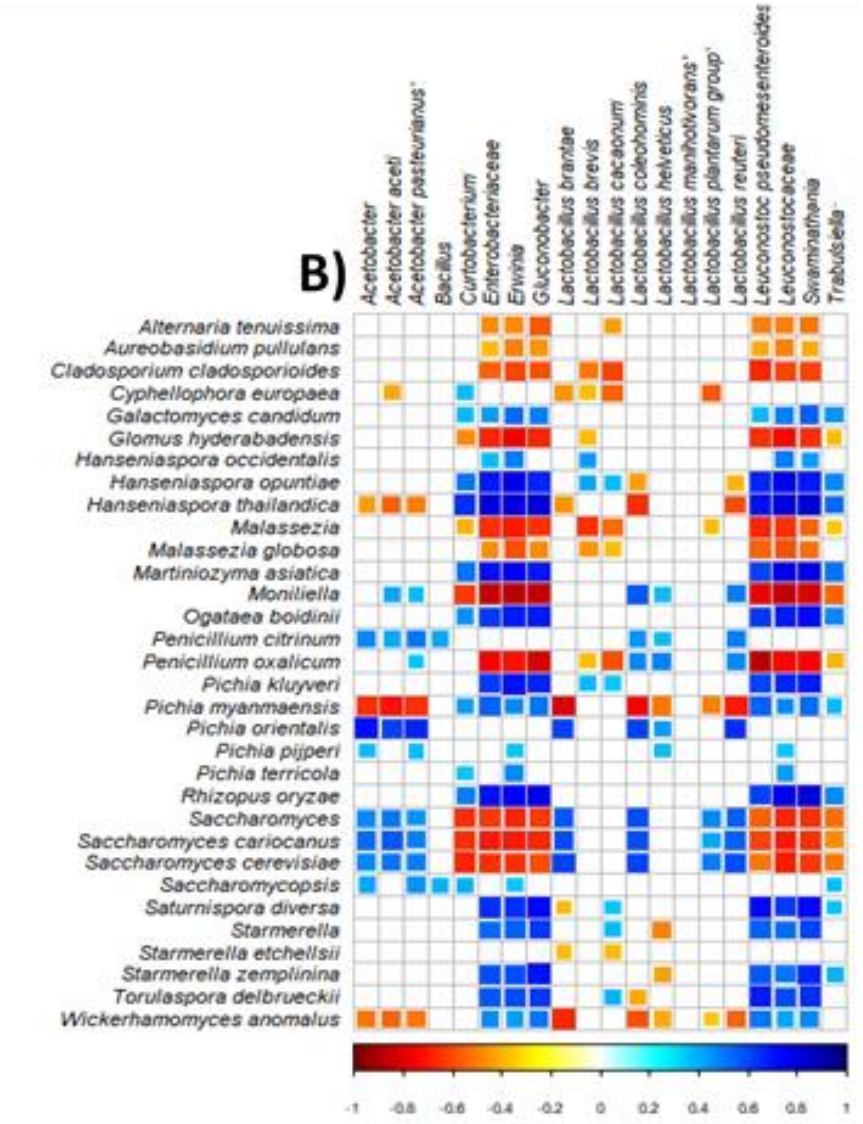


Figure 5.6 Correlation plot showing Spearman's correlation between microbial OTUs observed with an incidence above 1% in at least 2 samples. Figure are label according to the type of cocoa variety **A)** *Criollo* and **B)** *Forastero.* Significance associations between OTUs are only shown ($P < 0.05$). The intensity of the colors represents the degree of correlation between fungal and bacterial OTUs as measured by the Spearman's correlation, where the color blue represents a positive degree of correlation and red a negative correlation between microbial communities

5.3.9 Potential metabolic pathways of fermented cocoa microbiota

The PICRUSt analysis was used to predict the metagenomes from the 16S gene sequences database. The weighted nearest sequenced taxon index (NSTI) for fermented cocoa beans was 0.17 ± 0.08. However, we observed that the branch length that separates each OTUs in the cocoa samples over the fermentation time decreased (0.26 to 0.04). The pathway enrichment analysis (performed by GAGE) of the predicted metagenomes showed that the amino acid, carbohydrate, and energy metabolism were the most abundant during cocoa fermentation and a significant difference between cocoa varieties over a specific fermentation time was observed for some pathways (Figure 5.7A, $P < 0.05$).

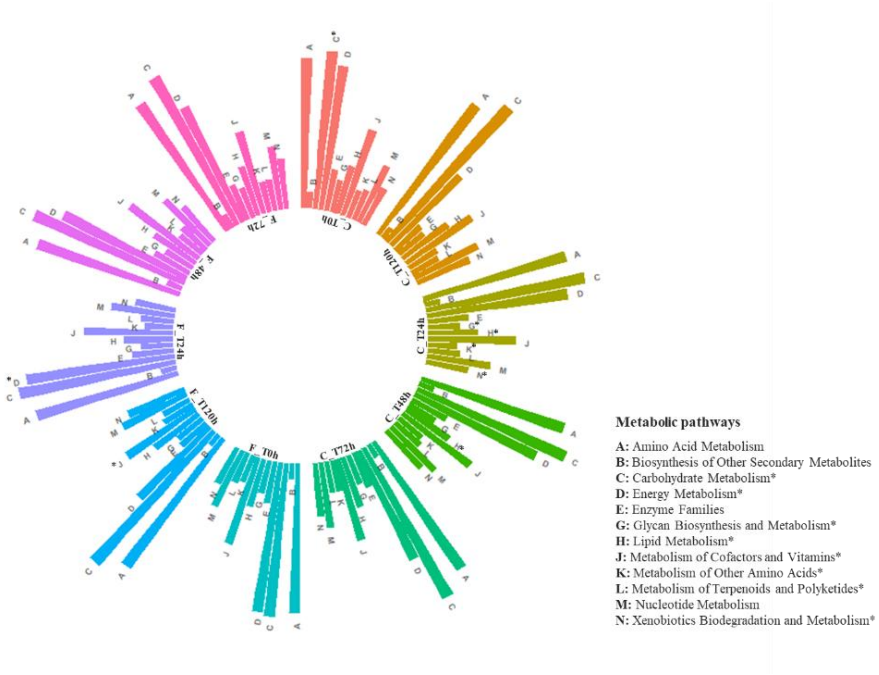


Figure 5.7A Relative abundance of the metabolic pathways identified in fermented cocoa beans. Asterisks indicate higher relative abundance at the specific fermentation time between cocoa varieties using the least significant difference test ($P < 0.05$). *P*-values were adjusted using Bonferroni's method. **Abbreviations:** *Criollo* (C) and *Forastero* (F)

A clear separation between the type of cocoa variety and the metabolism of the main pathways were also observed, in particular, more upregulated metabolic pathways in *Criollo* compared to *Forastero* varieties was observed (Figure 5.7B). Interestingly, *Criollo* varieties were characterized by the upregulation of several amino acid metabolisms such as alanine, aspartate, glutamate, phenylalanine, tyrosine, and tryptophan, while the degradation of valine, leucine, and isoleucine was characterized to *Forastero* samples. Besides the metabolism of the amino acids, *Forastero* samples were also characterized by the degradation of fatty acids, aromatic compounds, and synthesis of a short-chain fatty acid such as butanoate and propanoate (Figure 5.7B).

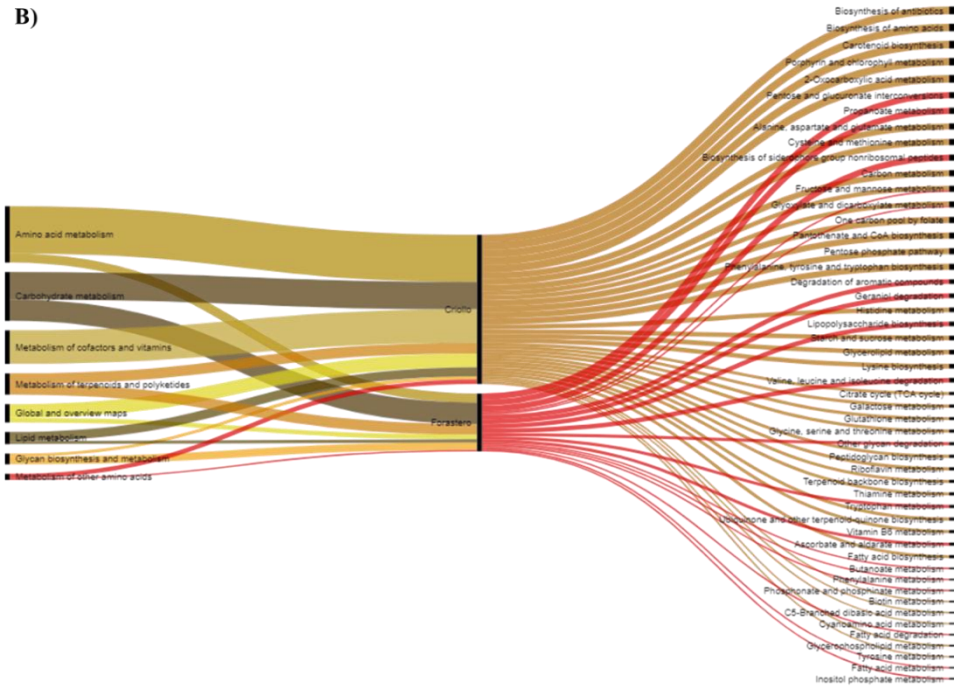


Figure 5.7B Sankey diagram showing the carbohydrate, amino acids and lipid metabolic pathways activated during the fermentation of *Criollo* and *Forastero* cocoa varieties (mustard: *Criollo* and red: *Forastero*)

Further, in the heatmap, we clearly observed two clusters positively correlated between bacterial communities and predicted pathways (Figure

5.7CD). In detail, we observed a positive correlation between *Acetobacter* and *Lactobacillus* species (*reuteri*, and *coleohominis*) with the metabolism of short-chain fatty acids (propanoate, and butanoate), amino acids, carbohydrate, and sulfur for both cocoa varieties (Figure 5.7CD). Interestingly, we observed less stronger correlations between the minor cocoa microbial group (*Gluconobacter*, *Erwinia*, *Swaminathania*, and *Leuconostoc*) and the predicted pathways of *Criollo* varieties compared with *Forastero*. However, both correlation plots linked positively to the minor bacteria group with the biosynthesis of unsaturated fatty acids, and protein metabolism (Figure 5.7CD).

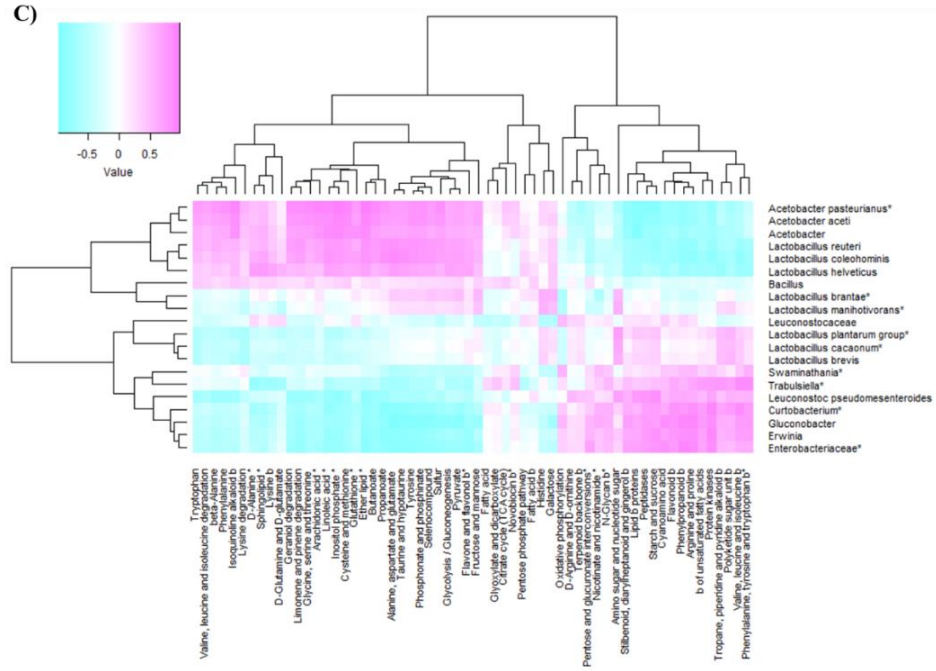


Figure 5.7C Spearman's correlation between metabolic pathways and microbial OTUs observed with above 1% in at least 2 samples. Figures are label according to the cocoa variety **C)** *Criollo* and **D)** *Forastero*. The intensity of the colors represents the degree of the correlation with pink (positive degree of correlation) and blue (negative degree of correlation) between metabolic pathways and bacterial OTUs as measured by the Spearman's correlation

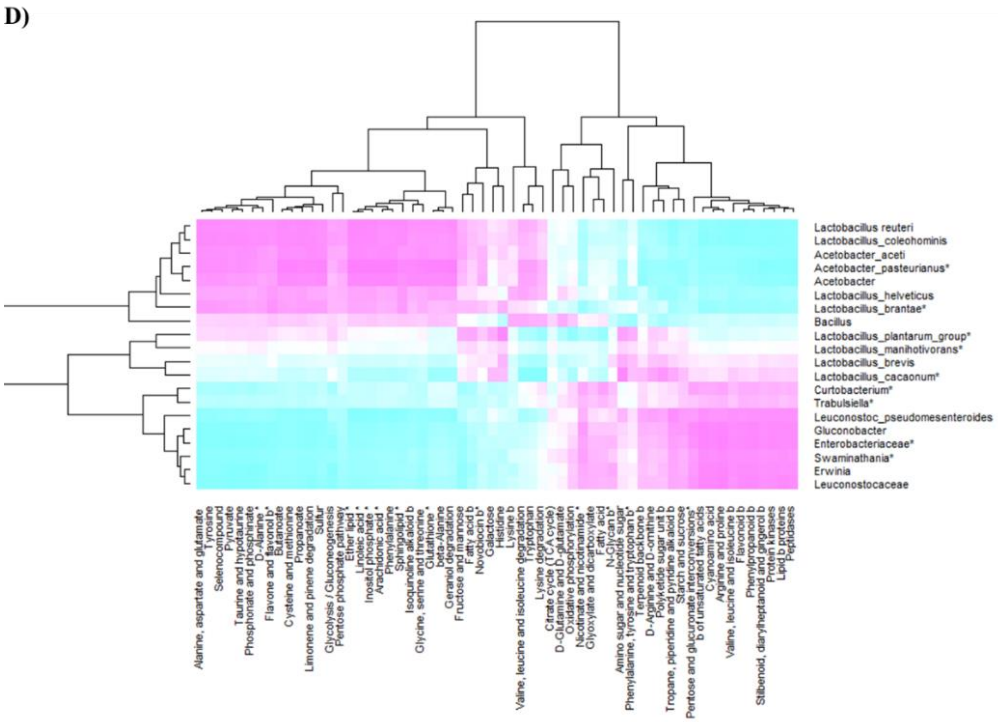


Figure 5.7D Spearman's correlation between metabolic pathways and microbial OTUs observed with above 1% in at least 2 samples. Figures are label according to the cocoa variety **C)** *Criollo* and **D)** *Forastero*. The intensity of the colors represents the degree of the correlation with pink (positive degree of correlation) and blue (negative degree of correlation) between metabolic pathways and bacterial OTUs as measured by the Spearman's correlation

5.4 DISCUSSION

The present chapter aimed at providing an integrated view on the microbial development and mechanism of actions of the metabolite production during cocoa fermentation in two different cocoa varieties (*Criollo* and *Forastero*). The selection of the two different cocoa varieties was used to clarify the differences in the fermentation management of cocoa beans. Therefore, it investigates the differences in microbial composition and functions during fermentation by using classical plate counts and amplicon-sequencing. It also

describes the environmental conditions (temperature, pH) that could affect the growth of microorganisms and the production of important desirable and undesirable metabolites for cocoa beans quantified by GC and perceived by humans.

The microbial succession in fermented cocoa beans can depend on the genetics of the cocoa variety, characteristics of the plant surfaces, environmental conditions, ecological factors, nutrient availability and composition, and harvesting and post-harvesting procedures [11,29–32]. However, classical plate counts revealed that the microbial load of the main microbial groups in the two different cocoa varieties was not different in our study, while the beta diversity calculation in 16S rRNA gene-based on amplicon-sequencing confirmed the impact of the environmental conditions in the composition of the bacterial communities between cocoa varieties. It is important to highlight that classical plate counts have been revealing a lower number of bacteria and yeast compared with independent-methods that could explain the contradictory results as also observed elsewhere [33,34].

Differences between the bacterial communities of the different cocoa varieties can be due to the poor standard hygiene procedures used during harvesting. According to previous literature, spontaneous cocoa fermentations are naturally inoculated from microorganisms found in the surface of the plant material (leaves, pods, and beans) or from the equipment (fermentation boxes, banana leaves, machetes, plastic covers, buckets, sacks, and platforms) [35,36]. However, *S. cerevisiae* one of the most abundant yeasts in fermented cocoa has not been detected in the fruit or the equipment, the presence of this important yeast could be induced by the hands of operators and/or by the air. The importance of the type of material of the vessel that contains the cocoa beans during fermentation is rising attention. Generally, cocoa beans are fermented in wooden boxes or whether place on heaps, which are less adequate for a thorough cleaning, as a consequence, it can increase the possibility of adherence by bacteria

and molds. In accordance with previous literature, cocoa containers made of stainless steel showed lower microbial diversity compared to plastic or wooden containers or heap fermentations, suggesting that wooden boxes should be replaced to improve the quality of the unfermented cocoa beans during fermentation [10,30,36,37].

Many molecular approaches have been used to identify the bacterial and fungal communities in fermented cocoa beans [38]. In this chapter, 30 fungal species and 27 bacterial species in cocoa beans during fermentation were identified using next-generation sequencing, revealing higher diversity than previous studies as also reported elsewhere [11,12,15,16]. The core of OTUs from the Mexican cocoa varieties was dominated by the presence of yeasts (*S. cerevisiae),* AAB *(A. pasteurianus),* and the heterofermentative LAB *(L. plantarum* group*),* as frequently found in fermented cocoa beans [11,12,15–17,33,36,39–41]. Interestingly, the most commonly identify *L. fermentum* from spontaneous cocoa fermentation in Nigeria [42], Ghana [17,43], Brazil [31,43,44], Malaysia [45], Ecuador [35], Nicaragua [12] and Australia [41] was not identified in any of the Mexican cocoa varieties, while *L. cacaonum* detected in our study has been only identified in few studies which highlight the importance of the environmental conditions to determine the denominated restricted microbial cocoa species [16]. Besides the differences in the composition of the major microbial group, it has been recently reported the presence of potential spoilers (*Pseudomonas*) on the surfaces of the microbial population when fermentations are extended beyond 6-8 days, which shed light on the importance of minor microbial groups and the environmental conditions [46]. Here, Miseq sequencing was used as a strategy to search for microbial markers characterizing the cocoa fermentation and to develop a strategy to monitor the process.

Interesting observations can be drawn regarding the statistical correlations plot between the potential interactions between microbial communities, in which the most abundant and minor bacteria group co-occurred with the most abundant yeast species (*S. cerevisiae* and *H. opuntiae*, respectively) in the cocoa environment, while a co-exclusion between these bacterial groups with *Wickerhamomyces* and the minor fungi group was noticed. From these results, we can speculate that the settlement of LAB in the environment could have a valuable effect on the protection against spoilage fungi as supported by our co-exclusion patterns, and positive effect on the activity of fermentative yeast as also supported form other studies [11,47]. To further support our theory, co-cultivation assays involving LAB and cocoa spoilage fungi would be valuable to study in depth their mechanism of competition.

As an attempt to gain more knowledge about the potential pathways that bacterial communities can enrich during cocoa fermentation, a correlation plot dataset was created. Our results revealed that two genera including *Lactobacillus* and *Acetobacter* were determined as functional core microbiota for the production of short-chain fatty acids (butanoate and propanoate), amino acids and sulfur. Our calculations have been also confirmed elsewhere and confirm the accuracy of using predictive models to examine the food ecosystem [17]. More interesting, a recent metagenomic study has reported the aerobic and anaerobic pathways used to synthesize flavor compounds by a mixed cocoa bean microbiome varieties in heap fermentations which was consistent with our results [17]. Further studies are needed to demonstrate the genomic potential and gene expression in the evolution of different fermented cocoa microbial environments through metagenomics and meta-transcriptomic studies.

The ecology of fermented cocoa beans as already observed is a complicated system and their effect on cocoa beans appeared to be related to the production of desirable and undesirable chocolate flavors [11,48,49]. Our results showed that the volatilome profile of both cocoa varieties during fermentation

was characterized by desirable cocoa aromas such as 2-methylbutanal, 3-methylbutanal, phenylethyl alcohol, benzeneacetaldehyde, ethyl acetate, and acetoin, as also previously identified elsewhere [10,20,50–55]. However, undesirable compounds such as isovaleric and isobutyric acids were also found in our samples. In terms of metabolic kinetics, herein acids such as isobutyric, isovaleric and acetic acid increased during the cocoa fermentation while aldehydes, such as 2-methylbutanal, 3-methylbutanal were gradually decreased. This succession tendency might be a potential indicator to ensure successful cocoa fermentation. To the best of our knowledge, this is the first report that assesses the relationship between the structure (genotype) and function (phenotype) of the microbial community in fermented cocoa beans and their role in quality assurance.

Available evidence on the kinetics of substrate consumption and aroma production lead from a successful microbial succession that is accompanied by the rise of temperature, pH, concentrations of acetic acid, lactic acid, aldehydes, esters and alcohols of this system has been extensively studied previously [30,31,45,56,57]. Besides the kinetics of organic acids and VOCs, the changes of protein and peptides, low and high molecular weight carbohydrates, lipids and polyphenols concentrations has been evaluated and proposed as indicators of cocoa fermentation statues [58–64]. However, further research is needed to understand the role of microbial communities on the metabolic changes of these compounds and the development of aroma compounds.

Interestingly, the quantitative data from GC-MS was correlated with the sensory descriptors generated for the two coca varieties over fermentation time and many compounds were correlated to the sensory descriptors. Judges perceived fermented beans as being fruity (apple, pineapple, and honey attributes), which corresponds well with the high concentrations of benzeneacetaldehyde, pheynylethyl alcohol, and ethyl acetate in fermented cocoa

beans. Further, tasters described the evolution of the cocoa fermentation as being more alcoholic and acidic (alcohol, vinegar, and tamarind notes) which corresponds well with the increase of concentrations of ethanol, n-dodecanol, and acetic acid. Therefore, a trained cocoa taster can be considered a useful tool to evaluate the quality and success of the cocoa fermentation. However, we should bear in mind that fermented and roasted cocoa beans are a complex matrix. In this chapter, the volatilome analysis allowed us to differentiate the VOCs profile between cocoa varieties but this was difficult to assess for the trained panelists.

Besides the difference in the bacterial composition and volatilome profile between the cocoa varieties, clear differences between the textural and physical characteristics between cocoa beans varieties were found in this chapter supported elsewhere [4,65]. Interestingly, from our fermentation index analysis, we found that each fermentation box reached a successful fermentation at a different time which shed light on the importance that each box has a unique rate of fermentation and monitoring the process is needed. In contrast, the quality assessment of the two cocoa varieties used in this chapter shows the limitation of the cut-test on *Criollo* varieties to predict indirectly cocoa bean quality, while the fermentation index demonstrated an adequate result to assess the degree of fermentation, which demonstrated the variability in results between boxes and significantly impact the homogeneity. Overall, both methods (cut-test and FI) have been proof that after 120 h cocoa beans were fully fermented which is in agreement with the results obtained in our study and elsewhere [21].

The experimental strategy following the evolution of microbial populations, physico-chemical and quality parameters used in this study provides new information regarding the discrimination of microbial development and aroma formation of two different cocoa varieties over fermentation. We demonstrated that the degree of fermentation and bacterial composition is influenced by the type of cocoa variety used and highlights the importance to generate a color surface descriptor for the fermentation of *Criollo* varieties to be

applied in the cut-test and to monitor bacterial communities during cocoa fermentation to ensure a successful cocoa fermentation. The evolution of the different parameters used in this study has a potential value in the chocolate industry to determine new fermentation management to standardize desirable aroma and flavor cocoa development.

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GENERAL

CONCLUSION

6.1 GENERAL CONCLUSION

This thesis contributes to the research field of microbial ecology as an indicator for quality assurance in fermented cocoa beans by demonstrating predictable microbial growth and development, as well as producing flavor and aroma attributes to determine unique product characteristics. Although more research on the effect and optimization of starter cultures is needed, the results of Chapter 2 are promising in terms of the possibility to alter the volatilome fingerprint of fermented cocoa beans introducing yeast cultures, and the potential of volatile compounds to have a health-promoting benefit. Furthermore, this thesis adds to the increasingly available data on microbial and quality aspects associated with fermented cocoa beans. The effectiveness of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* to guarantee the production of specific and reproducible features of fermented cocoa beans, as shown in Chapter 3, is controversial. More research should determine the effect of environmental conditions and the effect of starter cultures on cocoa fermentation. Chapter 4 shows that primer selection and identification bias are introduced by PCR amplification of fungal ecology. The capacity of 26S targeting region to provide greater fungal rRNA taxonomic depth compared with ITS2 is promising. However, a careful primer evaluation and validation, and comparisons with more region-specific primers for fermented food ecosystems are needed. Microbial ecology in fermented cocoa beans is undergoing a profound change due to the preference of studying microbial communities using next-generation sequencing technologies (NGS). In this context, Chapters 3 and 5 show differences in the microbial composition when fermentation was assessed from different countries using NGS. Although more research on the effect of microbial communities in the development of aromatic compounds and how are they produced it is needed, results of Chapter 5 are auspicious in terms of understanding the metabolic pathways activated by bacterial communities during spontaneous cocoa fermentation from two different cocoa varieties.

6.2 MICROBIAL DIVERSITY

The -omics sciences seem to be an excellent alternative to understand the diversity and functionality of the microorganisms involved in the fermented cocoa ecosystem. This thesis highlights the limited availability of -omics analysis to date, to compare microbial diversity and functionality involved in cocoa bean fermentation ecosystem around the world. Few studies have been conducted on the reconstruction of the microbial meta-pathways based on metagenomic data of fermented cocoa beans. In 2015, Illeghems and col. showed that the central metabolic pathways associated with lactic acid bacteria (LAB) were heterolactic fermentation and citrate assimilation, while acetic acid bacteria (AAB), was only partially reconstructed and were involved in responses toward stress [1]. However, this study only analyzed fermented cocoa after 30 h. Therefore, it is necessary to perform more research over the cocoa fermentation time to achieve a full panorama of the complex microbial functionality of the cocoa bean fermentation ecosystem. From a molecular perspective, concerning the identification of especially yeast, there is an evident need for more extensive identification of fungal species. Challenges and opportunities in understanding the complexity of microbial diversity and interactions in fermented cocoa beans under a control fermentation system guided by a dominated microbial species might help us to ensure fermented cocoa bean with homogenous quality.

6.3 CHALLENGES AND PERSPECTIVES CONTRIBUTING TO THE USE OF STARTER CULTURES IN COCOA FERMENTATION

The success of the starter culture is highly influenced by the environment and/or agricultural practices of the country where the cocoa is produced [2]. For this reason, it is recommended that each cocoa producing region characterize the

autochthonous microbial diversity involved in this process. To this regard, most studies of microbial diversity in fermented cocoa bean have been conducted in Africa (Ghana, Cameroon, Nigeria, and Ivory Coast) [2–16], Southeast Asia (Malaysia, and Philippines) [11,16–19], South America (Brazil, Bolivia, and Ecuador) [15,16,28–30,20–27], North America (Mexico) [31], Central America (Cuba, and Honduras) [32,33] and Oceania (Australia) [34,35]. Interestingly, in Mexico, a country with a deep-rooted tradition of production and consumption of cocoa, we found only one study describing the yeast diversity in fermented cocoa beans. More studies are needed to characterize the cocoa microbial diversity during fermentation to design a suitable mixed starter culture to conduct the fermentation process and to obtain a fermented cocoa bean with competitive quality in the international market.

Starter cultures in cocoa beans have an effect on the quality and safety of the end product and should be therefore taken into account when selecting the starter strains, especially when LAB or AAB are used as starter cultures. It is imperative to select appropriately the microorganisms that will be part of this culture, based on the functional, physiological, and biochemical criteria of each of the microbial strains, and assess which characteristics are expected to be relevant depending on the starter culture used. For example, yeast cultures would be more relevant to produce alcohols and esters, while LAB could overproduce lactic acid and therefore having a negative impact on the end product.

However, the addition of microbial cultures will not be able to standardize the whole fermentation process alone. Thus, the design and construction of a fermentation system that allows this process to be carried out under controlled conditions, and thus avoiding microbial contamination with a probable reduction of the fermentation time, need to be considered. A fermentation system could allow us to have a standardized, mechanized, and reproducible cocoa bean fermentation process. The design of stainless steel

bioreactors has been proposed to carry out this process [29]. Though, other process conditions must be taken into consideration, such as a specific rate of agitation, adequate control of temperature and pH following the microbial succession pattern and they must have a system that allows the elimination of waste from the process. It should be noted that the design of the starter culture and a suitable fermentation system may allow us to control and conduct this process. More search is needed into the effect of processing, shelf-life, and storage of dry cocoa beans previously inoculated with a starter culture during cocoa fermentation, since the microbial composition in these stages may also change, and as a consequence, nutrient composition, sensory parameters, and food safety, as well.

6.4 SCALING UP PRODUCTION

The growing demand for high-quality chocolate is increasing in recent years, as a result, much research has contributed to optimizing the production process, leading to the exploration of starter cultures during cocoa fermentation. In contrast, the use of starter cultures in large scale production is still at an early stage. Currently, chocolate companies together with research centers have started using commercial yeast cultures or autochthonous microorganisms to direct this fermentation process. However, the available knowledge highlights the lack of characterizing autochthonous microbial diversity by region of all or key producing cocoa countries might pose a barrier for chocolate companies. Research into microbial ecology together with the automation of large-scale fermentation is thus recommended. Starter cultures suitable for large scale fermentation production should among others have the capability to grow in sucrose and glucose, tolerate heat, oxidize lactic acid, survive in alcoholic and acidic environments and to produce a high rate of aromatic compounds. A critical step during this process is the fermentation mass of different cocoa varieties in

the system. Chapter 5 demonstrated that the degree of fermentation is influenced by the type of cocoa variety used and highlights the importance to generate a method or marker to monitor the fermentation degree. Reducing the fermentation time through the use of starter cultures or implementing new fermentation management should decrease the production cost of chocolate. Producing chocolates inoculated with starter cultures might aid in the process of cost reduction, however, more research is needed, to identify at strain level with analytic and sensorial data to evaluate the potential of each microorganism (yeast and bacteria), as well as more regulation, to explore the possibilities of using food cultures to conduct the cocoa bean fermentation.

6.5 CONSUMER AWARENESS AND LEGISLATION

Consumer perceptions are a major factor determining the success of the inclusion of starter cultures in chocolate. Therefore, studies on consumer acceptance of chocolate previously inoculated with microbial cultures, need to be assessed to guarantee food acceptability. As an attempt to contribute to this matter, Chapter 5 shows how a trained panel successfully perceived the sensorial changes during cocoa fermentation as a function of the microbial metabolic activity and development, while the difference between cocoa varieties was not achieved. However, positive sensory perceptions or consumer beliefs, such as acceptability taste and beliefs if the product might be harmful or unhealthy were arguments not consider in this thesis.

Consumer beliefs is an argument that deserves consideration, although several studies found that starter cultures enhance the quality of chocolate might not be enough to convince consumers to accept this product. A strategy that might motivate the acceptability of a new product is to provide information on the

recognition of the importance of starter cultures during fermentation. However, this strategy may also not convince the consumer.

Food cultures are defined as live bacteria, yeasts or molds used in food production in the meat, dairy, wine and health food industry [36]. In Europe, several entities are involved in the assessment of the safety of the use of food cultures. In this context, starter cultures are evaluated at the strain level, during production and throughout the shelf-life [37]. As a result of the assessment of a broad range of biological agents the European Food Safety Authority (EFSA) has published a qualified presumption of safety list to recommend the use of bacteria, yeasts and molds as sources of food and feed additives [38]. However, at present, no clear legislation regarding the use of starter cultures for the chocolate industry exists within the European Union. Industrial guidelines of quality control for starter cultures used in cocoa fermentation, including quality and food safety management, product information and methods of analyses are currently not included in food regulations.

6.6 CONCLUSION

A large number of volatile compounds are produced during microbial growth in cocoa fermentation. This production is dependent on the type of cocoa variety, fermentation time, and fermentation methods used, mostly explained by the metabolic pathways activated through microbial communities influenced by its own microenvironment. However, the addition of starter cultures to enhance the production of aromatic compounds in this thesis during cocoa fermentation provided no additional effect. These results shed light on the importance of the complexity of the microbial consortia and environmental conditions.

Food microbiology, food technology, food engineering, and consumer science are vital in the chocolate industry. Research determining a microbial marker for monitoring the quality and degree of fermentation and food safety as

well as optimization of the process of fermented cocoa beans could form the basis in the development of coherent guidelines and regulations on the use of starter cultures as an enhancer of chocolate quality. This is necessary not only to guarantee the quality but also to provide the consumer with a safe product and help cocoa farmers to reduce fermented cocoa loss. Chocolate products inoculated during fermentation that may appear on the food market in the present or the future should thus be clearly labeled with the food additive information to protect and increase consumer's awareness.

6.7 REFERENCES

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C ROMAN CHALO RUBEN AZU PRIMAVERA TIA CHATA TIA ESTELA TIA ROSI BETSY ANA

J H IRENE MYRTE **TO MY FAMILY** ABUE EMETIO ELIUD TIO LUIS TIO FER TIA NOHEMI

IVAN JESSI YURI ALEJANDRO DANI BENJA INGRID

"THERE WAS A TIME, I USED TO LOOK INTO MY FAMILY'S EYES MAYTE MARINANA

O A ERIK MAIKE ME TIO BENJA NADIA TIO RUBEN TIA ROSAURA TIO ALEJANDRO ANGELICA

IN A HAPPY HOME, I WAS STANDING IN THE LIGHT AND SEEN AS I AM. TIA MORE LUCY

ABRIL DANIEL PIZ CARMEN PATY IRIS PAO ARELY TIA SANJUANA CATERINA

R R THOSE DAYS ARE GONE, NOW THE MEMORIES ARE ON THE WALL CESAR LOLI HUGO THAN

TANIA ANITA LLUVIA MALOU PRIYA MAGDA SARA PHANIE ALONDRA ALBERTO LORENA

G I I HEAR THE SOUNDS FROM THE PLACES WHERE I WAS BORN" ISABEL RICHARD ARA TIO MARIO

NATALI SOL RUTH JULIANA ADRIAN ELY JORCH LUPITA FRANCISCO ALEJANDRO MAYRA ABRAHAM

E T TIO CHECO SILVIA LAURA ANDREA KARO TIA LALA KARLITA AMY ITALIA MARIO ABRIL

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JUAN NILOO PATY ZAVALA VERO RAMOS GERARDO HOMERO
"TO REACH FOR THE BRANCH AND CLIMB UP
FRANCISCO BRENDAN ILEANA ROSEL
LUCA MIRNA LETRI JOSUE LUZ DIXIA ALIDA KELLY
LEAVING EVERYTHING BEHIND

TO MY PROFESSORS

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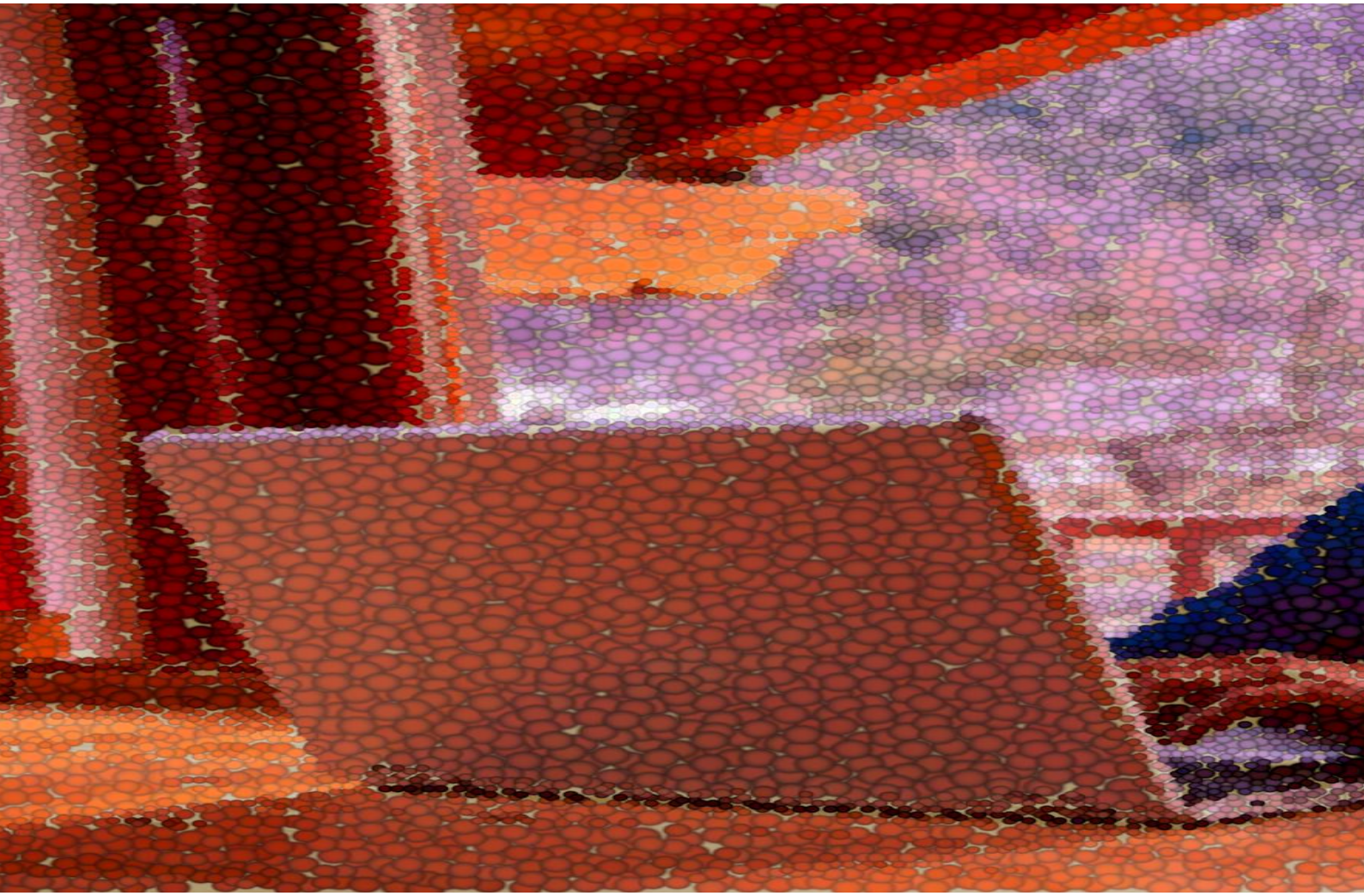
CRISTIAN G. ZEPPA HUGO CLAUDIA ILARIO YOLANDA
OSCAR NORBERTO JORDAN
FOR BELIEVING ON ME AND ABDEL
I CARRY THE MUSIC, THE MEMORIES AND KEEP THEM INSIDE
ARLETT SERGIO MIGUEL ARTURO ALEJANDRO JUDITH
SARAH V. BLANCA CHALLENING ME MANUELA
LAUGH EVERY DAY AND FEELING THE TEARS FALLING DOWN
PATY QUEVEDO DINDO MARTHA
CLAUDIA DANIELA GUSTAVO FAFI MIRIAM
GUILLERMINA CLAUDIA FOFO
I STILL HEAR THE SONGS, REMAINING ME OF MY FRIENDS"
DENIS NADIA ANNYA AMARA DANIELLE MARIO TRISH
MICHELLE CASANDRA PONCHO BLANCA

TO MY FUTURE

"I THINK OF YOU NOW AND THEN
DULAYM ISABELL BRENDA CRISTINA ANTONIO ELIDA FRANCIS
WITH ARMS OPEN WIDE"

WITHOUT ALL OF YOU, I WILL NOT BE STANDING IN THE LIGHT



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PUBLICATIONS

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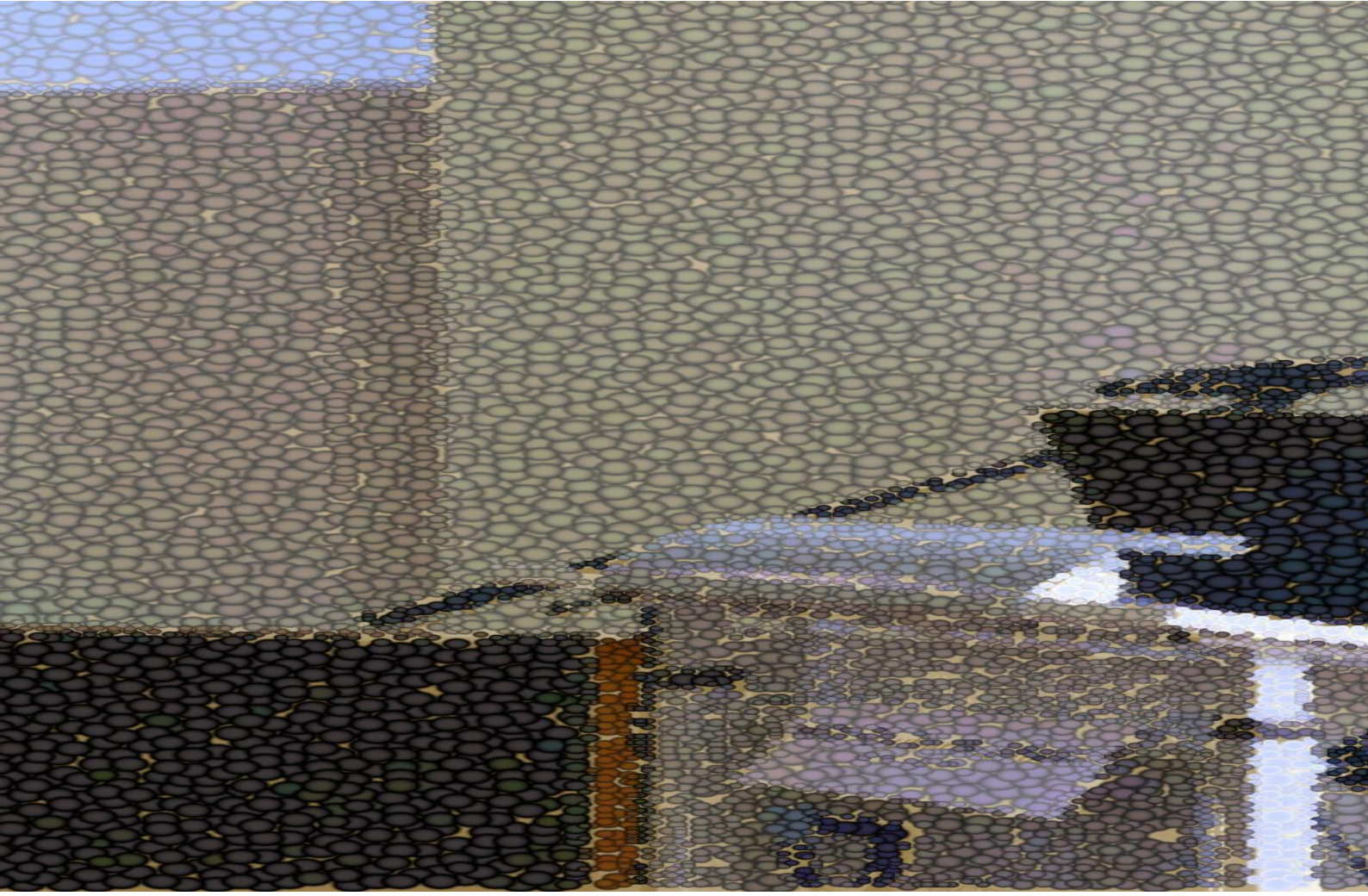
IN PREPARATION Mota-Gutierrez, J., Ferrocino, I., Hernández-Estrada, Z., González-Ríos, O., Cocolin, L., and Suárez-Quiroz, M. L. Bacteria and yeast identify during cocoa fermentation: exploring its functional capabilities and flavor formation



EDUCATIONAL

ACTIVITIES

FERMENTED COCOA MICROBIOME APRIL 2020

INTRODUCTION

With the completion of the study below described. This chapter described the activities the PhD student attended over the last 3 years, including academic courses, workshops, seminars, international and national congresses, study abroad, the publications successfully accepted by international journals, and extra-activities and awards.

EDUCATIVE ACTIVITIES AND CONFERENCES

Courses. A total of 49 credits were obtained after attending the courses described in Table 1.

Table 1. List of post-graduate courses attended

Year	Course's name	Organized	Dates	Place
1	Research writing	DISAFA	30th May 2017	Grugliasco, Italy
2	Research presentation	DISAFA	27th October 2017	Grugliasco, Italy
1	Bibliographic research	DISAFA	17th July 2017	Grugliasco, Italy
2	Food chemistry	Instituto Tecnologico de Veracruz	2nd – 6th July 2018	Veracruz, Mexico
2	Data Science:R basics	Harvard University	13th May 2018	Online-course
2	Summer School	SIMTREA	3rd – 7th September 2018	Florence, Italy
3	Summer School	UNITO	20th – 21st June 2019	Bardonecchia, Italy
3	Global Food Venture Program 2019 Summer School	EIT Food	2nd – 12th June 2019	Madrid, Spain
3	Global Food Venture Program 2019 European Bootcamp	EIT Food	14th – 19th July 2019	Laussane, Switzerland
3	Global Food Venture Program 2019 International Bootcamp	EIT Food	11st -25th August 2019	San Francisco, USA

Seminars. A total of 6 credits were obtained after attending the seminars described in Table 2.

Table 2. List of post-graduate seminars attended

Year	Seminar's name	Organized	Date	Place	Hour
1	NMR: New Applications in Chemical, Pharmaceutical and Agro-food Sectors	UNITO	1//12/16	Turin	7
1	Workshop on the transfer of knowledge for doctoral students	UNITO	14/12/16	Turin	4
1	An introduction to good practice for Investigators and site staff	St. James Hospital	20/01/17	Dublin	3
1	Building your career in the EU	Scienze della vita e della salute	08/03/17	Turin	8
1	Preparing your next professional step	UNITO	4/12/2017	Turin	9
1	Food Microbiology	DIFASA	3/21/2017	Turin	4
3	Seminar R	DISAFA	11/22/2018	Turin	7
3	Fundraising for research projects: an opportunity to be seized	UNITO	10/24/2018	Turin	4
3	The potential of the doctorate outside the academy	UNITO	12/18/2018	Turin	4

Conferences

- Poster presentation **(2019)**. Mota-Gutierrez, J; Ferrocino, I; Suarez-Quiroz, M.L; Gonzalez-Ríos, O; Cocolin L. Functional attributes of microbial communities of different fermented cocoa varieties and its influence on the aroma and flavor formation. *5th International Conference on Microbial Diversity, Catania, Italy*, September 25th to 27th, 2019. ISBN 978-88-943010-1-4
- Oral presentation **(2019)**. Mota-Gutierrez, J. Microbes in cocoa beans: What microbial communities can do on the formation of cocoa aroma during fermentation. *XXIV Workshop on Developments in the Italian Ph.D. research on Food Science, Technology and Biotechnology, Florence, Italy*, September 11th to 13th, 2019
- Poster presentation **(2019)**. Mota-Gutierrez, J; Ferrocino I, Botta, C; Giordano, M; Cocolin, L. The effect of yeast cultures on the microbial composition and fermented cocoa volatile compounds. *8th Congress of European Microbiologist 2019, Glasgow, Scotland*, July 7th to the 11th, 2019
- Oral presentation **(2019)**. Mota-Gutierrez, J; Suarez-Quiroz, ML; Gonzalez-Ríos, O; Ferrocino, I; Cocolin, L. A matter of chocolate aroma: what microbial communities can do on the formation of sensorial attributes in the early stages of the cocoa fermentation. *5th International Cocoa, coffee and tea 2019, Bremen, Germany*, June 26th to 28th, 2019
- Poster presentation **(2019)**. Mota-Gutierrez, J; Suarez-Quiroz M.L; Gonzalez-Ríos O; Cocolin, L. Dynamics of the moisture content of cocoa

beans during fermentation. *1st AISSA#under40, San Dona di Piave, Italy,* May 16th -17th, 2019

- Poster presentation **(2018)**. Mota-Gutierrez; J; Ferrocino, I; Botta, C; Giordano, M; Bertolino, M; Dolci, P; Cannoni, M; Luca Cocolin, L. Dynamics and biodiversity of bacterial and yeast communities during fermentation of cocoa beans. *26th International IDFMH Conference FoodMicro, Berlin, Gemany,* September 3rd – 6th 2018
- Poster presentation **(2018)**. Mota-Gutierrez J. Microbes in fermented cocoa beans: Evolution and chemical profile. *XXIII Workshop on the Developments in the Italian Ph.D. Research on Food Science Technology and Biotechnology, Oristano, Italy,* September 19th – 21st, 2018
- Poster presentation **(2017)**. Mota-Gutierrez J. Microbes in cocoa beans, fermentation and chemical profile: Yeast ecology in box and heap fermentations of cocoa beans in Cameroon. *XXII Workshop on the Developments in the Italian Ph.D. Research on Food Science Technology and Biotechnology, University of Bozen, Bozen, Italy,* September 20th-22nd, 2017
- Poster presentation **(2017)**. Mota-Gutierrez; J; Ferrocino, I; Botta, C; Giordano, M; Bertolino, M; Dolci, P; Cannoni, M; Luca Cocolin, L. Polyphasic comparison of yeast population's dynamics involved in box and heap fermentations of cocoa beans in Cameroon. *4th International Congress on Cocoa Coffee and Tea, Turin, Italy* July 25th – 28th, 2017
- Oral presentation **(2017)**. Mota-Gutierrez; J. Polyphasic comparison of yeast population's dynamics involved in box and heap fermentations of cocoa beans in Cameroon. Seminar, organized by *the School of Agricultural, Forest and Food Sciences, Grugliasco, Italy*. March 21st, 2017

RESEARCH ABROAD

- Instituto Tecnologico de Veracruz, Mexico **(2018).** Fermentative/roasted metabolites and volatile compounds in cocoa beans.

AWARDS

- Final Evaluation Attendance and Travel Grant **(2019)**. Global Food Venture Program financed by the *European Institute of Food Innovation and Technology* (EIT Food)
- International Bootcamp Attendance and Travel Grant **(2019)**. Global Food Venture Program financed by the *European Institute of Food Innovation and Technology* (EIT Food)

- European Bootcamp Attendance and Travel Grant **(2019)**. Global Food Venture Program financed by the *European Institute of Food Innovation and Technology* (EIT Food)
- Summer School Attendance and Travel Grant **(2019)**. Global Food Venture Program financed by the *European Institute of Food Innovation and Technology* (EIT Food)
- Congress Attendance Grant **(2019)**. 8th Congress of the European Microbiologist 2019 financed by *Federation of European Microbiological Societies*
- Social Media Course **(2019)**. Financed by the *Federation of European Microbiological Societies*
- Summer School Attendance Grant **(2018)**. Computational Analysis: From Genomic Diversity to Ecosystem structure financed by the *Societa Italiana di Microbiologia Agraria, Alimentare e Ambientale*

TEACHING ACTIVITIES

- 1.4 h seminar for master's students **(2019).** Cocoa fermentation. What microbial communities can do on the aroma formation? *University of Turin, Grugliasco, Italy*, December 18th, 2019
- 3.2 h seminar for master's students **(2019).** Current understanding of food microbiome in the era of big data. *University of Turin, Cuneo, Italy*, December 11th, 2019
- 1.5 h seminar for master's students **(2019).** Cocoa fermentation. What microbial communities can do on the aroma formation? *University of Turin, Cuneo, Italy*, December 5th, 2019
- 2 h seminar for bachelor's students **(2018)**. Steps for writing a project proposal: The case study of fermented cocoa beans. *Instituto Tecnologico de Veracruz*, *Mexico,* February 6th, 2018
- 2 h seminar for bachelor's students **(2018)**. How to study abroad? *Instituto Tecnologico de Tabasco*, *Mexico,* March 13th and 14th, 2018
- 1.3 h seminar for master's students **(2018)**. Microbes in fermented cocoa beans. *University of Turin*, *Grugliasco, Italy,* December 17th, 2018

INVITED REVIEW OF (UNPUBLISHED) JOURNAL MANUSCRIPT

- International Journal of Food Microbiology (2019)
- International of Food and Agriculture (2019)

DISCUSSION GROUPS

- University of California, Davis lunch meeting **(2017)**. Mycobiota in cocoa beans. *School of Agricultural, Forest and Food Sciences, Grugliasco, Italy.* 10th, October 2017
- Purato's lunch meeting **(2019)**. Fermented cocoa. *School of Agricultural, Forest and Food Sciences, Grugliasco, Italy.* 30th, April 2019

ORGANIZING SEMINAR

- Colloquium in Food Microbiology **(2017).** *School of Agricultural, Forest and Food Sciences, Grugliasco, Italy.* March 21st, 2017

SCHOOL OF AGRICULTURE, FORESTRY AND FOOD SCIENCE ANNUAL MEETINGS

- Oral presentation **(2017).** Microbes in cocoa beans. *School of Agricultural, Forest and Food Sciences, Grugliasco, Italy.* 15th, September 2017
- Oral presentation **(2018).** Metataxonomic comparison between internal transcribed spacer and 26S ribosomal large subunit (LSU) rDNA gene. *School of Agricultural, Forest and Food Sciences, Grugliasco, Italy.* 18th, September 2018

ACADEMIC ROLES

- Ph.D. representative of the academic board of the School of Agricultural, Forest and Food Sciences **(2019)**. University of Turin, *Italy*
- Ph.D. representative of the School of Agricultural, Forest and Food Sciences **(2019)**. University of Turin, *Italy*