# Functional diversity of microbial communities associated with fermentation processes in coffee (*Coffea arabica* L.)

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## ABSTRACT

Mucilage fermentation conducted to degrade and remove coffee mucilage, is an important stage to also define coffee quality, but each microorganism's contribution to the final quality is not yet known. Therefore, tools are needed to easily identify this relationship in order to be used to enhance coffee quality. The present manuscript describes the behavior of the microbial communities present in fermentations conducted under various conditions, which produced differences in the sensory quality of the coffee as assessed by the community-level catabolic profile approach. The coffee samples came from six different fermentation processes that produced coffee in two quality classifications according to the Specialty Coffee Association (SCA) protocol: very good and excellent. Functional diversity of the microbial communities and substrate consumption were compared through analysis of variance. The multidimensional scaling analysis was used to identify the similarities or differences between treatments. The indices of functional diversity revealed significant differences and direct proportionality with the quality rating. Diversity index (H) was between 2.09 and 2.71 and Evenness was between 1.75 and 2.21. The consumption of groups of substrates was different between fermentations, especially in carbohydrates and carboxylic acids, and the greatest consumption of these was found in the excellent-quality coffee. The different fermentative processes evaluated by this technique showed a high metabolic activity related to the great diversity of substrates given to the microbial communities and microorganisms involved, causing reactions that had influence on the final quality of the product.

**Key words:** Catabolic profiles; Coffee quality; Diversity indices.

## 1 INTRODUCTION

Fermentation is a necessary stage within the coffee's wet process that naturally removes mucilage. This process is spontaneously conducted by microorganisms that are found in the fruit and in the environment and perform a series of transformations that result in the formation of compounds that might influence the quality of the grain. Attributes such as highlighted acidity and aroma are related to the wet process that yields the coffee, in which fermentation has an important role (Joët et al., 2010; Gonzales-Rios et al., 2007; De Bruyn et al., 2017; Worku et al., 2018; Pereira et al., 2019). The aforementioned attributes, together with flavor, are key for the consumption of this product, especially in the specialty coffee sector, a sector that is under continuous evolution, driven by its higher prices compared to the standard coffee sector (Sepúlveda et al., 2016; Samoggia; Riedel, 2018).

Recent studies cited by Pereira et al. (2019) have focused on the effect of fermentation, since it is a key mechanism influencing the attributes of coffee. Hence, it is important to understand the role of the communities of microorganisms that participate in these transformations (Haile; Kang, 2019). The community structure is influenced by the fermentation method and implies variations in the chemical composition of the grains (De Bruyn et al., 2017; Silva et al., 2008). According to the way fermentation is conducted, different concentrations of organic acids and organic volatile compounds are produced in the grain influencing the resulting quality (Peñuela-Martínez; Zapata-Zapata; Durango-Restrepo, 2018). The changes in microbial population are reflected in the variations in the patterns of use of carbon sources, which can be measured through a technique called community-level physiological profile (CLPP) (Weber; Legge, 2010), using the system BIOLOG™. This method is considered fast, reproducible, and economical and can detect differences between microbial communities according to their use of substrates (Srivastava et al., 2019). The functional diversity of communities describes the role of microorganisms, the competition between them, the co-occurrence of species, and their interaction in a native environment under changing conditions (Srivastava, et al., 2019). The CLPP approach allows the identification of functional profiles in the communities as well as their preferences for the consumption of carbohydrates and other substrates under different conditions, allowing a better understanding of the microorganism-quality relationship, the metabolic activity of the communities present and the differences in their behavior between the given fermentation conditions.

This study compares the response of microbial communities in the form of their use of carbon sources under different types of fermentation. It aims to determine how the fermentation process influences microbial diversity and composition, as well as to evaluate the use of the CLPP technique as a method. In short, it aims to find an easy way to define the behaviors of communities in fermentation processes that enhance coffee quality.

# 2 MATERIAL AND METHODS

## 2.1 Coffee samples

Samples of fruits of coffee (*Coffea arabica* L.), Colombia variety, produced at 1,940 m altitude in Ciudad Bolívar, Antioquia, Colombia, were used. For the coffee process, the wet method was used, including the stages of pulping, size classification, fermentation, washing, and drying with forced air at 50 °C. The treatments described in Table 1 correspond to six fermentations, selected from a series of processes that were conducted in two stages: the first with two waiting times before pulping ($6 \pm 1$ and $16 \pm 1$ h) and four fermentation times (16, 24, 48, and 72 h) and the second with fermentation for 24 h at controlled pH (5.0 and 4.0) and controlled temperature (17 °C and 23 °C), both factors that significantly influence the final quality of coffee (Peñuela-Martínez; Zapata-Zapata; Durango-Restrepo, 2018). Therefore, the treatments performed in this analysis were selected according to the individual final quality, which allowed each coffee to be classified as very good (80.00 – 84.99 SCA points) or excellent (85.00 – 89.00 SCA points). These grades were obtained after following the international protocol for sensory evaluation established by the Specialty Coffee Association of America (Specialty Coffee Association of America - SCAA, 2015). In which an expert panel evaluated the samples, considering all the important attributes of coffee. Each cuppers evaluated five cups of each coffee sample. The attributes for the sensory profile were fragrance/ aroma, uniformity, cleanliness, sweetness, flavor, acidity, body, aftertaste, balance and overall impression. Each attribute was evaluated in the 10-point scale. The final score, which was obtained by adding the individual scores given to each attribute to represent the overall coffee quality. The highest final score indicates the best classification of coffee quality.

## 2.2 Microbial activity and Substrates use

The functional diversity of the microbial communities present in the fermentations was evaluated with the community-level catabolic profile (CLCP) approach using the Biolog Eco Microplate™ identification system (Biolog Inc., Hayward, CA, USA). These plates contain 31 different carbon sources, grouped into carbohydrates, carboxylic acids, polymers, amino acids, and amines, and all substrates are included in triplicate in each plate (Table 2).

The mucilage samples obtained at the end of the fermentations were prepared at a $10^{-3}$ dilution in sterile water, and a 150 µL was incubated in each of the 96 wells of each Ecoplate™ plate after standardization of the procedure. The plates were incubated at room temperature (~20 °C) in the dark. Color development was detected by reading the optical density at 590 nm ($OD_{590}$), wavelength of maximum absorbance of tetrazolium, at different times between 6 and 24 h, up to 160 h, to construct the community growth curves.

**Table 1:** Fermentative processes used for the analysis of functional diversity.

| Code | Time before pulping (h) | Fermentation time (h) | Controlled variables | | Score in cup | Classification SCA |
|------|------------------------|----------------------|----------------------|-----|-------------|--------------------|
| | | | Temperature (°C) | pH | | |
| F1 | $6 \pm 1$ h | 16 | No | No | 81.00 | |
| F2 | $6 \pm 1$ h | 24 | No | No | 81.75 | Very good |
| F3 | $16 \pm 1$ h | 24 | 23 | No | 80.88 | |
| F4 | $16 \pm 1$ h | 24 | No | No | 86.31 | |
| F5 | $16 \pm 1$ h | 24 | 23 | 4.0 | 86.00 | Excellent |
| F6 | $16 \pm 1$ h | 24 | 17 | 5.0 | 86.63 | |

**Table 2:** Carbon sources by chemical group supplied as substrates in Ecoplate™ plates.

| Carbohydrates | Carboxylic acids | Polymers | Amino acids | Amines |
|---------------|------------------|----------|-------------|--------|
| D-Cellobiose, D-Galactonic-γ-lactone, α-D-Lactose, β-Methyl-D-glucoside, D-Xylose, Erythritol D-Mannitol, N-Acetyl-D-glucosamine Glucose-1-PO$_4$ D,L-α-Glycerol phosphate | D-Glucosamine D-Galacturonic acid 2-Hydroxybenzoic 4-Hydroxybenzoic γ-Hydroxybutyric Itaconic α-Ketobutyric D-Malic Pyruvic methyl ester | Tween 40 Tween 80 α-Cyclodextrin Glycogen | L-Arginine L-Asparagine L-Phenylalanine L-Serine L-Threonine Glycyl-L-glutamic | Phenylethylamine Putrescine |

The microbial activity in each microplate on the carbon sources provided by the Ecoplate™ plates was determined by the color development (average well color development, AWCD), which indicates the production of NADH given by cellular respiration, which reduces the salt of tetrazolium to formazan, generating a color change detected photometrically. AWCD was calculated by the following Equation 1:

$$AWCD = \frac{1}{31} \sum_{i=1}^{31} (A_i - A_0) \qquad (1)$$

where $A_i$ is the absorbance reading in well $i$ and $A_0$ is the absorbance reading in the blank well.

To reduce any deviation caused by differences in the density of the inoculum between the samples, the data were standardized following Weber and Legge (2010) using Equation 2:

$$\bar{A}_k = \frac{A_i - A_0}{AWCD} \qquad (2)$$

where $A_k$ is the standardized absorbance in well $k$.

The relationship between the AWCD change and the incubation time was calculated to determine the growth curve of the community in each sample. Once the data were standardized, and to analyze the information obtained, the time in which there were no $OD_{590}$ values greater than 2.0 were selected, to reduce measurement errors. Higher values are considered outside the linear range of absorbance (Weber; Legge, 2010).

The use of substrates in each sample was defined as the average value of the activities on the substrates in each carbon sources group, as shown in Table 2.

## 2.3 Functional diversity indices

The indices of functional diversity were estimated with standardized data (Zak et al., 1994), again selecting incubation times in which no $OD_{590}$ values greater than 2,0 were present. These indices were estimated as explained below:

The Shannon substrate diversity index is used to track and understand changes in communities over space and time, and indicates the pattern of substrate use. It is an index to measure species diversity after considering the uniformity of them within a community. It includes the richness and evenness index and is estimated according to Equation 3:

$$H = - \sum p_i ln(p_i) \qquad (3)$$

where H = diversity index and $p_i$ = the ratio of the activity of a particular substrate to the sum of activities of all substrates.

The richness index (S) is calculated as the number of wells with blank-corrected absorbance greater than 0.25 and is related to the number of substrates that are used by a microbial community (Weber; Legge, 2010).

The substrate evenness (E) is defined as the equality of activities across all substrates used. It was calculated by Equation (4):

$$E = \frac{H}{log\ S} \qquad (4)$$

## 2.4 Metabolic profile of bacteria and yeast

In order to analyze microorganisms' ability to transform different carbon sources, bacteria and yeast involved in following the evaluated fermentative processes were identified. Then, pure cultures of these microorganisms (25 bacteria and 14 yeast) were used to inoculate GEN III Microplate™ and YT Microplate™ (MicroStation system) respectively, according to the procedure described by Ansari et al. (2019) and Tilahun et al. (2018). The carbon sources on the microplates allowed the identification of microorganisms to genus and species level, as well as to determine their metabolic profile.

## 2.5 Statistical analysis

For the data analysis, the 95% confidence intervals for AWCD with respect to the incubation time were constructed. For the set of treatments, analysis of variance was performed with a significance level of 5% for the use of groups of substrates and the indices of diversity (H), richness (S), evenness (E), and. In cases where there was a significant effect, the Tukey test was applied at the 5% level to identify the differences between the mean values, using the statistical program SAS (Statistical Analysis System-Cary, NC, USA).

To identify the similarity or difference in the use of substrates between treatments, multidimensional scaling analysis was applied to the median of the consumption of each substrate, and the distance plane was prepared from the matrix of these, based on the Euclidean distance between the activity values of the different substrates. For this, the statistical program R (version 3.3.1.) was used.

To maximize the homogeneity of the profiles of bacteria and yeast, dendrograms were constructed in R using the Euclidean distance with the hierarchical agglomerative clustering method.

## 3 RESULTS

Differences in the adaptation of the community to the substrates provided were initially detected colorimetrically. The relationship of AWCD with time (Figure 1) showed

microbial growth in all of the samples except for treatment F3, in which there was a low adaptation to the carbon sources provided, as growth began only at 96 h. The highest activity on the substrates occurred in fermentations F4, F6, and F5, all in the classification of excellent quality.

The microbial community's analysis showed that the main carbon sources were carbohydrates and carboxylic acids. They showed significant differences ($p < 0,0001$) by accumulating values of $OD_{590}$ than other sources (Table 3). Metabolic activity in the fermentations increased as the time between harvest and pulping increased (F4, F5, and F6), which marks the beginning of fermentation.

Regarding functional diversity indices, significant differences were observed in $H$ ($p = 0.0117$) and $E$ ($p = 0.0216$), while all treatments used the same average number of substrates according to the values of $S$ ($p = 0.1451$) (Table 4). $H$ and $E$ were significantly higher under F4 than F3 and F2, respectively, showing behaviors that were affected by the variations in the fermentative processes carried out and implying a high metabolic diversity.

On the other hand, the spatial map obtained for the different metabolic profiles of the microbial communities according to treatment allowed to understand the relationship between them (Figure 2). The communities that presented similar metabolic profiles are represented by nearby points. The map revealed two groups, which coincide with the quality scores according to the predefined SCAA scale and an outlier treatment (F2), which presented a different metabolic profile. The different profile of the F2 may be explained by its low $E$ index, which indicates equivalence in the community activity on the used substrates. This behavior can also be observed through the mean carbohydrates and carboxylic acids consumption during the F2, which is different to the low consumption observed in F1 and F3, and the high consumption observed in F4, F5, and F6, which in turn relates to greater values of the diversity index ($H$). This finding implies that the use of different carbon and nitrogen sources on the F2, led to a different behavior compared to the two distinguished groups.
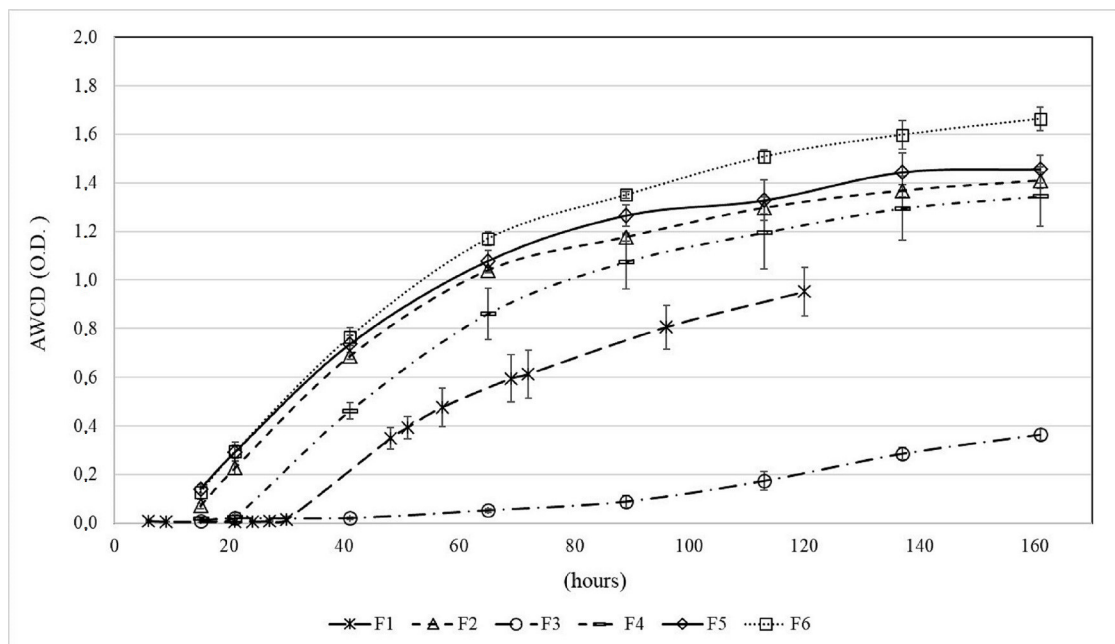


**Figure 1:** Color development (AWCD) in samples obtained from different fermentative processes in coffee.

**Table 3:** Average optical density ($OD_{590}$) obtained for the groups of carbon sources in different samples of fermented coffee.

| Code | Carbohydrates | Carboxylic acids | Polymers | Amino acids | Amines |
|------|---------------|------------------|----------|-------------|--------|
| F1 | 2.538[cd] | 1.308[b] | 0.779 | 1.208 | 0.303 |
| F2 | 4.357[bc] | 1.873[b] | 0.237 | 0.403 | 0.160 |
| F3 | 1.493[d] | 0.177[c] | 0.276 | 0.782 | 0.008 |
| F4 | 8.626[a] | 3.726[a] | 0.466 | 0.955 | 0.559 |
| F5 | 6.352[ab] | 2.170[b] | 0.060 | 0.325 | 0.158 |
| F6 | 5.733[b] | 2.121[b] | 0.668 | 0.550 | 0.044 |

* Different letters imply significant differences according to the Tukey test at a significance level of 5%.

**Table 4:** Average values of diversity (H), richness (S), and evenness (E) of the fermentations evaluated.

| Code | Diversity (H) | Evenness (E) | Richness (S) |
|------|---------------|--------------|--------------|
| F1 | 2.30± 0.27[ab] | 1.98±0.05[ab] | 14.7±3.2 |
| F2 | 2.20±0.23[ab] | 1.75±0.16[b] | 18.3±3.2 |
| F3 | 2.09±0.09[b] | 1.88±0.06[ab] | 13.0±2.0 |
| F4 | 2.71±0.11[a] | 2.21±0.02[a] | 17.0±1.0 |
| F5 | 2.40±0.15[ab] | 2.10±0.03[a] | 14.0±2.6 |
| F6 | 2.59± 0.20[ab] | 2.06±0.02[ab] | 18.3±3.8 |

Different letters imply significant differences according to the Tukey test with a significance level of 5%.
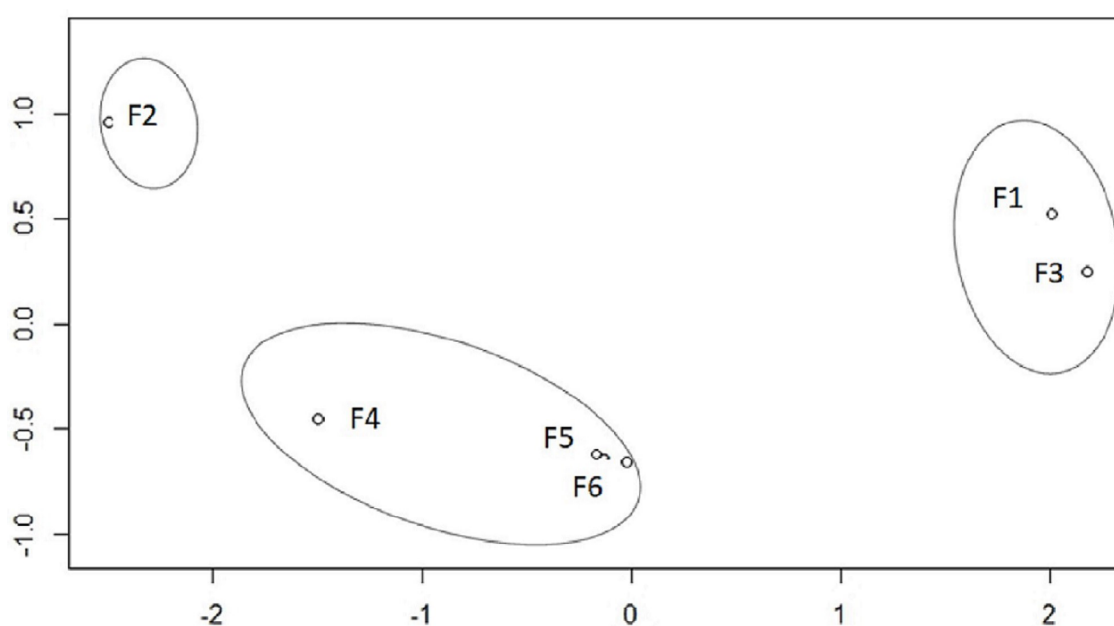


**Figure 2:** Map of the similarity of the metabolic profiles of the microbial communities of different fermentative processes in coffee.

## 3.1 Metabolic profile of bacteria and yeast

Finally, the classification of the metabolic profiles of the strains of bacteria and yeasts obtained by the hierarchical agglomerative clustering method (Figures 3 and 4) suggested the existence of four groups of bacteria with similar metabolic profiles and three groups of yeasts with similar metabolic profiles, given their positive, partial, or negative consumption of substrates. The bacteria that were classified individually mainly use organic acids and amino acids, so they had higher consumption of these substrates, especially polyalcohols, monosaccharides, and disaccharides, in addition to consuming pectin and galacturonic acid. These bacteria that show differentiated metabolic profiles were part of the populations identified in F1, and their participation did not seem to lead to a differentiation in the quality of the coffee obtained.

The group highlighted in the green box in Figure 3, including the majority of bacteria of the genus *Bacillus*, was characterized in general by having a lower total consumption of substrates, as well as lower average conversion of which the disaccharides D-trehalose, sucrose and D-maltose and D-fructose

were more representative. The group highlighted in the blue box was characterized by having the largest number of positive reactions in addition to the highest average conversion rate of substrates, monosaccharides being their main carbon source. None of the strains consumed pectin, but all could completely transform galacturonic acid. *Bacillus butanolivorans* was present in the samples whose coffee was classified as excellent (F4, F5, and F6) and could have fulfilled a differentiating function. Given its high capacity for substrate transformation, its role might be related to the formation of compounds that could be used as substrates by other microorganisms.

The group composed of bacteria of the genus *Ochrobactrum* (orange box) had similar amounts of substrates consumed and did not show a major difference in the type of substrates used between all chemical groups. The species of this genus did not show the ability to completely transform pectin. These bacteria were present in all fermentations, so their metabolic profile has a secondary role in the metabolism of the community and has no differentiating function on the others.
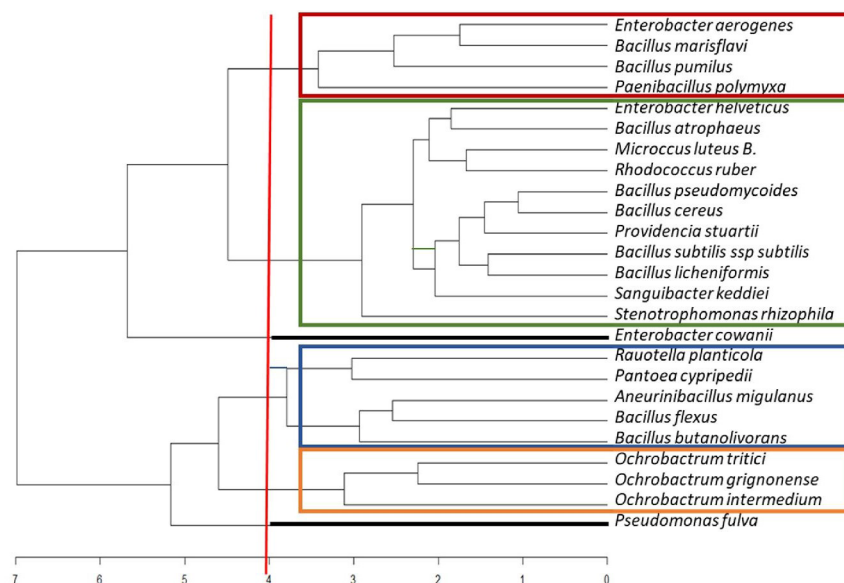
**Figure 3:** Dendrogram of the classification of bacteria associated with the different fermentative processes in coffee.
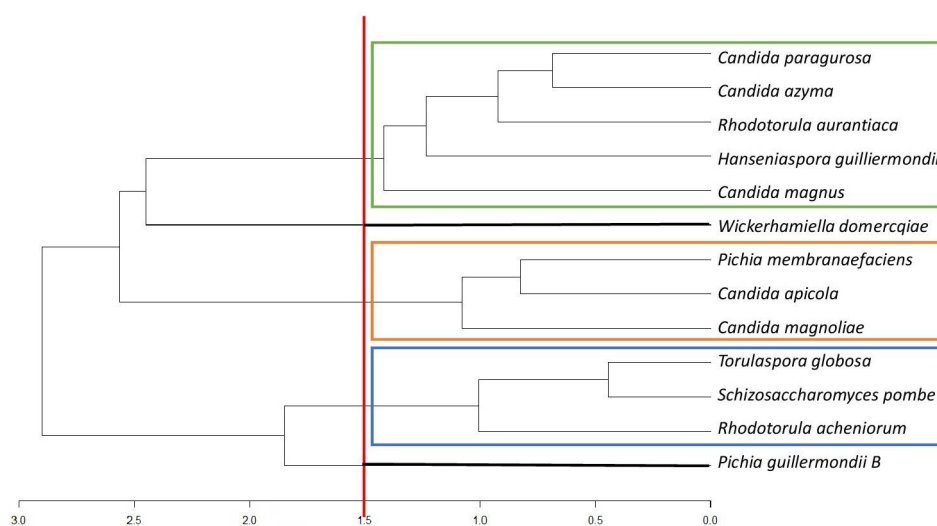


**Figure 4:** Dendrogram of yeast classification associated with the different fermentative processes in coffee according to their oxidation of substrates.

On the other hand, the metabolism of yeasts is considered heterogeneous and complex, since each one has different nutrient absorption pathways and presents regulatory differences between fermentation and respiration. The existence of three groups of yeasts with different oxidative metabolic profiles was suggested (Figure 4). In the group indicated in the green box, there was low or no consumption of glucose, but there was consumption of cellobiose and gentibiose, as well as salicin, an alcoholic glycoside whose oxidation produces glucose, the main carbon source of yeasts. The group highlighted in blue showed the lowest ability to oxidize the substrates, but with greater activity on them, indicating their high fermentative capacity, as also shown by their tendency to consume disaccharides.

## 4 DISCUSSION

The activity on the substrates observed allowed to quickly compare communities by detecting differential rates of color development in the plates, independent of the incubation time, implying different physiological profiles. Such behavior is normal for heterotrophic communities analyzed by this kind of approach (Pinzari et al., 2016), as is the case of the microbial communities that participate in the fermentation of mucilage.

The analysis of the consumption of substrates individually showed differences, especially in carbohydrates such as D-xylose and galacturonic acid, which are a monosaccharide and an acid sugar, respectively, and have been identified as two of the carbohydrates that make up the

coffee mucilage (Neu et al., 2016). The activity on glucose was lower in general, despite being the main molecule for conversion in this type of process. In contrast, the low activity of carbohydrates observed in F3 could indicate a low fermentative capacity of the microorganisms associated with this treatment. Joët et al. (2010) found that these types of compounds were the most affected by the wet process, in which fermentation represents most biochemical transformations.

The catabolic profile estimated using these indices showed differences based on the presence or absence of metabolic activity on the substrates and related to the quality classification. This agrees with the behavior of other products under the catabolic profile approach (CLCP), such as in the fermentation of wheat flour dough, which showed differences between treatments due to the catabolic profile of different microbial communities (Cavallo et al., 2018; Minervini et al., 2016; Siragusa et al., 2009), or in the inoculation processes of minimally processed pineapple, in which the ability of inocula from the fruit was demonstrated by applying the CLCP to control fermentation (Di Cagno et al., 2010).

Regarding the indices of functional diversity, the values obtained in this study for $H$ and $E$ are close to those found for communities of wheat flour fermentations (Minervini et al., 2016; Siragusa et al., 2009) and minimally processed pineapple (Di Cagno et al., 2010). The richness index obtained was greater than the values reported in the studies cited above, which could be related to a greater metabolic diversity of the microorganisms that make up the communities involved in coffee fermentation (Evangelista et al., 2015; Elhalis; Cox; Zhao, 2018) due to the wide variety of substrates that are supplied by the mucilage and consumed by a potential diversity of microorganisms, which in turn leads to complex interactions between them.

Similar results were obtained by Junqueira et al. (2019), who described the microbial communities in coffee fermentation using an Illumina-based amplicon sequence with a high index of microbial richness, which increased during fermentation. They reported $H$ values between 0.77 and 1.82, lower than those found under the approach proposed in this study. This discrepancy was probably related to the different conditions in which the fermentative processes were conducted. However, in both cases, a great microbial diversity was found, with a high number of interactions between microorganisms, which can ultimately enhance sensory attributes, as in other fermented products such as wine.

In the analysis of the functional diversity of the communities, differences were observed between them due to the consumption of carbohydrates, mainly D-cellobiose, N-acetyl-glucosamine and β-methyl-glucoside. These substrates were completely transformed by *Hanseniaspora guilliermondii* and *Pichia membranifaciens*, which were present in the treatments that corresponded the excellent SCA classification, so that the metabolic profiles of these yeasts confirmed the differences observed in the analysis of functional diversity and indicated that their function has a positive impact on the fermentation process and enhances coffee quality.

## 5 CONCLUSIONS

The classification of treatment quality was related to the structure of the microbial communities as determined from the changes in their functional diversity. The fermentation conditions modified the environment in which the microorganisms interacted, generating different transformations of the substrates, mainly carbohydrates and organic acids. These changes were evident in fermentation processes with coffee of the same origin, variety, and production conditions.

The CLCP approach helps to find differences in the behavior of the microbial communities associated with the fermentation process in coffee, and it can be used to detect differences in the microbial composition with respect to individual microbes. This technique has a high potential to be used in this type of process since it facilitates the identification of differences in metabolism between samples, as the microbes depend on organic compounds as an energy source for their development. Most researchers have concluded that this approach is useful for making rapid comparisons with a high degree of discrimination, which can help us better understand the roles of microorganisms in fermentation and their relationship with coffee quality.

## 6 ACKNOWLEDGMENTS

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