beverages

Article

Influence of the Biotechnological Process of Mezcal Fermentation on Yeast Diversity in Four *palenques* of Oaxaca, Mexico

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Abstract: Mezcal is an alcoholic beverage obtained by distilling musts and juices fermented by spontaneous or cultivated microorganisms, which are extracted from ripe stems of cooked *Agaves* and harvested in Mexico. Both raw material and production practices differ markedly between producing regions, locations, and even factories, resulting in a very distinctive set of products. The state of Oaxaca is the top producer worldwide of mezcal, and 35,000 families are involved in the production of this aromatic alcoholic beverage. Fermentation is the most important stage of mezcal production and is performed by different yeast and bacteria. In this study, the yeast strains were isolated from fermentation containers of four mezcal factories (*palenques*) in Oaxaca. Taxonomic determination was verified by ITS, and an analysis of the biotechnological process through personal interviews and principal component analysis was performed. Eighteen different isolates of eight different genera (*Candida*, *Clavispora*, *Meyerozyma*, *Metarhizium*, *Pichia*, *Saccharomyces*, *Torulaspora*, and *Yarrowia*) were identified. According to the biotechnological process analysis and principal component analysis, the artisanal *palenques* (*palenques* 1, 3, and 4) are more like each other than and differ radically from *palenque* 2, which is where the artisanal process has changed towards technical production.

Keywords: Agave angustifolia; Agave potatorum; mezcal; Ascomycota; tahona

Citation: Espinoza-Martinez, V.A.; Alvarez-Gutierrez, P.E.; Palma-Cruz, F.d.J.; Enriquez-Valencia, R.; Ramirez-Lopez, M.P.; Lopez-Sanchez, C.; Vazquez-Lopez, H.G. Influence of the Biotechnological Process of Mezcal Fermentation on Yeast Diversity in Four *palenques* of Oaxaca, Mexico. *Beverages* **2023**, *9*, 99. https://doi.org/10.3390/beverages9040099

Academic Editor: Antonio Morata

Received: 12 October 2023
Revised: 20 November 2023
Accepted: 23 November 2023
Published: 28 November 2023

1. Introduction

Mezcal is a Mexican alcoholic beverage obtained by the distillation of musts and juices fermented by spontaneous or cultivated microorganisms, which are extracted from ripe stems of cooked Agaves and harvested in Mexico and are included in the resolution of the denomination of origin mezcal [1]. The raw material or the *Agave* is a perennial plant with a life cycle ranging from 7 to 14 years; it adapts well to poorly fertile conditions and is resistant to extreme climatic conditions [2]. The artisanal mezcal process has five steps: the collection and cutting of the raw material, the cooking of the Agave, the grinding, the fermentation of the juice and the musts, and the distillation. The official Mexican standard NOM-070-SCFI-2016 [3] establishes that mezcal is a liquid with the aroma and flavor derived from the species of Agave used. The quality also varies due from the type of soil, topography, climate, water, the producer of mezcal, the region, and microorganisms, among other factors that define the character and organoleptic properties of each mezcal. Fermentation is the most important stage of the artisanal mezcal elaboration process since it is here where a large amount of pleasant aromatic, volatile, and non-volatile compounds are

produced that affect the quality of the mezcal produced, such as alcohols, ketones, terpenes, acids, phenols, and aldehydes [4,5]. It is performed by microbial consortia composed mainly of yeasts, acetic acid bacteria, and lactic acid bacteria [6,7]. The *palenque* is the factory where the mezcal production process occurs in the state of Oaxaca. There are basically two types: the artisanal and the technical or industrialized. The artisanal *palenque*, which is the most common, consists of four fully coordinated work areas, where cooking, grinding, fermentation, and distillation activities are conducted. The Agave is cooked in conical ovens dug into the ground, which can be used just as they were originally after excavating them or by lining their walls with refractory stones. The firewood used as fuel is normally placed in the center of the oven with many stones, also refractory, which is used to cook the Agave for at least 72 h during the process. After the Agave is cooked, it is ground in a *tahona* or Egyptian mill, which consists of a circular area with a stone-lined floor, where a stone wheel pulled by some animals crushes the prime material of mezcal. The cooked and shredded *Agave*, together with molasses, is fermented in cylindrical wooden containers with an average capacity of 2000 L, filled to 80% of their capacity with the raw material and water. The fermentation takes 5 to 7 days depending on the ambient temperature. Additionally, once the fermentation is complete, the musts are conventionally distilled in copper stills.

Yeasts have a very important role in the fermentation of fermented and distilled alcoholic beverages. The most recent studies by [8] show that the yeasts involved in the mezcal fermentation correspond to the genera *Pichia manshurica*, *Pichia kudriavzevii*, *Torulaspora delbrueckii*, and *Saccharomyces paradoxus*. Meanwhile, [9] discovered in Oaxaca that the yeasts in this distilled beverage are *Pichia kudriavzevii*, *Pichia manshurica*, *Saccharomyces cerevisiae*, and *Kluyveromyces marxianus*.

Non-*Saccharomyces* yeasts, which are present in spontaneous fermentations, can increase the diversity of aromas and flavors in fermented and distilled beverages [10]. The metabolism of these yeasts is not only responsible for producing ethanol but also for the formation of various chemical compounds that are part of the aroma and flavor [11]. The production and concentration of metabolites, desirable or unpleasant flavors, formed during fermentation will depend on the diversity of yeast species present [12].

The objective of this study was to determine the diversity of the yeasts responsible for the fermentation of artisanal mezcal, as well as the variables that influence the process in four *palenques* in Oaxaca. The most relevant results refer to the presence of seven genera of yeasts: *Candida*, *Clavispora*, *Meyerozyma*, *Pichia*, *Saccharomyces*, *Torulaspora*, and *Yarrowia*, which include thirteen species and two varieties. According to the performed principal component analysis, the artisanal *palenques* (1, 3, and 4) are more like each other and differ radically from *palenque* 2.

2. Materials and Methods

2.1. Sampling, Isolation, and Propagation of Yeast

The samples of the fermentation musts were aseptically collected directly from the fermentation container in each of the four *palenques*.

The sites in which the study was performed were as follows: *palenque* 1 located in the municipality of San Dionisio Ocotepec, with an altitude of 1620 m.a.s.l. (16°46′11″ N, 96°22′11″ W); *palenque* 2 in San Pablo Etla with an altitude of 1726 m.a.s.l. (17°09′14″ N and 96°44′49″ W); and *palenque* 3 in the Río de Las Palmas Coixtlahuaca belonging to the municipality of Concepción Buenavista with an altitude of 1734 m.a.s.l. (17°57′43″ N and 97°27′25″ W). Finally, *palenque* 4 corresponded to the municipality of San Pedro Teozacoalco with an altitude of 1586 m.a.s.l. (17°01′01″ N and 97°17′12″ W). The temperature, pH, and total soluble solids of each of the fermentation containers were determined in situ. The samples were refrigerated at 4 °C until processing. The must samples were taken to the environmental control laboratory of the Department of Chemical and Biochemical Engineering of the Technological National of Mexico, on the Oaxaca campus.

The yeast colonies were isolated in Petri dishes with Sabouraud dextrose agar medium (ADS) supplemented with ampicillin (200 mg/L), through serial dilutions in 1% peptone broth. The 1×10^{-6} dilution was inoculated by a spread plate in Petri dishes and incubated at 28 °C for 48 h. Single isolated colonies were reseeded until pure cultures of yeast strains were obtained and propagated in the same medium.

2.2. DNA Extraction and PCR Amplification

The genomic DNA was extracted with the Zymo Research's YeaStar Genomic DNA kit, following the manufacturer's instructions.

Amplification of the 5.8S gene was performed using the polymerase chain reaction (PCR) technique using the oligos ITS1 (5′- TCCGTAGGTGAACCTGCGG -3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC -3′) [8], under the following conditions: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, with a final extension of 10 min at 72 °C [13]. The sequence analysis was performed using the same primers (Macrogen, Seoul, Republic of Korea). The ITS sequences were edited in the BioEdit software package version 7.2.5. These sequences were compared in the GenBank database at the National Center for Biotechnology Information (NCBI) using the nucleotide BLAST tool [14].

2.3. Phylogenetic Analysis

All the proposed 5.2 S rRNA ITS4-identified fragments and a *Rhodotorula* sp. 5.2S rRNA ITS4 partial homologous sequence, which was used as an outgroup, were compared, and reported using the MAFFT alignment software [15]. By using this bioinformatic tool, we obtained an fst file that enabled us to compare all the sequences in a maximum likelihood phylogenetic analysis with IQ-TREE shell version [16]. All the sequences were analyzed using a model test mode to recognize the corresponding substitution model of all sequences. Additionally, we selected an ultrafast bootstrap model with 10,000 repetitions to sustain the topological position and generate bootstrap values for the phylogenetic tree. The obtained consensus tree was used to represent the sequence relation and bootstrap for each node. The obtained contree file (nwk) was read using the Fig Tree software [17]. This software enabled transforming, rooting, and rotating the branches' position to create a png image and the obtained bootstrap result. To evidence the origin of each sequence (*palenque*), the png images were modified and colored using the GNU image-modifying program GIMP [18], to use the same color pattern presented in the map corresponding to Figure 1. To obtain a homologous outgroup sequence, a random query element was used in the web version of BLAST. The search was modified by using a filter to only recognize all homologous sequences except for all those belonging to Ascomycetes sequences. A *Rhodotorula* sequence was obtained to use it as an outgroup and to root the phylogenetic tree.

2.4. Multivariable Analysis of Biotechnological Variable Process

Data from mezcal production processes were obtained from mezcaleros through personal unstructured interviews with their consent. For a complete comparison among the categorical and discrete variables measured in different *palenques*, we grouped all the data into a table with binary and numerical data respectively into a spreadsheet from Microsoft Excel software® (2019). The variables analyzed in the work of the different *palenques*' localities were categorical. The variables analyzed involved the following: the cooking (use of an earth or stone oven), the shredding, the containers' material used in the fermentation (wooden and plastic container), the water used in the fermentation (river or tap water), the material of the distiller (copper or pottery), and the species used for the fermentation (*Agave angustifolia* or *Agave potatorum*). The discrete variables analyzed were pH, total of soluble solids' content, and temperature. All the variables were scaled into a common number value to analyze in RStudio [19] using the corresponding packages. All the data were captured in "xls files", and were loaded as an R language object, working with it in the RStudio IDE. The data were arranged into a data frame, allowing an analysis

with the heatmap package. The protocol and code modification used an R language guide [20]. Employing this heatmap package enabled us to compare the scale and generate the comparison among all variables. The cluster method applied in the graphical analysis was ward.D2 [21] because of its preference for recognizing variance in all the localities. We gave preference to only analyzing how variables could link and group the *palenque* sites instead of recognizing the hierarchy among variables. The graphical result using a color Ramp Palette compiler [21], with yellow for low scale values and red for high values, made it possible to recognize patterns and relationships among the *palenques*' corresponding analyzed samples. The obtained plot from RStudio was captured and optimized in the GNU image-modifying program (GIMP).

The same data frame object that originated in RStudio for analyzing the heatmap was used to generate the covariance matrix, and the eigenvectors and eigenvectors used in the principal component analysis (PCA) were calculated using the factoextra and factoMineR packages [20] from the R CRAN repository. The PCA graphical result is represented in a biplot graphic. All significant values are reported in green, with blue for all the eigenvectors with a variance and covariance that do not allow separating and providing significant relationships and arrangement in the analysis.

3. Results

3.1. Study Site

Oaxaca is a state in southern Mexico with the most rugged topography in the country: altitudes range from sea level in the coastal region to mountains of 3200 m asl in Cempoaltepetl in the Sierra Norte and 3800 m asl in Cerro Nube in the Sierra Sur. This variation leads to diverse weather and microclimates associated with diverse ecosystems in one of the most biodiverse regions in Mexico. The study site was four *palenques* in Oaxaca (Figure 1). *Palenque* 1 is in the municipality of San Dionisio Ocotepec in the Tlacolula district in the central valley 50 km from Oaxaca City. The predominant ecosystem around *Palenque* 1 is xerophilous scrubland in the foothills and low hills at 1800 m asl, whereas the mountain zones are between 1800 and 2200 m asl with *Quercus* and *Pinus* Forest. *Palenque* 2 is in the central valley 15 km from Oaxaca City in the municipality of San Pablo Etla. The surroundings are seasonal agriculture with summer rainfall and semi-urban zones. The temporary runoff from the northern mountains provides enough tap water for human activities. *Palenque* 3 is located at Río Las Palmas, in the municipality of Concepción Buenavista in the Coixtlahuaca district. This region is in La Cañada, where the predominant ecosystems are xerophilous scrubland and thorny forest with 500 mm of annual precipitation. These are extreme conditions for human survival, so there is a low population density in the zone. *Palenque* 4 is located at the municipality San Pedro Teozacoalco in the district of Nochixtlan in the Mixteca region. This zone lies 120 km from Oaxaca City. The topography of the zone is extremely complicated, and there are xerophilous scrubland and *Brahea dulcis* palms at low altitudes. *Quercus* forest exists in areas up to 2000 m asl, whereas there is coniferous forest in the high mountains.

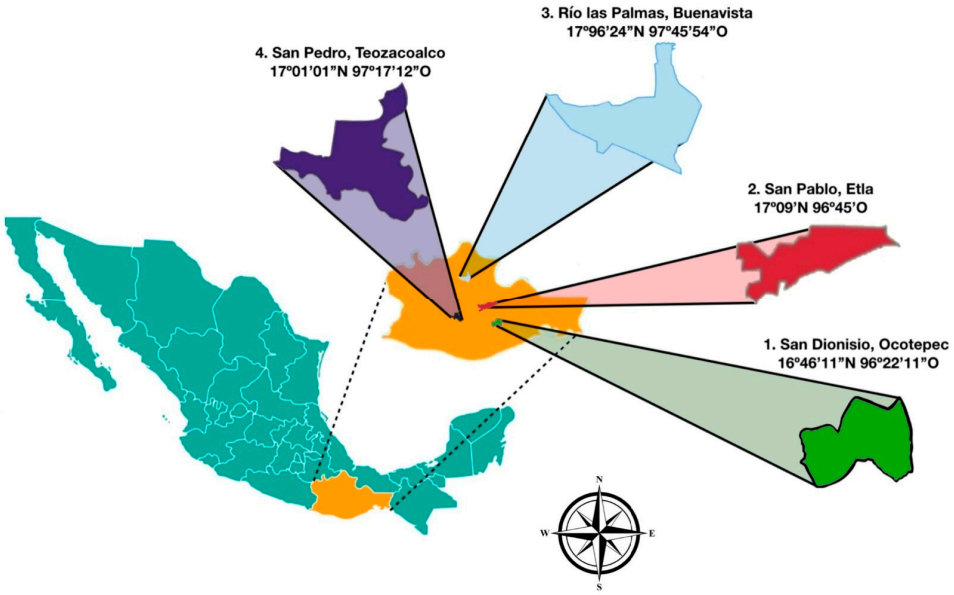

Figure 1. Sampling sites of four *palenques* for mezcal production in Oaxaca, Mexico. Green: *Palenque* 1 in San Dionisio, Ocotepec. Red: *Palenque* 2 in San Pablo Etla. Blue: Palenque 3 in Río de Las Palmas, municipality Concepción Buenavista. Purple: Palenque 4 in San Pedro Teozacoalco.

3.2. Mezcal Production Process

The mezcal production process has two important elements: the *Agave* spp. used and the people who transform the *Agave* into mezcal. This process has five critical unit operations: raw material preparation, hydrolysis of the polymers of the piñas (or cooking), the shredding of the cooked material, the fermentation, and distillation. The information provided in the following sections was obtained through personal interviews and empiric observation of at least five 3- to 7-day visits by some of the authors.

3.2.1. The *Agave* spp.

Mexico has the greatest diversity of Agaves in the world, and, in the state of Oaxaca, three to seven species of Agave grow naturally, which represents 22% of the national total and makes Oaxaca the territory with the most species of the aforementioned genus [22]. In Mexico, 42 Agave species, 7 subspecies, and 7 varieties have been registered in 24 states for mezcal production, although for the denomination of origin of mezcal, only 6 taxa and 7 states of the country are recognized [23].

Agave angustifolia (*palenques* 1, 2, and 4) and *A. potatorum* (*palenque* 3) were the species used to make mezcal in the study sites (Figure 2A).

Agave angustifolia is a highly variable species, has many cultivated and wild varieties, and is the species with the widest distribution among the Agaves in the world. It is common in dry climates, surrounded by xerophilous scrubland and *Quercus* and *Pinus* forests. It is the most widely cultivated species in Oaxaca for mezcal production and this is why its plantations are found in the districts of Tlacolula, Etla, and Nochixtlán, for *palenques* 1, 2, and 4, respectively, and the plants are generally interspersed with staple crops or with other Agave species. The maguey espadín, which is the common name for *A. angustifolia*, easily adapts to adverse nutritional and climatic conditions. In the locations of *palenques* 1 and 4, this species is cultivated in sites with steep slopes that were previously covered by local ecosystems. Conversely, in the region where *palenque* 2 is found, *A. angustifolia* has been cultivated for more than three human generations in extensive plantations. The stems of the mature plants that are used in the production of artisanal mezcal usually weigh 60 to 80 kg.

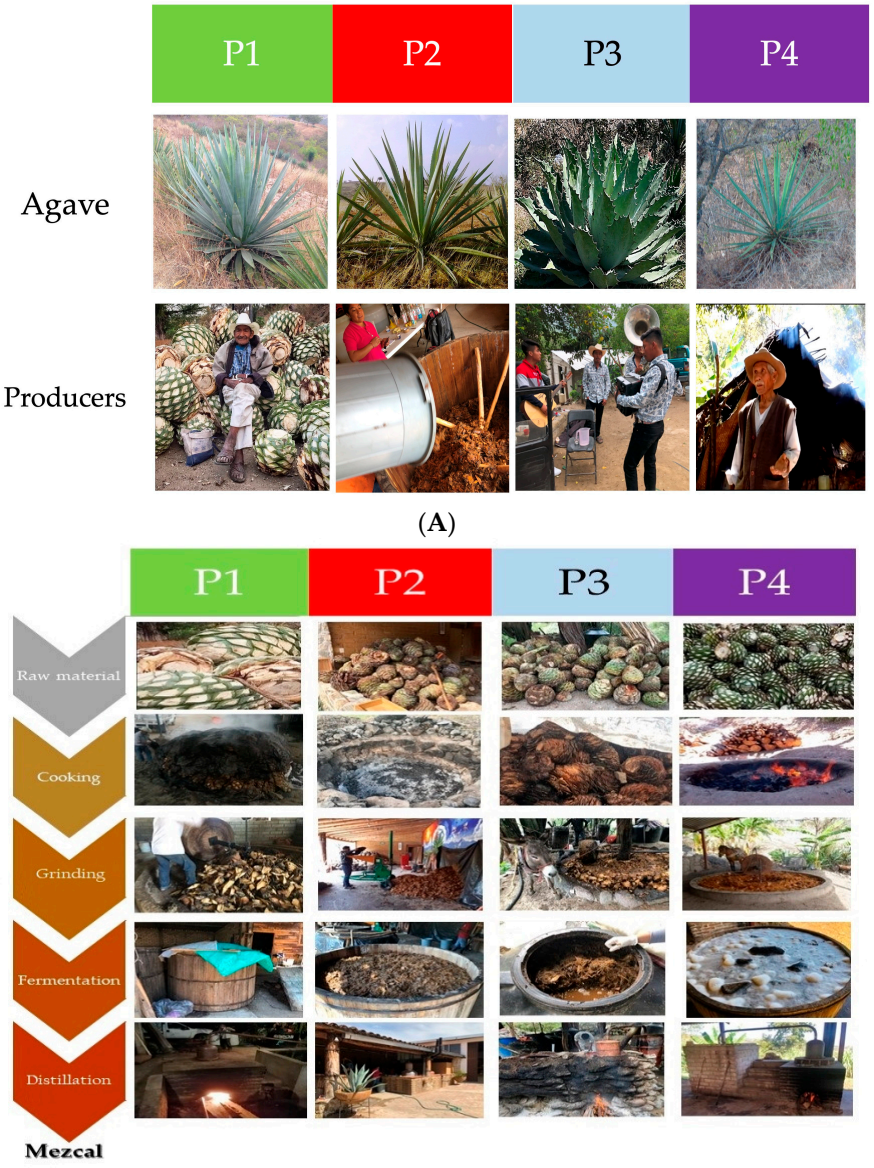

Figure 2. Biotechnological process of mezcal production in four palenques in Oaxaca, Mexico. (**A**). Mezcaleros (mezcal producers) and species of *Agave* used in the *palenques*: *Agave angustifolia* (*palenques* 1, 2, and 4) and *A. potatorum* (*palenque* 3). (**B**). Elements and equipment of the biotechnological production of mezcal: raw material (piñas), cooking process in stone oven (*palenque* 2) or earthen oven (palenques 1, 3, and 4); shredding in gasoline-powered blend mill (*palenque* 2) or "*tahona*" (*palenques* 1, 3, and 4); fermentation step in wooden fermentation container (palenques 1, 2, and 4) or plastic container (*palenque* 3); distillation in copper distiller (*palenques* 1, 3, and 4) or pottery distiller (*palenque* 2).

Agave potatorum is a wild species of Agave that reproduces solely and exclusively by seeds and has been used in the production of mezcal since the end of the twentieth century in *palenque* 3, although its use has increased greatly in recent times. It develops naturally in the ecotone between the xeric shrubland and the *Quercus* Forest, where it is harvested. It is commonly identified as *tobalá* or *papalometl*. In addition to being raw material for mezcal, its flowers are used as food, and its leaves are used in traditional medicine. Its high content of total reducing sugars has made it one of the preferred Agaves to produce artisanal mezcals in Oaxaca. The stems of the mature plants that are used in the production of artisanal mezcal are normally small, barely 20 to 40 kg.

3.2.2. The *Mezcaleros*

The people who make mezcal in Oaxaca are called *mezcaleros* (Figure 2B). The culture and idiosyncrasy of *mezcaleros* are what define the style of work throughout the production process of artisanal mezcal. Ethnically, the producers are different: those of *palenque* 1 are Zapotec; those of *palenques* 3 and 4 are Mixtec, while those of *palenque* 2 are mestizos who are native Spanish speakers. The producers of *palenques* 1, 3, and 4, who belong to the original groups of Oaxaca, are peasants, who dedicate most of their work during the year to the production of corn (*Zea mays*), beans (*Phaseolus vulgaris*), squash (*Cucurbita peppo*), chili (*Capsicum annuum*), and *Agave* spp., mainly for self-consumption. Additionally, some *mezcaleros* are musicians (Figure 2A, *palenque* 3). The production of artisanal mezcal begins in the harvest season at the end of the rainy season and the beginning of the season with warm and/or cold temperatures. By then, the ripe *Agaves* spp. can also be harvested and are ready to be transformed into mezcal. Mezcal production can last 4 to 5 months. In the regions where the palenques 1, 3, and 4 are located, the work in the mezcal factory is essentially family-oriented, with the indistinct participation of children, women, and the elderly. At times, members of the extended family participate uncles, nephews, brothers-in-law, godparents, etc. This depends on the amount of work, and there is no economic remuneration. Said participation corresponds to a type of community work, el *tequio*, which will be reciprocated by the *mezcalero* when his family requires it. The clothing of the peasant *mezcaleros* is basically ordinary, although there are elements that still preserve their original heritage, such as the huaraches, which are a type of very rustic sandals made with leather straps and mounted on a sole made from reused automobile tires (Figure 2A, *palenque* 1, 3, and 4). In contrast, the mezcal producers of *palenque* 2 got involved in the production of the distilled beverage often as one of the alternative sources of economic income and very rarely to continue a family tradition. Naturally, as in many modern companies, the work of these types of *palenques* requires the participation of personnel hired for that purpose, and, except for the owner, all other people are outside the production process. Hence, sometimes, the final distillate does not share organoleptic properties with the distillates produced in the original towns.

3.2.3. Treatment to Raw Material

The selection and harvest of the raw material to produce artisanal mezcal begins when the producer chooses the mature plants, either in the plantation, in the case of *Agave angustifolia* (Figure 2B, *palenques* 1, 2, and 4), or in the natural ecosystems, as is in the case of *A. potatorum* (Figure 2B, *palenque* 3). In both cases, the agaves are chosen in the beginning of their reproductive stage, when the floral scape emerges. The flower, fruits, and seed-producing organs are removed with a panga (machete). This action is recognized as the *capado* of the plant. After the elimination of the floral scape, the Agave requires the rest of the living plant for at least 8 more months. Once that period is completed, the producer can then harvest the plant. To do this, all the leaves that cover the stem of the *Agave*, also known as the piña, are removed until the part where both organs meet, which is called the *rasurado*. Finally, the *Agave* is separated from the soil with the help of a slasher. Afterwards, the stems are transported to the mezcal factory called a *palenque.* When the stems weigh more than 30 kg, such as with *Agave angustifolia* (Figure 2B, *palenques* 1, 2, and 4), they are divided into two or three parts, depending on their size, to facilitate their handling, although the stems of *A. potatorum* (Figure 2B, *palenque* 3) can remain whole if their weight does not exceed that mentioned. At the *palenque*, the harvested agaves wait until the cooking oven is ready to be used.

3.2.4. High-Temperature Hydrolysis of *Agave* spp. Polymers (Cooking)

The agaves harvested to produce artisanal mezcal are cooked in an inverted conical oven. This oven is dug into the ground to hydrolyze the carbohydrate polymers of the *piñas*. The oven dimensions on average can be from 4 to 10 m in diameter and 1 to 2 m in depth. These dimensions are due to the availability of raw material and the workforce of

the mezcaleros. These ovens can be used without any additional excavation, and they have refractory stones. The walls of the inverted cone are covered with them to improve the conservation and dissipation of heat energy. The fuel, consisting of firewood from tree or shrub species with good dendro-energetic capacities, is placed in the center and bottom of the oven. A lot of refractory stones are then placed on top of it that serve as the accumulator and heat sink once the fuel has been consumed. The oven can reach 900 °C after 12 to 16 h. The residual bagasse from the distillation, normally humid, is placed to prevent the *Agave* that will be cooked from charring. For cooking, the piñas, harvested and divided or not, are placed on top of the protective layer until the oven is filled. Immediately, the raw material is covered with *Brahea dulcis* leaves or mats made with the leaves of the same palm, known locally as *petates*, or failing that with blankets or any non-synthetic material fabric. This avoids contact between the raw material and the earth that is placed on top of it and with which the cooking oven is completely sealed. With this part of the process, and after 72 to 120 h depending on the ambient temperature, the hydrothermal transformation of the inulin contained in the *Agave* stems is achieved and includes fructans, disaccharides (sucrose), and monosaccharides (glucose and fructose) [5,24].

3.2.5. Shredding and Fermentation Steps

The next phase of the artisanal mezcal production process is grinding or shredding, which consists of dividing the cooked *Agave* into small fragments so that the microbial action during fermentation is achieved. Therefore, the traditional *palenques* (*palenques* 1, 3, and 4) use a *tahona* or Egyptian mill. The *tahona* consists of a metamorphic stone wheel weighing up to 1 ton, 1.0 to 1.6 m in diameter, and 0.4 to 0.6 m wide. The stone has a circular opening in the center where a piece of wood or a metal tubular structure is placed. The stone wheel turns thanks to the impulse of a horse, donkey, or other animal (Figure 2B *palenques* 1, 3, and 4). The dimensions of the main wheel of the *tahona* depend on the amount of *piñas* available per season or the hardness and resistance of the raw material to being transformed. It should be noted that the cooked stems of *Agave angustifolia*, such as those used in *palenques* 1, 2, and 4, are harder and more resistant to grinding than the stems of *A. potatorum*, like those of *palenque* 3. The factories that have modernized their process, such as *palenque* 2, have replaced the *tahona* with a gasoline-powered blade mill, which was originally used to chop corn stubble, but which crushes the cooked *Agave* too much and can alter the aeration of the substrate during its fermentation.

Once the grinding of the cooked *Agave* is complete, the next phase of the process is fermentation. For this purpose, cylindrical wooden containers with iron belts of 2 to 2.2 m in diameter by 1.2 to 1.5 m in height are used, with a capacity of 2000 to 2500 L (*palenques* 1, 2, and 4). In some mezcal factories (like *palenque* 2), however, the wooden containers have been replaced by plastic water tanks with a capacity of 1200 to 1500 L. In these containers, commonly called tinas, the shredded *Agave* is poured and left to rest for at least 20 to 24 h, with a slight increase in the temperature of the substrate, which reaches 40 °C. This is where the fermentation takes place.

The tina is filled with water (up to 80% of the container capacity) and *Agave*. The ratio of shredded *Agave* to water is 2:1, and thus formally begins the fermentation. The water used throughout the process comes from two sources: normally from a natural current (river or stream) (*palenques* 1, 3, and 4) or tap water for the development of many of the productive activities (*palenque* 2). This phase of the process lasts 5 to 7 days on average depending on the climatic conditions of the *palenque*. The fermentation of the cooked *Agave* musts and liquids is spontaneous. From the beginning of the transformation process, *Drosophila melanogaster* fruit flies attracted by the exquisite aroma of the sugary must visit the containers, although prior to fermentation. These Diptera very frequently visit the flowers of the plants cultivated in the orchards and the natural vegetation around the *palenque*. All this suggests that these flies inoculate the ferment with the microbiota of the surroundings. It is also pertinent to mention that the fermentation containers, the utensils, and the tools used during the transportation of the cooked *Agave* to the fermenters are never washed,

which also suggests that the remains of the fermenting microbial consortium have been stored there and are recovered as inoculates at the very moment of starting the described process again. From the second day of fermentation, the containers begin to produce gasses because of the transformation of the musts, and, over time, this phenomenon is perceived because of the loud sound it emits. Two days after this, the production of gasses ceases completely, and the fermentation enters another phase, possibly microaerophilic. Occasionally, and when the ambient temperature drops drastically, the fermenters can be covered with a mat or a 4 m^2 sheet of polystyrene to prevent the fermentation from stopping. The end of the fermentation time is decided by the *mezcalero* after testing the odor, the flavor of the ferment, and, according to its sweet or acid taste, decides to start with the distillation phase. During the fermentation, temperature varies from 26 to 28 °C, and the metabolic process of microbiota acidifies the must from pH 4.8 to 4.3.

3.2.6. Distillation

The distillation is the last phase of the process. Conventionally, a copper alembic is used, which is composed of a 200 L pot, the "*montera*" or steam concentrator, and the turban. The last element is the tube that conducts the steam to the coil where it achieves condensation and it is totally submerged in water (Figure 2B, *palenques* 1, 3, and 4). Some *palenques* use other metal distillation devices with two clay pots (Figure 2B, *palenque* 2). The clay pots are superimposed one on top of the other, with a plate-shaped copper condenser. This element is cooled with a constant flow of water. The upper pot is in direct contact with the vapors and condenses them. Then, a drip is produced and led to the outside of the clay container by a grooved wooden structure. Generally, this wooden structure is made with *Arundo donax* or the hollow inflorescence of some *Agave* species. The two devices described are heated with firewood for the entire duration of the activity (30 to 50 h), depending on the volume of the fermentation. In the copper still, when the pot has been heated perfectly, after approximately 2 h, its walls can reach 900 °C; that is when the production of steam and its condensation as mezcal is almost continuous. When the metal still is heated beyond the referred to temperature, however, the process can cause extremely hot liquid to pass through the entire still apparatus and contaminate the distillate, which is why the regulation of the heating is extremely important. While the clay still does not overheat because of the seal between each of the pieces that compose it, they fail to cause the steam to exert great pressure.

3.3. Yeast Diversity in Four Palenques for Mezcal Production in Oaxaca, Mexico

The obtained phylogenetic analysis is represented in Figure 3. The maximum likelihood phylogenetic tree allows us to identify that all the identified species from *palenques* 1, 3, and 4 are distributed in all the branches without forming a particular cluster. The only species that are separated from all the sampled diversity are those from *palenque* 2. It is in this group where it is possible to recognize three sequences from the same locality but only representing two different species: an endophytic fungal species and a species from the *Pichia* genus. The only element of this cluster that does not correspond to *palenque* 2 in the cluster is an element recognized as an organism from the same *Pichia* genus. The arrangement presented in the figure enables us to recognize that although the diversity is low, its phylogenetic signal is clear. This arrangement proposal is significant because the bootstrap value is calculated for this position. The species related to *palenque* 2 are different because of their phylogenetic signal and their reduced representation.

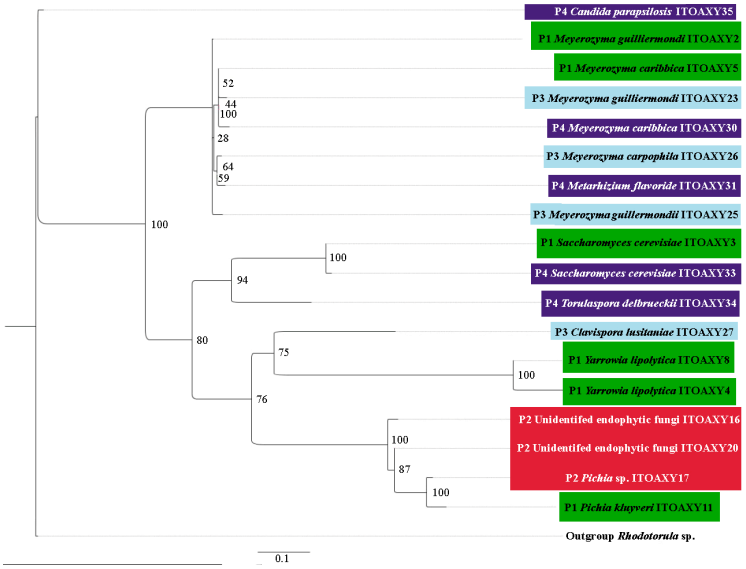

Figure 3. Maximum likelihood phylogenetic tree of yeast strains isolated from four *palenques* in Oaxaca, Mexico. Each branch corresponds to a resulting sequence obtained from the different localities of *palenques*. The name of the sequence species is based on the identification made with specific BLASTN. The color code is green: *palenque* 1 in San Dionisio, Ocotepec, and red: for *palenque* 2 in San Pablo Etla. Blue corresponds to palenque 3 in Río Las Palmas, in the municipality Concepción Buenavista and purple for *palenque* 4 in San Pedro Teozacoalco. The node number is the support probability for each topology region. It was defined as the result of an ultrafast bootstrap made with IQ-TREE with 10,000 repetitions.

3.4. Relation between Yeast Diversity and the Biotechnological Variables

The obtained heatmap graphically presented in Figure 4 represents a hierarchical cluster analysis where it is possible to link all variables with the different analyzed localities. Firstly, a shared signal combined in the clustering grouping can be recognize, in addition to the differential signal of variables; only temperature and pH are elements that are isolated from the rest of the cluster. The data of all localities enable us to propose a dendrogram that can demonstrate two different types of *palenques* with differences focused on the *Agave* species and the container used for fermentation. With this figure, it is possible to show that among all the localities, because of their particular processing, *palenque* 2 possesses values that are dissimilar from the entire group. The majority of the values that create the difference and cause this separation are those linked to the cooking and distillery processes. These results are coupled with the final mezcal products and are comparable with the total soluble solids' values.

Figure 4 represents the analysis to integrate all variables and recognize which are most significant to compare the different locations and corresponding elements in the elaboration of mezcal. In this case, the modifications performed in the R code allow us to use all variables in a whole complete comparison with additional data to all the studied parameters presented in the locality comparison.

In this case, the graphical representation shows that the oven material and the grinder method are the methods that skew and provide the significant values for all the *palenque* processes and the resulting products. In this study, the variance defines that the earth grinder process and the clay oven have the highest values and make it possible to differentiate all the localities by combining the following: a secondary level of comparison, the variables of total soluble solids, variables involved in the cooking (clay and stone oven), and the quality of water (river or tap *Torulaspora* water). The variables less related to the variance for all the samples were temperature and pH. These values seem almost identical for all the localities (Figure 5).

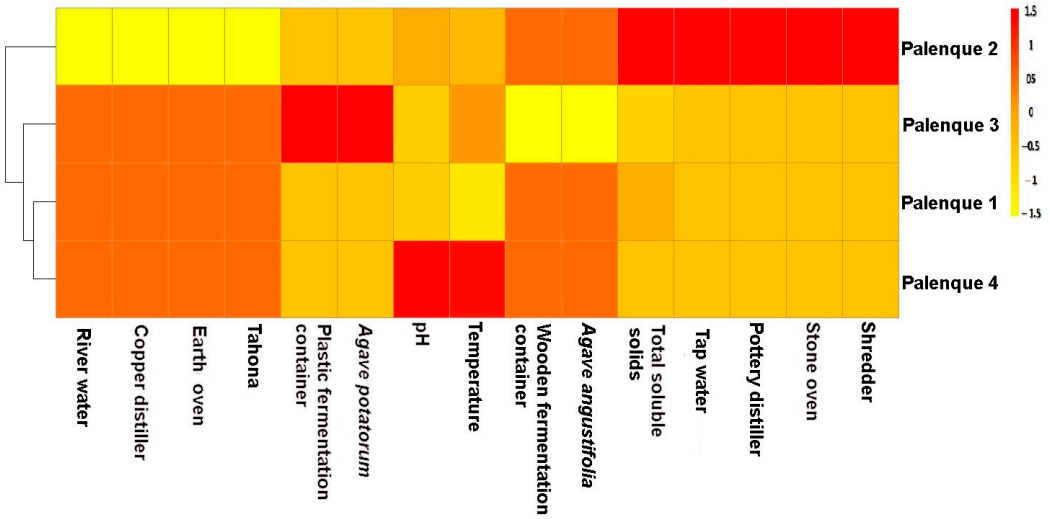

Figure 4. Heatmap of biotechnological process elements and equipment used in four *palenques* of Oaxaca, Mexico after scaling all the aspects and discrete features recognized in this study. Using the heatmap from R language and defining yellow as low numbers on the scale and red as the higher values proposed for each variable, it is possible to arrange the *palenques'* relationships using a Ward.D2 clustering method defined on the left side of the figure.

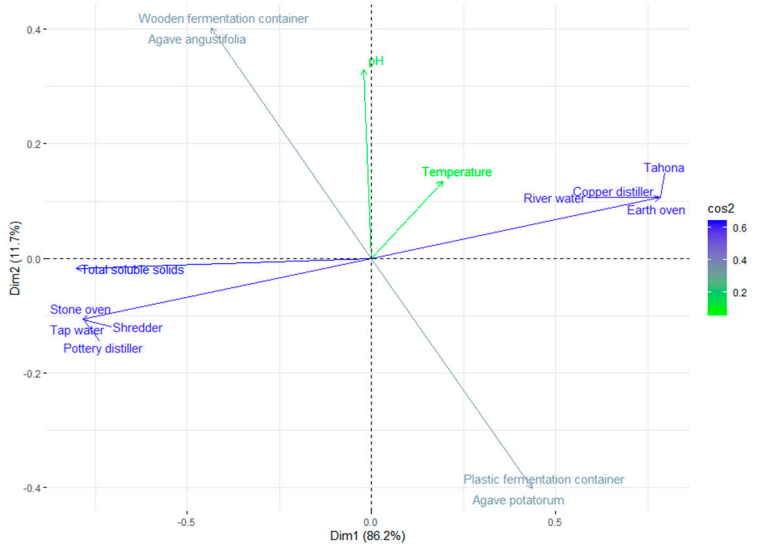

Figure 5. Principal component analysis of biotechnological process elements and equipment used in four *palenques* of Oaxaca, Mexico. This biplot defines in red all the variables with variances that are significant and enables us to select them among the rest of the other principal components. Green defines all the most significant variables, including all those components of intermediate tones. Blue components might be subtracted to make a comparison (Figure 5).

4. Discussion

Regarding the *Agave* species destined to produce mezcal [23,25–27], it is recognized that *Agave angustifolia* and *A. potatorum* are indeed used in mezcal production processes and that the various mezcals essentially differ by the *Agave* species used, as well as by the traditional cooking, fermentation, and distillation conditions during the production process, which they evidently share with the conditions of the Oaxacan *palenques*.

Agave angustifolia and *A. potatorum* have organic compounds that contribute to the organoleptic features of mezcal. Both *Agave* species have diverse carbohydrates such as mono- and disaccharides, fructooligosaccharides, and fructans (including agavins) [24]. These carbohydrates and other prebiotic compounds are the substrates for the fermenting

microorganisms. In addition, *Agave angustifolia* has monoterpenes and sesquiterpenes like limonene, linalool, geraniol, and *trans* nerolidol, among others [28]. The microbiota transforms *Agave* compounds into alcohol and metabolites that contribute to organoleptic features of mezcal. So, *Agave* spp. are suitable for mezcal fermentation.

Hernández-López [26] also described the environmental, technological, and productive conditions of the *palenques* in which his study was performed; he also mentioned that these same conditions are recognized in the Official Mexican Standard NOM-070-SCFI-2016 [1], which does not specify that these are traditional mezcal production processes. The author stated that artisanal mezcal "is a deeply rooted drink with a direct connection to the indigenous world".

According to NOM-070-SCFI-2016 [1], ancestral mezcal is produced with basic technology, particularly the use of wood mallets in the step of shredding for crumbled cooked agave. This step is carried out in holes dug in the soil. The fermentation is spontaneous in leather, wood, or stone containers where the cooked agave is transformed into mezcal. The clay or wood distillers are used with a copper condenser (Figure 2A,B, *palenque* 3). All these equipment and processes contribute to the complexity of ancestral mezcal and the genuine complex flavors and tastes due to the contact with the soil, the clay minerals, and the natural fuel from which it is made. Meanwhile, the artisanal mezcal (according to the NOM), is a beverage produced with modern technology. The shredding step is carried out with a *tahona* (stone mill with animal or mechanical traction). The fermentation takes place in wood containers and the distillation is carried out with copper distillers. The artisanal mezcal has a higher ethanol concentration compared with ancestral mezcal, but it still has a lot of flavors and taste. The artisanal mezcal has a weaker relationship with natural elements of the *palenque* environment compared with the ancestral process. Both types of mezcal have the invaluable richness of knowledge of the Oaxacan producers and have the microbial diversity of the community. Finally, according to NOM, the mezcal is the most technified distilled beverage. It is produced at different scales from pilot plant to industrial scale. In this process, the shredding is carried out with stone or mechanical metal mills, with hammers or blades. The fermentation process is controlled with only *Saccharomyces* as inoculum. So, the flavors and tastes are limited. The volume of ethanol is higher compared with the other two types of mezcal, and the distillation is carried out in copper *alambiques* or stainless-steel distillers. This alcoholic beverage has less flavor but a higher ethanol concentration.

It is recognized that spontaneous fermentation determines the presence of a very diverse microbial community, which in turn is responsible for many metabolic transformation processes of the sugars contained in the cooked agave, which produce an infinite number of fruity, herbal, caramelized, mentholated, smoky, dairy, and acidic flavors, etc. Also, there are a wide range of aromas, mostly recognized by their similarity to quite defined chemical compounds, such as various alcohols, a multiplicity of terpenes, esters, aldehydes, and ketones (Supplementary Table S1). All of them leave a unique chemical imprint on traditional distillates; in contrast, most commercial distillers, since they control the fermentation and therefore the microorganisms that carry it out, are more committed to producing distillates with higher alcohol contents with very few flavors and aromas [29]. That is why these organoleptic characteristics place distillates in three different categories according to their chemical contents.

Black and Thoms [30] have found that earthen ovens, such as those currently used in the study areas for the *Agave* cooking process, have archaeological precedents, referenced in other parts of the continent, being built with the same method and functional structure as current ovens and having been used for cooking food for just over 10,000 years. It must be recognized that the literature has barely considered the rustic conditions in which the traditional mezcals of Oaxaca have been produced.

The phylogenetic tree presented in Figure 3 represents a clear distribution from diverse genera involving fermentation alcoholic beverages. The presence of *Saccharomyces* in not all the processing sites shows that although the time and conditions of the processes are similar,

their diversity might be due to different conditions, and the fermenting containers, ovens, and grinders are probable components of enrichment in all processes. It confers a unique evolution and succession among them. The *Saccharomyces* genus is present in previous reports [31,32] as elements in the fermenting biodiversity in diverse alcoholic beverages. It is possible to assume that these elements could prevail even in the late-fermenting phases because of their previously reported tolerance in high-alcohol environments, but they are particularly recognized features present in *palenques* 4 and 1 and absent in *palenques* 2 and 3, even though *palenque* 2 creates mezcal with higher values of total soluble solids. The prevalence of *Saccharomyces* in this case is not related to strains with tolerance to higher alcoholic values but interactions and even an initial contact with organic substrates and an interaction with strains possibly originating from the microbiome of Insecta, particularly because of the contact with *Drosophila* spp. [33]. This contact with *Drosophila* species is confirmed by observations recorded in different workshops where it has been recognized that this contact is significant in the early process recognized in the fermenting containers.

The diversity of yeasts involved in the mezcal fermentation is very large, and normally varies from one place to another, mainly due to the complex molecules present in the agaves, the environmental conditions, and the variations in the production process, which decisively influence about the presence of these unicellular fungi. These microorganisms involved in the fermentation process, and their metabolic capacities, are directly responsible for the transformation of the raw material into a wide range of volatile and non-volatile compounds that give distinctive flavors and aromas to this distilled beverage from Oaxaca and from other places in Mexico, which are also related to the quality of the final product. This direct relationship between the chemical nature of the raw material, the physicochemical conditions of the fermentation process, and the activities during mezcal production is so particular that it is the reason why there is a huge range of very different mezcals in the country [7,27,34–36].

One feature in the topology of the phylogenetic tree is the appearance of the *Candida* genus. According to [37], the incidence of this genus might be involved in later fermenting phases, where complementary reactions of oxidation and reduction of organic compounds combine different second-order compounds only to generate a variety of chiral synthons with the increase in secondary metabolites by *Candida*.

The incidence of the *Meyerozyma* genus might have a role in the diversification of biochemical reactions present in the diverse fermenting microbial communities [38]. *Meyerozyma guilliermondi* is reported as a species involved in the fermentation of sugars and even in the uptake of acetic acid that limits its metabolic interaction only if the pH is lower than 3.5 which is similar in the fermentation reactions in every locality that is present in the study. The *Meyerozyma* species are reported as fermenting yeasts that might be involved in sugar-enriched media and conditions with an increasing acetic acid and furfural concentration. These latter compounds are elements that need to be measured in later studies to have a complete view and of conditions possibly present in other mezcal fermentation processes. On the other hand, *Torulaspora* spp. are species recognized in *palenque* 4. Their prevalence and input might be a feature in the enrichment of mezcal because these organisms in wine making are some of the most important in the enrichment and production of ethanol, glycerol, volatile compounds, succinic acid in the medium, anthocyanin processing, and other compounds involved in sensory perception of the beverage [8].

Clavispora spp., unlike the previously described species, have been previously described in studies [39,40], where species directly linked to the elaboration and fermentation of mezcal are reported. The prevalence of these species only in *palenque* 3 gives a notion about how diverse the artisanal process analyzed in localities 1, 3 and 4 is.

Yarrowia spp., on the other hand, are possibly general fermenting elements, which previously had only been linked to the fermentation of food [41]. Their prevalence in a single location leads to recognizing that their presence can have a complementary ecological role as a modifier in the environment and a synthesizer of molecules which promotes the construction of biofilms.

The proposed phylogenetic tree is evidence of the aerial enrichment of fermenting species received in the containers for the elaboration of mezcal. This diversity is similar in all the localities, and the reception of species is a stochastic process. According to personal communications with *mezcaleros*, the fermentation of the mezcal could change its condition, notes, and particular flavors because in all the localities a fermenting seed or basic organism is not used. The diversity comes from the seasonal conditions and the aerial contact brings a wide diversity and that is why there are no skews within the analyzed *palenques* except for *palenque* 2.

The recognition of this main skew in the phylogenetic tree might be defined because of the elaboration that is mentioned in Figure 4, where the biotechnological properties in making mezcal, such as the type of water that is used in the fermentation process, the stone oven, and even the change in distiller, might be factors that reduce the number of species presented in the analyzed samples.

The biodiversity of the yeast in mezcal fermentations of the four *palenques* of this study included the genera *Candida*, *Meyerozyma*, *Yarrowia*, *Torulaspora*, *Clavispora*, and *Pichia*. The species *Saccharomyces cerevisiae* and *Clavispora lusitaniae* have already been reported in mezcal fermentation [6]. *S. cerevisiae*, *Clavispora lusitaniae*, *Torulaspora delbrueckii*, and *Pichia kluyveri* have been reported in mezcal fermentation of *Agave salmiana* in San Luis Potosí [42]. *S. cerevisiae*, *T. delbrueckii*, *C. diversa*, and *Pichia fermentans* have been reported in mezcal fermentation in Durango from *Agave duranguensis* [43]. *Candida parapsilopsis*, *Clavispora lusitaniae*, *Pichia kluyveri*, *S. cerevisiae*, and *Torulaspora* were reported for mezcal fermentation in Oaxaca [44]. *S. cerevisiae*, *Pichia kluyveri* var. *kluyveri,* and *Torulaspora delbrueckii* have been reported in other fermented beverages, such as "*champús*" [9].

Fermentation performed with non-*Saccharomyces* species included the following: *Candida*, *Clavispora*, *Meyerozyma*, *Pichia*, and *Torulaspora* for alcohol fermentation. Other authors have reported *Zygosaccharomyces*, *Torulaspora*, *Pichia*, *Debaryomyces*, *Clavispora*, and *Candida* [12]. Microbiota is one of the main factors for the organoleptic properties of mezcal including volatiles [4].

5. Conclusions

The unconventional environmental, technological, and cultural conditions of the production of artisanal mezcals in Oaxaca, Mexico are why the diversity of yeasts involved in the microbial consortia that ferment the musts of *Agave angustifolia* and *Agave potatorum* are so exceptionally original and unique, which is closely related to the megadiversity that surrounds the localities where the research was performed and that definitely provides sensory characteristics that distinguish this class of mezcal today, without forgetting that the technology used largely depends on the ancestral knowledge that the native people of Oaxaca still preserve, where the distilled beverage under study is produced daily. The presence of seven genera, thirteen species, and two varieties of yeasts is reported here, some of which are reported for the first time in a mezcal production process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/beverages9040099/s1, Supplementary Table S1: Yeasts present in *Agave* distilled beverages and the most prominent volatile and non-volatile compounds. Supplementary Table S2. Data used to generate the heatmap in Figure 4. Supplementary Table S3. Where is grouped the corresponding data from the correlation matrix used to generate the PCA analysis. This table is the result to apply the "corr" R function, originated in the "corr" and the "ggcorrplot" packages. All the data is escalated and to use this table as a matrix, the table present the variable in the first line and in the first column [45–50].

Author Contributions: Conceptualization, C.L.-S. and F.d.J.P.-C.; methodology, V.A.E.-M., P.E.A.-G., F.d.J.P.-C. and C.L.-S.; software, V.A.E.-M. and H.G.V.-L.; validation, P.E.A.-G. and F.d.J.P.-C.; formal analysis, V.A.E.-M. and H.G.V.-L.; investigation, F.d.J.P.-C., R.E.-V. and M.P.R.-L.; resources, C.L.-S., P.E.A.-G. and F.d.J.P.-C.; data curation, V.A.E.-M. and H.G.V.-L.; writing—original draft preparation, V.A.E.-M., C.L.-S. and F.d.J.P.-C.; supervision, C.L.-S.; project administration, C.L.-S.; funding

acquisition, C.L.-S. and F.d.J.P.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Public Education Ministry of the Mexican government (PRODEP 511-6/2019.-8513). The authors thank the Mexican Council of Science and Technology (CONACyT) for the financial support through V.A.E.-M's student grant number 811304.

Data Availability Statement: ITS sequences of yeast isolates are available in the GenBank database under accession numbers OR242522 (P1, *Pichia kluyveri*); OR242523 (P1, *Yarrowia lypolitica*); OR238518 (P1, *Meyerozyma guillermondii*); OR238911 (P1, *Saccharomyces cerevisiae*); OR2378916 (P1, *Yarrowia lypolitica*); OR239058 (P1, *Meyerozyma caribbica*); OR244474 (P2, Uncultured endophytic fungus); OR239766 (P2, *Pichia* sp.); OR244475 (P2, Uncultured endophytic fungus); OR239800 (P3, *Meyerozyma guillermondii*); OR239863 (P3, *Meyerozyma guillermondii*); OR239865 (P3, *Meyerozyma carpophila*); OR239874 (P3, *Clavispora lusitaniae*); OR239875 (P4, *Meyerozyma caribbica*); OR240083 (P4, *Metarhizium flavoviridae*); OR240087 (P4, *Saccharomyces cerevisiae*); OR240088 (P4, *Torulaspora delbrueckii*); OR240089 (P4, *Candida parapsilosis*).

Acknowledgments: The authors thank Rigoberto García Bautista (*palenque* 1), Armando Isidro Aquino (*palenque* 2), Antonio Trujano Ramírez and Javier Trujano Ramírez (*palenque* 3), and Jesus Velasco, Pedro Cruz Franco, and Imelda Ramirez Cuevas (*palenque* 4) for the facilities provided for personal interviews and bioprocess sampling.

Conflicts of Interest: The authors declare no conflict of interest.

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