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Review

Unveiling the Microbial Ecology behind Mezcal: A Spirit Drink with a Growing Global Demand

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Abstract: The advent of omics has expanded our knowledge of microbial ecology behind Mezcal, a fermented spirit made from the juices of cooked Agave plants (*Agave* spp., Asparagaceae). Mezcal has been produced in Mexico for over 200 years, however, has been in high demand since its discovery by international markets in the last decade. Mezcal is appreciated for its diverse and complex sensory profile, which is tied to the geographic and environmental diversity of the different Mezcal-producing regions. This regional typicity is brought about by spontaneous fermentation consortia that act in loosely controlled artisanal fermentation processes. Previous works have mainly concentrated on microorganisms involved in the biosynthesis of alcohol and other volatile compounds, or from a different perspective, on culturable microorganisms (mainly yeasts) influencing the taste profile. Attention has been aimed at the richness of microbial populations in point events or under laboratory conditions, which leaves much of the biological richness out of account. Omics techniques have become powerful tools for characterizing the composition of autochthonous fermentation microbiota, regional or endemic features, and ecological processes that determine the dynamics of Mezcal fermentation. The analyses of genetic material, proteins, and metabolites allow disentangling the biological complexity of Mezcal production. This review presents the reader with an up-to-date overview of publications that discuss microbial communities in Mezcal fermentation, metabolic pathways regulated by microbial interactions, and the application of omics to characterize the spontaneous fermenting microbiota conformation and dynamics considering the subjacent ecological processes.

Keywords: agave; aroma volatiles; fermentation; microbial ecology; microbial interactions

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1. Introduction

Mezcal is a traditional Mexican alcoholic spirit rapidly gaining popularity in the global market thanks to profile and quality optimization [1,2]. In 2021, more than 8 million liters were produced, of which about 70% were exported to 65 countries [3]. Mezcal carries the Denominación de Origen Mezcal (DOM, Appellation of Origin Mezcal) label [4]. The production of Mezcal involves the—usually spontaneous—anaerobic fermentation by undetermined microbial consortia, followed by the distillation of the fermented must to concentrate the alcohol and other less abundant volatile compounds. Mezcal's wide variety of sensory characteristics are derived substantially from the fermentation step.

Various studies have focused on the microbiota composition of Mezcal, Raicilla, Bacanora, Tequila, and pulque (all Agave-derived beverages) (reviewed previously by [5–7]. However, few scientific publications on Mezcal consider the vast process diversity (discussed further) without considering the origin of fermenting microbiota and related ecological phenomena involved during Mezcal production. The available work on Mezcal mainly

concentrates on the identification of dominant microorganisms, mostly ethanol-producing and functional yeasts, and is limited to discrete fermentation phases or sampling only near the surface of the tank [8–12]. In addition, although to a minor extent, few works have considered the characterization of the non-yeast microbiota members such as bacteria based on using culture-independent techniques [13,14]. Identifying minority microorganisms in spontaneous fermentation is vital for investigating their impact on acting communities and exploring their influence on the sensory characteristics of the final product [4,15], in addition to understanding the ecology of the community, including unexplored members such as viruses, protozoans, and algae, among others.

Many research opportunities remain unexplored, as knowledge generation around Mezcal production has mainly focused on process improvement [16]. Identifying natural reservoirs acting as the microbiota's source in spontaneous fermentation, the wide microorganism diversity, ecological interactions, and successional dynamics occurring during different fermentation phases are still pending issues.

The microbiota acting in the spontaneous fermentation of Mezcal, derived from unique cultural practices and local environmental biodiversity, make every production batch practically unique and unrepeatable, even in the same factory [17]. The most experienced Maestros Mezcaleros (MMs) (empiric-skilled Master distillers) recognize this situation (MMs, personal communication). Mezcal is, hence, a vibrant heterogeneous spirit. Disastrously, most technical recommendations are exclusively oriented to homogenizing the sensory profile and increasing ethanol yield by incorporating industrial practices such as sanitization and yeast inoculation instead of more robust approaches such as synthetic ecology previously explored in wine fermentation [18,19]. Cleanliness will limit the essential cross-contamination paths for microbiota incorporation and establishment in fermenting consortia. Yeast inoculation may limit the development of non-*Saccharomyces* yeasts and other minority microbes contributing to sensory complexity. It is desirable that Mezcal follows a different path that of Tequila, whose industrialization has impoverished the sensory diversity of the spirit [20]. To maintain the complexity and diversity of Mezcal, the use of high-throughput analytical techniques such as the omics sciences has demonstrated to link sensory profiles to microbial components and geographical differentiation in diverse fermented products such as wine, Sichuan Paocai, or Chocolat [21–23], among others.

In the following sections, the ecological factors influencing the composition and progress of fermenting microbiota for Mezcal production are analyzed, particularly those taking advantage of the 0mics sciences, and possible future lines of investigation are suggested that may help to preserve Mezcal diversity, environmental richness, and cultural practices of original production processes.

2. Diversity of Mezcal Production

As mentioned before, the production of Mezcal is a hyper-diverse process and a testimony to the environmental heterogeneity of Mexican geography. It includes highly phenotypically/genotypically diverse Agave plants. In addition to this, the complexity of the Mezcal production process depends on several variables, such as environmental temperature (from 5 to 40 °C), altitude (400 to 2500 m a.s.l.), and climate (Aw, Bw, and Cw), vegetation (tropical deciduous forest, savanna, and desert shrubland). MMs have empirically determined that environmental variables and (even slight) seasonal changes are responsible for substantial quality variation between production batches and regions. This includes high phenotypic variation from different Agave species that can be used as raw materials [24]. Mezcal diversity also encompasses a multitude of different and producer-specific cropping, cooking, milling, fermenting, distillation, and aging techniques. Production practices, moreover, may vary between the different production regions, localities, and neighboring factories [4,17,25]. Even different family members from the same factory may introduce cultural practices resulting in a particular variation in product quality (MMs from Oaxaca and Michoacán, personal communication). This diversity is acknowledged by the Official Mexican Standard NOM-070-SCFI-2016 [26]. It is undoubtedly

approved by countless consumers who discover a sensorial experience worthy of further exploration in each production batch. For further information in this regard, a recent review on marketing, production practices, and sustainability of the artisanal process has recently been published [16]. The Mezcal process may be labeled as "Mezcal *ancestral*," "Mezcal *artesanal*" (artisanal Mezcal), or just "Mezcal" (non-compliant procedures established from the other two categories), depending the different processes, illustrated on Figure 1. All this diversity makes Mezcal a distilled beverage of broad variability and extensive sensorial profile. The further sections of this paper focus primarily on artisanal Mezcal, as it is the dominant category representing 88.92% of bottled production in 2021 [3].

Figure 1. Schematic representation of Mezcal production process diversity and natural reservoirs/sources of microbiota. Different stages in the process are shown: (**a**) monoculture-produced

plants under a technological package involving agrochemical applications in a perturbed environment prone to low microorganism diversity; (**b**) wild Agave plants in contact with highly diverse populations of macro- and microorganisms; (**c**) "bleeding Agave" plants whose floral meristem has been removed—these plants show signs of in-field fermentation, changing their aspects and producing a reddish resin from the injury; (**d**) plant harvesting and transport to factory; (**e**) *piñas* lay in bare soil for few days starting to produce evident signs of microorganism growth; (**f**) cooking procedures; (**g**) microorganism cross-contamination vectors. Large arrow represents the putative microorganism flux occurring from the environment to the cocked *piñas*; thick arrows represent bi-directional flux of microorganisms between concomitantly occurring processes. (**h**) Evident microorganism growth in cooked *piñas* is desired by some producers; (**i**) milling practices involving manual or bare feet crushing, use of animal-driven mills, or mechanized alternatives; (**j**) wooden, plastic, or rawhide fermentation containers; and (**k**) distilling in ceramic or metallic stills.

3. Spontaneous Fermentation: Origin of Microbiota and Environmental Effects

Some Mezcal production (primarily industrial) is based on plants from monospecific plantations, promoting long-term land-use change and altering the local biodiversity with the consequent increase in phytosanitary problems. This production system is usually linked to excessive agrochemical use (mainly pesticides and fungicides) that may as a consequence affect soil and endophytic microbial diversity [27] (Figure 1a). In contrast, the artisanal Mezcal production process begins with the harvesting of mature wild Agave plants. Wild Agave exploitation has a significant environmental impact, as producers open new paths to harvest plants from the wild and to gather the fuelwood necessary for the cooking of Agave heads. However, those plants are growing in pristine environments, as the soil and endophytic microbiota are expected to be more diverse (Figure 1b). Microbial diversity loss in domesticated crops has been demonstrated in several models [27–31]. In the case of Agave, however, the geographic comparison of microbiota structure suggests the persistence of well-adapted bacteria [32], including some fermenting microorganisms in tissues from commercially produced plants such as *Acinectobacter* sp., *A. baumanii*, *A. bereziniae*, *Cronobacter sakazakii*, *Enterobacter hormaechei*, *Bacillus* sp. *Klebsiella oxytoca*, *Pseudomonas* sp., *Enterococcus casseliflavus*, *Leuconostoc mesenteroides*, and *Gluconobacter oxydans*. [33], suggesting that plant and associated microbiota domestication has had an unclear impact on the holobiont communities.

The empirical knowledge of MMs regarding the role of microbiota is reflected in the harvesting process. This person sources the plants and decides whether wild or cultivated Agave will be used. Wild Agave is generally favored because it yields a better-tasting end product (MMs from Oaxaca and Michoacán, personal communications). MMs also determine at which stage of maturity the plants are harvested. In some cases, when mature Agave plants start the reproductive phase, the floral meristem is cut in the field, triggering a deliberate mid- to long-term decay process of in-field fermentation lasting up to a year, leading to a so-called "bleeding Agave," for its characteristic reddish resin production (Figure 1c). The vegetative to flowering phase change in Agave plants carries the mobilization of complex carbohydrates to low-molecular-weight skeletons able to undergo long-distance allocation [34]: from the stem to the floral meristem up to 10 m high. This shift in carbohydrate content may affect the abundance of endogenous or environmental saprophytic microbial communities, contributing in a different way to the microbial input to the factory. Cropped Agave heads or *piñas* are transported to the factories, dropped in the bare soil, and cut in half, remaining outside to be exposed to insects, animals, dust, and other microbial sources until processed (Figure 1d). Following the same empirical reason as the "bleeding Agave", in some cases, cropped *piñas* are deliberately allowed to decay once cropped until the formation of visible microbial colonies (Figure 1e). These practices must be considered as relevant methods of input and reproduction of environmental microbes in the receiving factory; most of them are widely appreciated by MMs for producing specific sensory profiles (MMs from Jalisco, personal communication).

The processing of Agave *piñas* starts within a few days, with cooking in earthen pit ovens or in some cases using industrial autoclaves (Figure 1f). Cooking is expected to sterilize the *piñas*; however, cross-contamination begins immediately when the oven is open after cooking. As artisanal methods are employed, several vectors for cross-contamination inoculation can be identified, involving hand and feet manipulation of cooked *piñas*, the use of unsterilized water and tools, and the presence of domestic animals and insects, among other sources (Figure 1g). Again, in this phase, cooked *piñas* are allowed by some MMs to decay near the oven pit until evident microorganism colonies appear (Figure 1h). Cooked Agaves are milled to reduce their size and extract the juices to facilitate fermentation. Milling is traditionally carried out by hand or by an animal-driven mill pulled by horses, bulls, or donkeys (Figure 1i). Production processes that apply modern techniques use mechanical shredders powered by gasoline engines or electric motors [35]. These milling techniques may contribute in different ways to cross-contamination, in which more mechanization tends to lower local biodiversity that may act as a reservoir for autochthonous microorganisms. Cross-contamination is also expected to occur between raw and cooked Agave when the workers handle at the same time fresh, cooked, and fermented material with their bare hands or the same tools from separate batches at different production stages (Figure 1k).

The cooked and crushed fibers and extracted juices are transferred to wooden containers for the fermentation stage, although rawhide, brick, stone, and even plastic containers are also used [36] (Figure 1j). The vessels are filled to about 75% capacity with crushed fibers and accommodated with a fork, shovel, or wood stick, frequently manipulated by the MMs or a family member standing inside the containers with bare feet. Most of the cross-contamination has already occurred at this stage, cooked fibers and juices are inoculated, and the "formal" fermentation begins. The initial fermentation phase lasts two to three days. The MMs determine its duration by considering the bubbling sound as a guide (empirical observations by authors) (Supplementary Video). Then, crude, untreated water is added until the volume reaches 90% of the vessel's capacity. Juices and fibers are sometimes agitated as an empirical practice to accelerate fermentation [6]. Fermentation generally takes approximately 8 to 20 days to complete and depends on various factors, which are explained in the following paragraphs. During all fermentation phases, containers remain open with free access to insects, primarily bees and fruit flies. Once bubbling ceases, fermented must is transferred to a copper still in the case of artisanal Mezcal, in clay pots for ancestral Mezcal, in copper stills with wooden parts, or stainless-steel stills and distilled (Figure 1k). The distilled spirit remains in separate batches and is sold for blending or normalization and bottling to private companies that own the trademarks for national and international commercialization.

It is thought that microorganisms associated with artisanal fermentation have been empirically "domesticated" in distilleries over the centuries, as has been described for other fermented foods and beverages [37,38]. It should not be surprising that spontaneous fermentation in traditional methods relies on the unconscious bidirectional transfer of microorganisms from natural reservoirs such as tools, containers, water, domestic animals, insects, plants, or even the workers to the fermenting containers. In each batch, a fermentation consortium with unique properties is formed by combining microbiota (each with its specific characteristics) from diverse sources [17]. Previous works demonstrate that the spontaneous fermentation consortium comprises microorganisms derived from various environmental provenances surrounding production factories [17,39].

In spontaneous fermentation, the dynamics in autochthonous microbial consortia are closely related to the richness and abundance of the species involved [15]. The function of each consortium member and their metabolic or ecological contribution depends on their genetic potential and capacity to adapt their metabolism to microenvironmental conditions (including the Agave substrate and accompanying microorganisms) [40]. Environmental factors influence the formation of particular consortia and confer specific sensory notes

to the final product [41]. These factors are covered by the term terroir, which reflects the expression of these specific conditions within a designated geographic area [42,43].

Different microbial communities develop synergistic or antagonistic relationships that drive ecological successions during fermentation. These processes positively or negatively affect the community's structure, the growth of certain species, the capacity to produce specific sensory-active compounds, and ethanol yield [44,45]. The diversity of synergistic and antagonistic relationships is fascinating from microbial ecology and bioprocess design perspectives. Bioprocess studies can translate the observations from the production of Mezcal to other models [12]. In this idea, the high heterogeneity of Mezcal production allows insight into the plasticity of microorganisms that occupy similar ecological niches in variable environments (MM from Oaxaca and Michoacán, personal communications).

The omics characterization of biological diversity, physiology, metabolism, genetic properties, and interactions of fermenting consortia in many separate production batches may help to identify critical regulatory actors within all complex communities. The eventual control of such regulators can be used to develop more stable or even increased yields throughout the year and create a more homogeneous (but no less complex) taste profile. Furthermore, microbiota modulation has been demonstrated to reduce the formation of unwanted and toxic metabolites linked to low-quality fermented beverages [46,47]. Autochthonous microbiota modulation [48] may help to overcome a significant production problem when batches with unacceptably high levels of regulated molecules such as methanol or furfural have to be discarded, leading to an economic loss for the producer family. These low-quality batches are sometimes sold to bottling companies below the production cost. Bottling companies blend Mezcal from various origins and mix norm-compliant with non-compliant production lots, producing low-quality Mezcals that negatively affect Mezcal's reputation among non-connoisseur consumers.

4. Composition of Fermenting Consortia and Regional Diversity

The number of publications that describe the members of a microbial consortium in Agave fermentation is minimal in proportion to the diversity of the process [15,44]. The first study that documented the diversity of microorganisms involved in the fermentation of Agave for Tequila production goes back to 1995 [39]. The production of Tequila is the most studied process. For Tequila, the members of a native fermentation consortium were characterized for the first time in the fermenting must of *Agave tequilana* Weber var. Azul. The dominant species reported since then are *Candida intermedia*, *C. krusei*, *C. milleri*, *Brettanomyces anomalus*, *B. bruxellensis*, *Kluyveromyces marxianus*, *Pichia membranifaciens*, *P. anomala*, *Torulaspora delbrueckii*, *Hanseniaspora* spp., *Saccharomyces cerevisiae*, *S. ludwigii*, *Zygosaccharomyces bailii*, and *Z. rouxii* [10,39,49]. These yeasts, except for *S. cerevisiae*, are considered secondary yeasts. *S. cerevisiae* has been shown to improve ethanol production during the fermentation of Agave must [8,50]. Table 1 summarizes by production region the microorganisms identified in Mezcal fermentation. Some of the identified yeasts have also been documented in Tequila (*S. cerevisiae*, *Brettanomyces* sp., *Hanseniaspora* sp., and *K. marxianus*) [8]. Rapidly growing colonizers are known to establish early populations at the beginning of fermentation such as *Hanseniaspora uvarum*, *H. guilliermondii*, *Candida zemplinina*, *LacSShancea thermotolerans*, and *Torulaspora delbrueckii* [39]. As has been reported in other fermenting processes, oxygen depletion enables a population succession by microaerophilic, facultatively anaerobic, or strictly anaerobic microorganisms, responsible for the formation of aroma and flavor, where the profile of the microbiota and its interactions can vary, mainly in the interactions between species and within species [23,51]. In the case of alcoholic fermentation, the accumulation of secondary metabolites such as ethanol also triggers a succession process leading to the progression of species with a higher tolerance to this by-product [44].

Table 1. Regional differences between identified microorganisms in Mezcal fermentation (Durango, Michoacan, Guerrero, Oaxaca, San Luis Potosi, and Tamaulipas Mexican federal states). Check symbol (✓) means that the microorganism was reported for the corresponding region.

Microorganism/Region	Dgo. [7,9,52]	Mich. [44,53]	Gro. [40]	Oax. [11,17,49]	S.L.P. [13]	Tams. [54]
Brettanomyces sp.				✓		
Candida apicola				✓		
C. boidinii				✓		
C. coliculosa				✓		
C. intermedia				✓		
C. lusitaniae		✓				
C. parapsilosis				✓		✓
C. rugosa				✓		
C. sp.				✓		
C. utilis		✓				
C. zemplinia				✓		
Citeromyces matriensis				✓		
Clavispora lusitaniae		✓		✓		
Cryptococcus albidius				✓		
C. humícola		✓				
C. kuetzingii	✓			✓	✓	✓
C. laurentii		✓				
C. uniguttulatus				✓		
Debaryomyces hansenii				✓		
Dekkera anómala				✓		
Hanseniaspora guilliermondi	✓			✓		
H. osmophila				✓		
H. uvarum		✓				
Issatchenkia orientalis				✓		
Kloeckera sp.				✓		
Kluyveromyces lactis				✓		
K. marxianus	✓	✓	✓	✓	✓	✓
Lactobacillus farraginis					✓	
L. kefiri					✓	
L. plantarum					✓	
L. pontis					✓	
Meyerozyma guillermondii				✓		
Pichia fementans					✓	
P. guilliermondii						✓
P. kluyveri	✓		✓	✓		✓
P. kudriavzevii				✓		
P. manshurica				✓		
P. membranifaciens				✓		
P. mexicana						✓
P. sp.		✓		✓		
Pseudozyma prolífica				✓		
Rhodosporidium fluviale				✓		
Rhodotorula glutinis				✓		
R. mucilaginosa		✓		✓		
Saccharomyces cerevisiae	✓	✓	✓	✓	✓	✓
S. unisporus	✓					
Schizosaccaromyces pombe				✓		
Schwanniomyces castelli	✓					
Sporidiobolus salmonicolor				✓		
Torulaspora delbrueckii	✓		✓	✓		✓
Weissella cibaria					✓	
W. paramesenteroides					✓	

Table 1. *Cont.*

Microorganism/Region	Dgo. [7,9,52]	Mich. [44,53]	Gro. [40]	Oax. [11,17,49]	S.L.P. [13]	Tams. [54]
Wickerhamomyces anomalus				✓		
Zygoascus sp.				✓		
Zygosaccharomyces bailii				✓		✓
Z. bisphorus						
Z. rouxii				✓		
Z. sp.				✓		
Zymomona mobilis					✓	
Z. mobilis subsp. pomaceae					✓	
Reported species by region:	5	10	4	39	12	9

Even though fermenting consortia may comprise a wide diversity of microorganisms, within the context of fermented Agave-based beverages, the yeast *S. cerevisiae* has received the most detailed attention because of its tolerance to relatively high ethanol concentrations. In winemaking, *S. cerevisiae* is appreciated for its high fermentation capacity and ethanol tolerance, contributing to yield [55]. As we mentioned before, the initial consortium may contain other yeasts, although their presence is undesirable because they affect yield, resulting in production batches containing unwanted levels of acetate and butyrate esters (among other metabolites) [56,57]. This limitation has been overcome by using mixed culture inoculants, including *S. cerevisiae* strains mixed with other microorganisms, to modulate ethanol yield and flavors in fermented beverages [4,58–60]. Particularly for Agave-fermented distillates, a mix of indigenous strains belonging to the genera *Saccharomyces* and *Kluyveromyces* [61], *Saccharomyces*, *Kluyveromyces*, and *Torulaspora* [62,63], or more complex mixes involving *Saccharomyces* and other non-*Saccharomyces* yeasts [64], have been evaluated as inoculants for improvement yield or sensory profile of resulting beverages.

On the other hand, several studies have recognized that the microbial communities responsible for the fermentation of pulque (a non-distilled alcohol beverage made from fermented Agave sap) varied in response to regional, seasonal, and environmental differences [65]. Exploration of the microbial diversity revealed the presence of a wide variety of Agave-borne bacteria, yeasts, and fungi, such as lactic and acetic acid bacteria, and ethanol-producing microorganisms such as *S. cerevisiae*, *Zymomonas mobilis*, and *Leuconostoc mesenteroides*; the last is a bacterium that produces dextran, the compound responsible for pulque's viscous appearance [33,66,67]. Moreover, previous evidence suggests that depending on the successional dynamics of the environment in which plants develop, endophyte structure changes, influencing the taste and quality of the raw juice [25].

Omics approaches applied to the characterization of fermenting consortia members have been used with greater frequency as technology becomes more accessible, from culturomics [9], DGGA-based [52] and amplicon-based [14] methods, genome-wide studies [10], to metabolomics [41]. Using high-throughput analytical tools is vital to propel research on Mezcal production systems to map and understand microbial diversity before market, cultural, and political decisions transform ancestral or artisanal processes [16] to the point of the loss of microbial diversity [28].

5. Microbial Contribution to Alcohol Yield, Aroma Compounds, and Sensory Profiles

In winemaking, plant endophytes and environment-borne autochthonous microorganisms associated with spontaneous fermentation are responsible for the main sensory characteristics, yield, and quality of the end product [68–70], and are even used as oenological biomarkers or terroir [21,71]. Mezcal represents a similar case, which has been recognized as the only beverage besides wine that was reviewed by the prestigious Michelin group [72]. Local small-scale and family-owned distilleries have been refining the traits of this beverage for more than 200 years. Emerging evidence suggests that in artisanal

fermenting processes, repeating cycles of empirical selection may result in the local domestication of microbial communities [8,37]. Several works have focused on the metabolic characteristics of some microorganisms and the compounds they produce during fermentation, demonstrating how environmental and geographic conditions significantly alter the molecular profile of analyzed batches [41]. The influences of these interactions on the terroir-bound sensory properties of Mezcal from different production conditions represent another vast opportunity for future research.

In the past, it was thought that metabolic products derived from the more abundant species define the final composition and sensory profile of fermented beverages [73–75]. However, less-represented species also contribute to the taste profile by releasing volatile aroma compounds with floral and fruity flavors, such as higher alcohols (1-propanol, isobutanol, amyl, and isoamyl alcohol), acetate esters, or ethyl esters [76,77]. As mentioned, the metabolic profile has been characterized in some Mezcal batches, revealing the presence of different compounds, including ethyl esters, acetate esters, organic acids, higher alcohols, and volatile fatty acids; these compounds are shown in Table 2. Chromatographic fingerprinting has made it possible to link certain aroma compounds to particular sensory profiles. Ethyl acetate, for instance, is responsible for fruity and floral notes in Tequila and wine. Higher alcohols, moreover, improve the aroma of fermented food and beverages [22,77–79], whereas fatty acid ethyl esters and acetate esters add a fruity tone to the end product [80–82].

Table 2. Aromatic compounds determined by GC-MS and HPLC in different batches of Mezcal and some non-*Saccharomyces* microorganisms that positively correlates with compound presence in Mezcal and other fermented beverages. Check symbol (✓) means that the compound was reported in the corresponding work.

Compound/Ref.	[83]	[45]	[84]	[17]	[77]	[53]	[12]	[85]	Microorganism/Ref.
1,1-Diethoxyheptane					✓				
1,1-Diethoxynonane					✓				
1,1-Diethoxyoctane					✓				
1,1-Diethoxypentane					✓				
1,3-Diethoxypropan-1-ol					✓				
1-Butanol			✓						*Clostridium acetobutylicum* [86]
2-Butanol					✓				
2-Furfuraldehyde			✓						
2-Methyl-1-propanol		✓			✓				
2-Methylbutanoic acid					✓				
2-Methylbutanol					✓	✓			
2-Methylbutyl acetate					✓				
2-Methylpropanoic acid					✓				
2-Methylpropanol					✓				
2-Methylpropyl acetate					✓				
2-Phenylethanol					✓				*Torulaspora delbrueckii* [87]
2-Phenylethyl acetate								✓	*Hanseniaspora vineae* [88]
3-Methylbutanoic acid					✓				
3-Methylbutyl acetate					✓				
5-Methyl-furfural	✓								
Acetaldehyde			✓	✓					
Acetic acid		✓			✓	✓		✓	*Cronobacter* sp. [89]
Butanoic acid					✓				
Butyric acid					✓				
Citronellol	✓		✓						
Cresol						✓			
Decanoic acid					✓				
Diethyl acetal						✓			
Dodecanoic acid					✓				
Ethanol	✓	✓		✓		✓	✓		

Table 2. *Cont.*

Compound/Ref.	[83]	[45]	[84]	[17]	[77]	[53]	[12]	[85]	Microorganism/Ref.
Ethyl 2-methylbutanoate					✓				
Ethyl 3-methylbutanoate					✓				
Ethyl acetate	✓	✓	✓	✓	✓	✓	✓	✓	
Ethyl butanoate						✓		✓	
Ethyl decanoate[8]								✓	
Ethyl dodecanoate[8]								✓	
Ethyl hexanoate								✓	
Ethyl lactate						✓			*Saccharomyces* sp. [48]
Ethyl octanoate	✓				✓				
Ethyl propionate						✓			
Ethyl valerate					✓				*Saccharomyces* sp. [48]
Furfural						✓			*Myceliophthora* sp. [22]
Furfuryl alcohol	✓								
Geraniol			✓						*Saccharomyces* sp. [48]
Glycerol					✓				
Hexanoic acid					✓				
Hexyl acetate					✓				
Isoamyl acetate								✓	
Isoamyl alcohol		✓	✓	✓	✓	✓	✓	✓	
Isobutanol			✓	✓			✓		
Isobutyric acid					✓	✓			
Isocresol					✓				
Lactic acid						✓			*Lachancea thermotolerans* [90]
Limonene			✓						
Linalool	✓		✓			✓			*Kazachstania gamospora* [91]
Menthol		✓			✓				
Methanol	✓		✓	✓		✓			
Methionol					✓				
Methyl acetate						✓			
Nonanoic acid						✓			
Octanoic acid					✓				
Oxalic acid						✓			
P-cymene			✓						
Pentanoic acid					✓				*Enterococcus* sp. [22]
Pentanol						✓	✓		
Phenylethyl acetate								✓	*Kazachstania gamospora* [91]
Propanoic acid					✓				
Propanol			✓	✓		✓	✓		
Propyl acetate					✓				
Pyruvic acid					✓				
Succinic acid					✓				
Valeric acid					✓				
α-ketoglutaric acid					✓				*Candida utilis* [92]

Mezcal shares some minority volatile compounds with other spirit beverages such as brandy, whiskey, rum, gin, baijiu, and vodka such as acetaldehyde, lactones, methanol, ethyl hexanoate, acetaldehyde, iso-butanol, 2-methylpropanol, and octanol, among others [93]. However, some compounds, such as limonene and pentyl butanoate, apparently are specific to Mezcal and can be used as markers [94]. Raw materials contain varying amounts of pectin and different species of microorganisms and will therefore influence the formation of higher alcohols, acetaldehyde, esters, and terpenes during fermentation. For this reason, microorganisms other than *S. cerevisiae* in mixed culture inoculants are appreciated for their distinctive flavor to the beverage, producing aldehydes with flavor profiles reminiscent of green apples, fresh-cut herbs, and dried fruits. Mezcal harbors a wide diversity of compounds that create a more complex profile than other alcoholic spirits such as vodka, whiskey, or rum [11,17,84,94].

6. Genomics and Transcriptomics in the Study of Microbial Fermentation Communities

The integration of omics or multi-omics approaches allows one to study in detail factors influencing microbial communities' structure, gene expression, or enzymes participating in metabolic routes resulting in Mezcal's components. High-throughput analysis of samples deepens in biological phenomena not yet studied in Mezcal production. In other systems, transcriptomic and metabolomic studies have recognized associations between products of gene expression and the presence of aroma compounds in fermented must from various origins and technological applications [79]. One such example is the expression of genes related to the metabolism of tetracyclic and pentacyclic triterpenes. These compounds are precursors to steroids and modulate cell membrane fluidity. They allow for efficient control of membrane transporters and higher tolerance to alcohol, which in the case of *S. cerevisiae* is correlated with higher fermentation yields. Another example is the positive regulation of the ADH7 and AAD6 genes, which regulate the biosynthesis of higher alcohols in the Ehrlich pathway [77,95]. The application of omics technologies may ease the selection of yeast and bacteria consortia directly or indirectly responsible for specific organoleptic profiles.

On the other hand, transcriptome data from *S. cerevisiae* and *L. thermotolerans* cocultivation experiments have shown that oxygen availability is the principal limiting factor to growth for *L. thermotolerans*, and not, as was previously thought, ethanol concentration [96,97]. Given the characteristics of these species, cell wall structure and adhesion properties may play an essential role in the modulation of ecological interactions. The investigation of microbial interactions sheds light on the molecular basis of microbial community dynamics during fermentation but also offers a better general understanding of interclade ecological interactions. However, data are limited on the impact of mixed cultures on yeast physiology and genetic and metabolic regulation [97].

Massive sequencing techniques have become more affordable, opening up new possibilities for analyzing fermentation communities from unexplored environments such as raw and cooked Agave fibers and juice. Quantifying the microbiota composition and gene expression shifts during different fermentation stages is a crucial step. Eukaryotic diversity can now be easily explored through amplification of the rRNA internal transcribed spacer (ITS) region, whereas bacterial communities can be investigated by 16S rRNA gene amplicon massive sequencing [98,99]. Previous work reporting amplicon-based massive sequencing data from Tequila, pulque, and Mezcal fermentation communities [67,100] reveal that the composition of bacterial populations is highly process-dependent, as has been reported for culturable yeasts (Figure 2).

Microbial interactions are vital to the production process of Mezcal. Understanding the microbial diversity and its interactions during the fermentation process will provide insight into the underlying mechanisms that lead to Mezcal's complexity. Computational tools would help to identify potential positive or negative regulating microorganisms, quorum sensing mechanisms, metabolic modeling, or fluxomics [101–103]. The complementary use of culture-dependent methods and culture-independent molecular tools has the potential to produce insightful data, which will enable us to gain a better understanding of the environmental interactions and their influence on the production process of not only Mezcal but also of other fermented spirits [85,95,104].

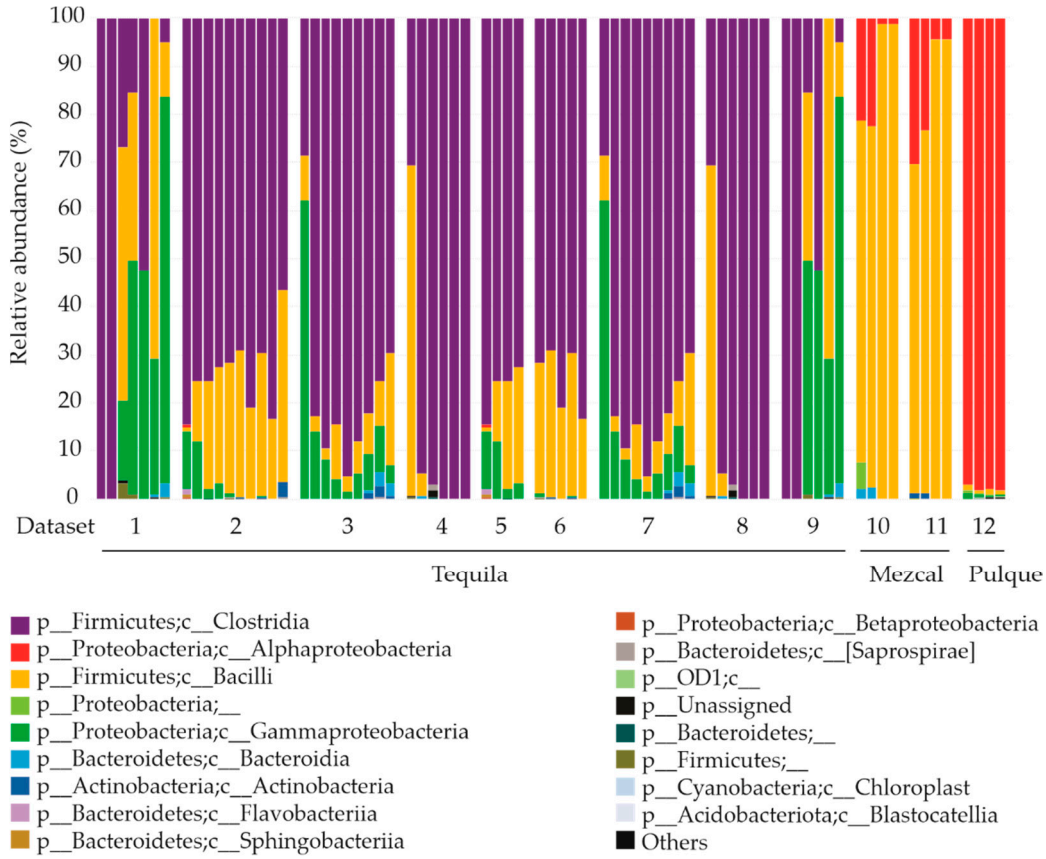

Figure 2. Previously reported bacterial microbiota comparison during Agave fermentation. Dataset comparison of fermentation process of *Agave tequilana* bagasse (1 to 9), artisanal Mezcal (10 and 11), and pulque (12). Time-course shifts in relative abundance at the phylum (p) and class level (c).

7. Conclusions and Perspectives

There is currently only limited hard evidence about the behavior and activity of microorganisms during Mezcal fermentation under natural conditions. The existing reports on alcohol-fermentation-associated microbiota are frequently limited to analyzing point events or experiments under controlled laboratory conditions. Future work based on high-throughput omics data analysis will benefit researchers who want to explore other strains and metabolic processes involved in the production of Mezcal. In addition, those who want to focus on ecological interactions between fermentation consortium members and the production of metabolites that influence the sensory profile of the final product will find information in this review that is helpful for their research. There are still many questions about the impact of the environment on the formation and functioning of spontaneous fermentation consortia. Understanding microbial diversity and interactions occurring during fermentation and identifying genes and metabolites that have not been analyzed could be linked to particular sensory profile features essential for their conservation in end products. Detailed biological and physiological knowledge revealed by genomics, transcriptomics, proteomics, metabolomics, fluxomics, and interactomics would help explore the microbial richness of many unexplored traditional distilleries, which can be recognized as essential reservoirs for microbiota bioprospection. The microbial richness of these distilleries constitutes a resource suitable for protection and benefit-sharing for legitimate owners as stipulated under the Nagoya protocol signed and ratified by Mexico [105]. Its implementation will undoubtedly contribute to increasing Mezcal sustainability, fair trade, biodiversity, and cultural preservation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8110662/s1.

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