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Microbes in Cocoa beans: What Microbial Communities can do on the formation of Cocoa Aroma during Fermentation

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Cocoa fermentation is a complex process addressed by a vast number of autochthonous microorganisms. The present dissertation asses the microbial diversity found in fermented cocoa beans to determine effective parameters for optimizing this process. Two different cocoa varieties, fermented in Cameroon and Mexico were used to describe the physicochemical, microbiological, and sensorial quality of fermented cocoa beans. These biological samples were also used to establish the development and optimization of targeted molecular method to asses' fungal communities. Overall, this work highlights the perspectives and challenges of the standardization of the fermentation process and the selection of starter cultures for fermented cocoa beans.

Microbi nelle fave di cacao: quali comunità microbiche possono fare sulla formazione dell'aroma del cacao durante la fermentazione

La fermentazione del cacao è un processo complesso affrontato da un vasto numero di microrganismi autoctoni. La presente tesi analizza la diversità microbica trovata nelle fave di cacao fermentate con l'obiettivo di determinare parametri efficaci per l'ottimizzazione di questo processo. Due diverse varietà di cacao, fermentate in Camerun e in Messico, sono state utilizzate per descrivere la qualità fisico-chimica, microbiologica, e sensoriale delle fave di cacao fermentate. Questi campioni biologici sono stati anche usati per stabilire lo sviluppo e l'ottimizzazione del metodo molecolare mirato per valutare le comunità fungine. Nel complesso, questo lavoro evidenza le prospettive e le sfide della selezione delle colture starter per la fermentazionedelle fave di cacao.

Key words: Criollo, forastero, bacteria, yeast, quality, starter cultures

1. Introduction

In accordance with the Ph.D. thesis project previously described (Mota-Gutierrez, 2017), this oral communication reports the main results of the following five activities directed to:

1) describe the physicochemical, microbiological and sensorial evolution of fermented cocoa beans in box and heap fermentations
2) assess the co-occurrence and/or co-exclusion relationships among the cocoa microbial communities through a pairwise correlation matrix adjusted for multiple comparisons
3) correlates the concentration of metabolites produced during cocoa fermentation and the presence of microbial communities
4) explores the potential metabolic pathways and interactions between bacterial communities

5) compare two different target regions through next-generation approaches to assess fungal communities

2. Functionalities of Microbial Communities in Fermented Cocoa Beans

Microbial communities are responsible for the unique functional properties of 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) (Mota-Gutierrez *et al.,* 2018; Nielsen *et al.,* 2007). 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 this fermentation process, producing ethanol, organic acids, and VOCs, that contribute as precursors of chocolate flavor (Schwan & Wheals, 2004). 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 thesis aims to describe the diversity and evolution of the microbes during cocoa beans fermentation carried out spontaneously and in presence of yeasts starter cultures, either in boxes or heaps to acquire deeper knowledge about the relationship of microorganisms between each other and their surroundings. In this thesis, we also proposed the measurements of associations between microbial communities and the development of microbial 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 to produce high-quality cocoa.

3. Experimental Procedure

In this Ph.D. thesis a transdisciplinary approach was set up by performing in sequence the following independent tests, that is i) physicochemical analysis including the measurement of temperature, pH, and the identification and quantification of volatile and non-volatile compounds, using HPLC and GC-MS analysis; ii) classical and molecular microbiology analysis using plate counts and amplicon-based sequencing targeting 16S and 26S rRNA, respectively; iii) sensory evaluation by quantitative descriptive analysis; iv) correlation analysis between the chemical and molecular analysis and between microbial communities; v) metataxonomic comparison between two different target regions for the identification of fungal communities using next-generation sequencing. A sixth approach was added to explores the potential metabolic pathways and interactions between microbial communities to investigate the microbial metabolic activity and development of fermented cocoa beans.

4. Materials and Methods

Two different cocoa varieties (*Criollo* and *Forastero*) inoculated or non-inoculated from Cameroon and Mexico were used to identify the microbes that could contribute to the aroma generation of the cocoa flavor during box or heap fermentations (B and H). The

lyophilized strains S*accharomyces cerevisiae* ID67 and *Torulaspora delbrueckii* ID103 were provided by Lallemand, (Canada, Quebec, Montreal) and were used as starter cultures in farmer-scale cocoa bean fermentations carried out only in Cameroon, while non-inoculated cocoa fermentations were asses in Cameroon and Mexico. The dynamics of non-volatile and volatile organic compounds (VOCs) of fermented cocoa beans-pulp under different conditions were obtained applying chromatographic methods (Rodriguez-Campos *et al.,* 2011). The assessment of the microbial population was performed by culture-independent analysis described elsewhere (Klindworth *et al.,* 2013; Tedersoo *et al.,* 2015). Last but not least, a mock community containing twenty fungal species was prepared to validate the performance of the two target regions (Mota-Gutierrez *et al.,* 2019).

5. Statistical analyses

Statistical analyses were carried out using generalized linear mixed-effects models for non-normally distributed data set and anova for normally distributed data. Mixed models were chosen for their ability to capture both fixed (fermentation condition: inoculated with S, ST and non-inoculated and fermentation time: 0-120 h) and random effects (fermentation type: B and H or cocoa varieties: C and F). The *P*-values were adjusted using Bonferroni's method when the test revealed significant differences ($P < 0.05$) the Duncan honestly significant difference (HSD) test was applied. Mixed models were built using R version 3.3.2.

6. Bioinformatics

Paired-end reads (2x250 bp) were first merged using the FLASH software (Magoč & Salzberg, 2011), with default parameters. Joint reads were further quality filtered (Phred < Q20) using the QIIME 1.9.0 software (Caporaso *et al.,* 2011). 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 (Edgar, 2010) 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. For the 16S data, each cluster was used to assign taxonomy by mapping against the Greengenes 16S rRNA gene database, version 2013 as described recently (Ferrocino *et al.,* 2017). Sequences were double-checked using the BlastN search tool (http://www.ncbi.nlm.nih.gov/blast/) to confirm the taxonomy assignment. Weighted and Unweighted UniFrac distance matrices, as well as OTUs table, were used to find differences between fermentation processes (B and H), under different conditions (inoculated and non-inoculated) and between cocoa varieties by Adonis and Anosim statistical test in R environment in order to avoid biases due to different sequencing depths, all samples for each dataset were rarefied at the lowest number of reads after raw read quality filtering. QIIME was used to produce a filtered OTU table at 1% in at least 2 samples. The OTU table displays the higher taxonomy resolution reached when the taxonomy assignment was not able to reach the species level, genus or family name was displayed. As a measure of the association between microbial OTUs occurring in at least 2 samples and chemical variables, the Spearman's rank correlation coefficient was obtained through the function *corr.test* and *corrplot* and plotted through the *psych* and *corrplot* package of R, respectively. OTUs occurring in at least 2 samples from microbial communities were conglomerate by hierarchical clustering analysis using Ward's method acquired through the function *heatmap.2* plotted through the *gplots* package of R.

7. Results and Discussion

7.1 Physical and microbiological changes throughout cocoa fermentations

No significant difference ($P > 0.05$) between the conditions used (inoculated and non-inoculated) or cocoa varieties was observed through physical and microbiological analysis, while temperature and pH observed during B and H fermentations significantly increased the end of the fermentation (data not shown). During fermentation, cocoa beans constitute an ecological niche for a wide range of microbes. The advances in studying the dynamics of cocoa microbial communities have shown that the composition of these communities follows predictable patterns reporting a rapid decline in yeast counts after 48 hours when the sugars are depleted, the temperature rise and LAB and AAB increase.

7.2 Dynamics of non-volatile and volatile organic compounds during cocoa beans fermentation

The evolution of non-volatile compounds was determined during B and H fermentations of different cocoa varieties. No significant differences between inoculated and non-inoculated fermentations were observed through non-volatile and volatile compounds analysis at the end of the cocoa fermentation from Cameroon (data not shown). However, the volatilome Box fermentations showed a faster carbohydrate metabolism and higher production of organic acid compounds than in heap fermentations, which boosted the formation of alcohols and esters. As expected statistical difference between the volatilome profile of two different cocoa varieties were observed (data not shown).

7.3 Performance of primers targeting ITS2 and 26S rRNA in mock communities

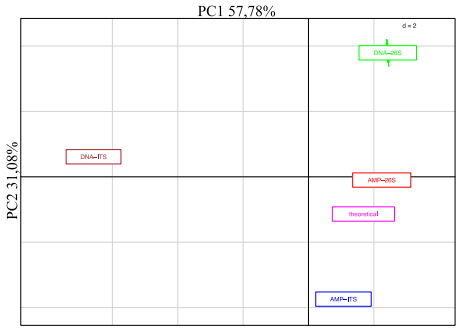

Figure 1. *Principal component analysis (PCA) based on mock mycobiota composition*

A significant difference in mycobiota composition ($P < 0.05$) by using the two target regions or mock communities (DNA or AMP) was observed by Principal Component Analysis (Figure 1). 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 estimation is not valid for all the species analyzed. The variability in the amplification lengths observed in our results might be contributed to the preferential amplification phenomenon.

7.4 Microbial communities and their correlation with microbial volatile compounds of cocoa beans during fermentation

Hanseniaspora opuntiae, Saccharomyces cerevisiae, and *Acetobacter pasteurianus* were the most abundant OTUs during the box and heap fermentation processes (B and H) from both countries. However, *Pichia pijperi* and *Lactobacillus fermentum* were also driven the cocoa fermentation in Cameroon, while *Lactobacillus cacaonum,* and *Lactobacillus*

plantarum group for the Mexican fermentation. Microbial richness analysis showed that only the diversity of fungal species indicated a higher level of complexity in B compared to fermentations ($P < 0.05$) and also revealed a statistically significant difference between starter cultures initially inoculated from the Cameroon samples ($P < 0.01$). In contrast, the bacterial community showed a higher level of complexity in *Criollo* compared to *Forastero* fermentations from Mexico and a significant difference across fermentation time was observed ($P < 0.05$). The difference between the microbial communities found in two different cocoa-producing countries highlights the importance of the environmental conditions to determine the denominated restricted microbial cocoa species (Figure 2).

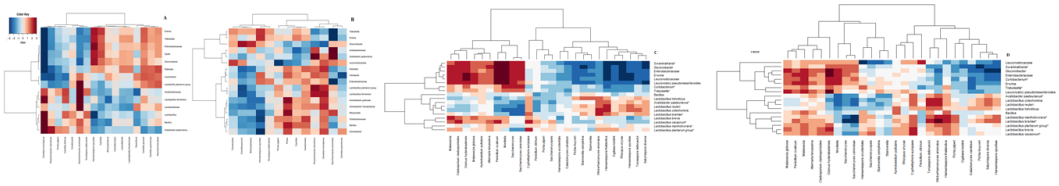

Figure 2. *Significance associations between microbial OTUs observed with an incidence above > 1% in at least 2 samples are described. Samples are label according to fermentation method* ***A)*** *Forastero Box from Cameroon* ***B)*** *Forastero Heap from Cameroon,* ***D)*** *Forastero Box from Mexico* ***C)*** *Criollo Box from Mexico. The intensity of the colors represents the degree of correlation between yeast and bacterial OTUs as measured by the Spearman's correlation.*

Overall, the microbial dynamics and associations between bacteria, yeast, and metabolites were found to depend on the type of fermentation and cocoa varieties, as shown in Figure 3. We observed that the major bacterial group found in our study increased the concentration of alcohols, esters, and acetaldehydes. Overall, the biochemical contribution in food ecosystems might change by the complexity of the microbial consortia (Fleet, 1999). 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 (Lima *et al.,* 2011; Thompson *et al,* 2001).

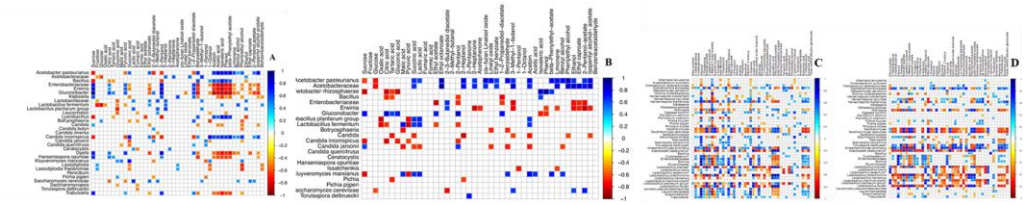

Figure 3. *Correlation plot showing Spearman's correlation between microbial OTUs and metabolites observed with an incidence above >1% in at least 2 samples. Samples are label according to fermentation method* ***A)*** *Forastero Box from Cameroon* ***B)*** *Forastero Heap from Cameroon,* ***D)*** *Forastero Box from Mexico* ***C)*** *Criollo Box from Mexico. The intensity of the colors represents the degree of correlation between microbial OTUs and metabolites as measured by the Spearman's correlation*

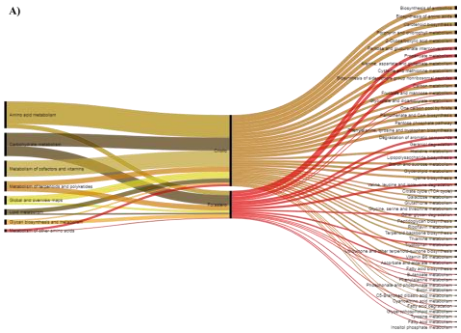

Figure 4. *Sankey diagram showing the carbohydrate, amino acids and lipid metabolic pathways activated for Criollo and Forastero varieties*

7.5 Potential Metabolic Pathways of Fermented Cocoa Microbiota

The pathway enrichment analysis of the predicted metagenomes showed that the amino acid, carbohydrate, and energy metabolisms were the most abundant during cocoa fermentation and significant difference between cocoa varieties over a specific fermentation time was observed for the amino acid, aromatic compounds and fatty acids degradation pathways (Figure 4, $P < 0.05$). In addition, a clear separation between the type of variety and the metabolism of the main pathways are observed (Figure 4). The inferred metagenomes confirmed differences between the cocoa varieties and indicated that the minor bacteria group (*Erwinia, Gluconobacter, Curtobacterium, Trabulsiella*, and *Leuconostoc*) have a negative association with the metabolism of short-chain fatty acids and amino acids.

7.6 Sensorial Evaluation

The quantitative descriptive analysis performed using a trained panel showed that no influences were observed between cocoa varieties, but the roasting process had a positive influence on the sensorial profile of the cocoa beans (Figure 5). Interestingly, the sensorial perception of fermented cocoa beans changes over fermentation time as a function of the microbial metabolic activity and development.

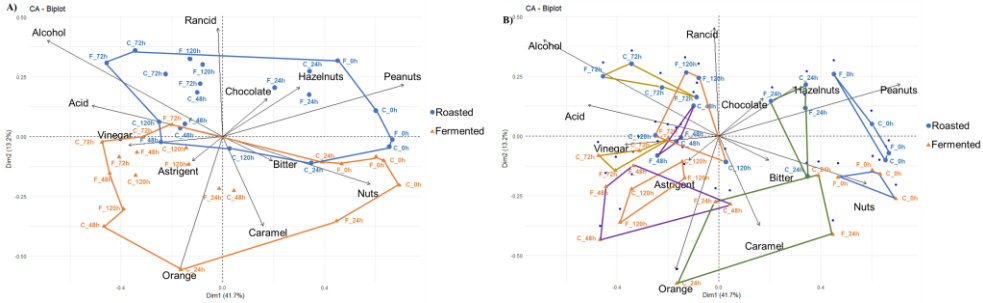

Figure 5. *Correspondence analysis factor (CA) map showing the flavor profile of fermented and roasted cocoa beans **A)** Criollo box from Mexico and **B)** Forastero box from Mexico*

8. Conclusions and Future Perspectives

The experimental strategy following the evolution of microbial populations, physicochemical 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 two different fermentation methods. We demonstrated that the degree of fermentation and bacterial composition is influenced by the type of cocoa variety and fermentation method used. 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. In spite of the limited effectiveness of starter strains, the information so far available suggests that microbial communities have been an important factor in the evolution of aroma compounds.

Understanding the pathways taken place during the formation of aroma by microorganisms could be used to improve fermentation processes and to enhance chocolate quality. However, the 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 obtain high-quality chocolate.

9. References

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