SUSTAINABLE PRODUCTION OF ETHNIC ALCOHOLIC BEVERAGES

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SUSTAINABLE PRODUCTION OF ETHNIC ALCOHOLIC BEVERAGES

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EDITORIAL
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Editorial: Sustainable Production of Ethnic Alcoholic Beverages

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Keywords: traditional fermentation, regional drinks, ancestral knowledge, raw materials preservation, traditional lifestyles

Editorial on the Research Topic

Sustainable Production of Ethnic Alcoholic Beverages

Scientific and technological research around ethnic alcoholic beverages (EAB) has experienced different degrees of progress. Almost all human cultures have EABs, which are distinctive of their food heritage, traditions, and, in many cases, of ritualistic significance. Many EABs are for local consumption, and their economic impact may be limited. However, they can probably increase their contribution to the local economy. On the other hand, some EABs have achieved international relevance, and their commercialization has grown. However, there are two general problems. First, in the case of beverages for local consumption, it is essential to preserve the knowledge and tradition of their production as a part of cultural heritage and ethnic identity.

Nevertheless, producing these beverages could benefit the economy and quality of life of the producing communities. The problem is different for beverages that have reached increasing production and marketing. In these cases, increased production may be causing problems in the supply of raw materials, endangering biodiversity and even the survival of the species used. Depending on the industries' size and degree of development, there may be other environmental problems derived from the generation and disposal of waste from the production process. All the cases require planning for sustainable production in the long term.

This Research Topic includes five reviews and four original research articles analyzing aspects related to EAB from different geographic origins. First, Sawadogo-Lingani et al. reviewed the production of dolo, an African traditional sorghum beer, finding that production remains artisanal and faces many sustainability challenges. They suggest that strategies must focus on sustainability challenges such as assuring the supply of quality raw materials, and optimizing and standardizing processing techniques for malting and brewing, among others. There is also the need to control fermentation using starters formulated with selected strains while preserving the biodiversity of the fermenting microorganisms associated with African sorghum beers.

Rawat et al. revised EABs from Indian Himalayan Region. Their work highlights that this geographic region has alcoholic beverages enriched with nutritional components such as vitamins and proteins. They concluded that traditional alcoholic beverages play a significant role in preserving the ancient traditions of the tribes. Therefore, it is necessary to preserve the ancestral heritage of traditional alcoholic beverages for the future. Likewise, research is needed to add value to these ethnic beverages to enhance the economic sustainability of tribal communities. Nath et al. discus the diversity and interactive association of yeasts to produce EAB by indigenous communities of northeast India. It is underscored the relevance of studying yeast-yeast association and the role of chemical communication by quorum-sensing molecules like tyrosol in controlling the quality of fermented products. The commercial potential of these products could

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benefit indigenous people through the commercialization of these alcoholic beverages to impart sustainable livelihood options.

The subject of the review done by Valdivieso Solís et al. is the production of pulque, a non-distilled EAB obtained by fermentation of the agave sap called aguamiel in Mexico. It emphasizes the disappearance of large agave plantations and the long time required (at least 5 years) to reach the plant maturation for pulque production. Then, the authors also underscored the relevance of sustainable plantation models to ensure the raw material supply. They describe successful examples of beverage industrialization and potential applications of several microorganisms isolated from this production system to produce high-value bioactive products. On the other hand, Arellano-Plaza et al. argue that the substantial production increase of mezcal, a distilled EAB from Mexico, is pressing on resources for its production, particularly agaves. The manuscript reviews the current state of mezcal production, the sustainability aspects in a very artisanal process, and the challenges of the production chain in the context of increasing demand.

One of the original research articles was written by Ghosh et al. It reports the physicochemical characteristics and diversity of lactic acid bacteria of haria, a rice fermented alcoholic beverage from India. Molecular techniques allowed them to identify four species of *Lactobacillus* and uncultured *Bacillus* sp. The bacteria activity enriched the beverage with lactic and acetic acids and some vitamins and essential minerals, enhancing the nutritional characteristics of the beverage. On the other hand, Núñez Caraballo et al. investigated the interaction between yeast cells and nanostructures of chitosan-coated manganese ferrite during ethanol production by *Saccharomyces cerevisiae*. They found that biomass immobilization on nanoparticles beneficed ethanol production since fermentation time was reduced, and higher ethanol yield and productivity were obtained. Larralde-Corona et al. made a yeast selection based on a characterization protocol to identify strains with good sugar consumption and ethanol productivity, but also a good profile of esters production. Their results allowed them to propose a rational methodology to select strains for starting agave fermentations during tequila and mezcal production. Finally, Ambrocio-Ríos et al. investigated the elaboration of the beverage called taberna (Chiapas, Mexico) from a social and cultural perspective. They applied semi-structured interviews to the producers, identifying the biocultural importance for the communities and the families involved in the beverage production.

Several manuscripts included in this compilation coincide with the importance of preserving ancestral knowledge and the traditions associated with the production of each EAB. Another aspect of coincidence is the need to preserve the raw materials used to produce beverages, considering both their biodiversity and a safe and quality supply for production. Furthermore, improving production methods, ensuring compliance with quality standards, labeling, and marketing of beverages are also considered. Finally, emphasis is placed on ensuring that the production and commercialization of EABs positively impact the economy and standard of living in the producing regions. Therefore, collaborative strategies must be implemented by raw material producers, EAB producers, entrepreneurs, and governments. Those strategies must contemplate a comprehensive approach that considers the social, ecological, technological, and economic dimensions to achieve sustainable production systems.

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All authors contributed equally to the article and approved the submitted version.

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REVIEW
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Sustainable Production of African Traditional Beers With Focus on *Dolo*, a West African Sorghum-Based Alcoholic Beverage

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Spontaneously fermented sorghum beers remain by far the most popular traditional cereal-based alcoholic beverages in Africa. Known under various common names (traditional beers, sorghum beers, opaque, native or indigenous beers) they are also recognized under various local names depending on the region or ethnic group. *Dolo* and *pito* are two similar traditional beers popular in West African countries including Burkina Faso, Mali, Ghana, Benin, Togo, Nigeria and Ivory Coast. These low-alcoholic beers are nutritious and contribute to the nutritional balance of local populations, as well as to their socio-cultural and economic well-being. The production of African traditional beers remains one of the major economic activities, creating employment and generating substantial income that contributes to livelihoods as well as the countries' economic systems. Their processing (malting and brewing) is still artisanal, based on traditional family know-how. The brewing process involves either an acidification and an alcoholic fermentation phases, or a mixed fermentation combining LAB and yeasts. *Saccharomyces cerevisiae* has been identified as the dominant yeast species involved in the alcoholic fermentation, with a biodiversity at strain level. LAB involved in the processing belong to the genera of *Limosilactobacillus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, *Streptococcus,* and *Enterococcus*. Molds (*Aspergillus*, *Penicillium*, *Rhizopus*, *Geotrichum*), and acetic bacteria are often associated with the malting and brewing processes. Challenges for sustainable production of African sorghum beer include inconsistent supply of raw materials, variability in product quality and safety, high energy consumption and its impact on the environment, poor packaging and short shelf-life. For sustainable and environmentally-friendly production of African sorghum beers, there is the need to assess the processing methods and address sustainability challenges. Strategies should promote wider distribution and adoption of improved sorghum varieties among farmers, prevent losses through the adoption of good storage practices of raw material, promote the adoption of improved cook stoves by the brewers, develop and adopt starter cultures for controlled fermentation,

regulate the production through the establishment of quality standards and better valorize by-products and wastes to increase the competitiveness of the value chain. Appropriate packaging and stabilization processes should be developed to extend the shelf-life and diversify the channels for sustainable distribution of African cereal-based alcoholic beverages.

Keywords: ethnic alcoholic beverages, African traditional sorghum beers, fermentation, sustainable production, pito, *Saccharomyces cerevisiae*

INTRODUCTION

Fermentation is one of the oldest methods used by man to process and preserve agricultural products. In addition to prolonging shelf-life, fermentation confers many beneficial effects such as probiotic attributes, enrichment of essential nutrients, improvement in safety, organoleptic characteristics, digestibility and edibility, and reduction of volume and cooking time of fermented products (Taur et al., 1984; Uzogara et al., 1990; Steinkraus, 1995; Holzapfel, 1997; Simango, 1997; Oluwafemi, 2020). Depending on geographical area, the dietary habits and cultural practices of the populations, various fermented products are produced from raw agricultural materials (Campbell-Platt, 1987; Steinkraus, 1996). In Africa, there is a diversity of traditional fermented beverages produced from starchy or sweet raw materials including cereals, tubers, fruits, palm sap, cane sugar, honey etc.). However, traditional beer remain by far the most commonly produced fermented beverage that stands out among the cultural food heritage of African societies. Commonly called sorghum or millet beers, opaque beers, autochthonous or indigenous beers, these drinks are known under various local names depending on the region of production or ethnic groups (Kayodé et al., 2007; Sawadogo-Lingani et al., 2007; Aka et al., 2008; Osseyi et al., 2011; Lyumugabe et al., 2012; Bayoï and Djoulde, 2017; Touwang et al., 2018). West African sorghum beers such as *dolo* and *pito* produced in Burkina-Faso and Ghana respectively are continuously fermenting unfiltered drinks containing insoluble substances and yeasts, mildly alcoholic and acidic with a characteristic taste, aroma and flavor appreciated by the consuming populations (Odunfa, 1985; Sefa-Dedeh and Asante, 1988; Yao et al., 1995; Sawadogo-Lingani et al., 2007; Djè et al., 2008; Sawadogo-Lingani, 2010; Kouame et al., 2015). Consumers also attribute therapeutic properties (laxative, anti-malarial and anti-hemorrhoidal) to African opaque beer (Enou, 1997; Amané et al., 2005; Aka et al., 2010). Although these therapeutic properties are mostly not scientifically proven, it has been extensively reported that sorghum grains and its subsequent food products including sorghum beers are excellent sources of nutrients (sugars, proteins, amino acids, vitamins, organic acids, minerals), contain health promoting constituents (polyphenols, bioactive lipids, policosanols, phytosterols, dietary fiber) and contribute to the well-being of local populations (Chevassus-Agnes et al., 1979; Nout, 1987; Leguizamón et al., 2009; Maoura and Pourquie, 2009; Abdoul-latif et al., 2012, 2013; Lee et al., 2014; Pontieri and Del Giudice, 2016; Oluwafemi, 2020). Additionally, these beverages play important socio-cultural and economic roles in the African society. The production and sale of traditional beers remain a significant sources of income-generating activity in the agricultural value chain, contributing to the economic systems in Africa (Odunfa, 1985; Sefa-Dedeh and Asante, 1988; Maoura et al., 2006; Pale et al., 2011; Aka et al., 2017).

The production of *dolo* and *pito*, as well as other traditional sorghum beers is generally artisanal and a traditional family skill passed down from one generation to another. In general, the production of African sorghum beer consists of malting sorghum grains and brewing of the beer. A spontaneous uncontrolled lactic fermentation has been reported to occur during the soaking of sorghum grains in the malting process. The dominant LAB species involved in the acidification phase of *dolo* production have been identified as *Limosilactobacillus fermentum* (Basonym: *Lactobacillus fermentum*), *Pediococcus acidilactici, Weissella confusa, Enteroccocus faecium, Pediococcus pentosaceus* and *Lactococcus lactis* ssp. *lactis* (Sawadogo-Lingani et al., 2010; Zheng et al., 2020). Similarly, the lactic acid bacteria generally involved in the brewing of traditional African beers include the genera *Limosilactobacillus, Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, *Streptococcus* and *Enterococcus* (Sawadogo-Lingani et al., 2007; N'Guessan et al., 2011; Lyumugabe et al., 2012; Adimpong et al., 2013; Greppi et al., 2013; Coulibaly et al., 2014; Oriola et al., 2017; Zheng et al., 2020). Molds of the genera *Aspergillus*, *Penicillium*, *Rhizopus* and *Geotrichum* as well as acetic acid bacteria are often associated with malting and brewing of African sorghum beers (Ilori et al., 1991; Ogundiwin et al., 1991; Lyumugabe et al., 2012; Zaukuu et al., 2016; Touwang et al., 2018). Alcoholic fermentation of *dolo* and *pito* in Burkina-faso and Ghana respectively are dominated by the species *Saccharomyces cerevisiae* (45–99%) with a biodiversity of strains of other species such as *Torulaspora delbrueckii*, *Geotrichum candidum*, *Kloeckera apiculata, Candida tropicalis, C. krusei, C. albicans, C. glabrata, C. utilis, Pichia* spp., *Kluyeromyces* spp. (Demuyakor and Ohta, 1991; Sanni and Lonner, 1993; Konlani et al., 1996; Sefa-Dedeh et al., 1999; van der Aa Kühle et al., 2001; Jespersen, 2003; Glover et al., 2005; Kolawole et al., 2007; Kayodé et al., 2011; Dossou et al., 2014; Zaukuu et al., 2016). Previous attempts have been made to develop starter cultures to control and optimize the lactic fermentation of sorghum wort as well as the alcoholic fermentation of African sorghum beer (Sefa-Dedeh et al., 1999; Orji et al., 2003; Sawadogo-Lingani et al., 2008a,b; Glover et al., 2009; Yao et al., 2009; N'Guessan et al., 2010, 2011, 2016; Adewara and Ogunbanwo, 2013; Coulibaly et al., 2014), but these have not been successful at an industrial scale and still remain at the laboratory or pilot production stage.

Development of the beer manufacturing sector in Africa is constrained by a number of factors such as the artisanal nature of the process, low output of production, variability in quality from one production batch to another and short shelf life. In order to overcome these constraints, it is necessary to analyze the available data and knowledge, assess the level of resolution of these constraints for sustainable production and to make appropriate recommendations for further developments. It is within this framework that the present review was undertaken to present an inventory of the available scientific data and information on the physico-chemical, nutritional and organoleptic characteristics, fermentation practices, microbiological characteristics and valorization of by-products. Furthermore, constraints and strategies for sustainable production of African traditional beers have been presented with focus on *dolo* production in West Africa.

OVERVIEW OF AFRICAN TRADITIONAL SORGHUM BEERS

Typology of African Traditional Beers

African traditional sorghum beers vary slightly in manufacturing processes and product characteristics according to the geographical location of production. Common sorghum-based traditional beers produced in different African countries with variations in alcohol contents are shown in **Figure 1**. Throughout Africa, sorghum beers with similar or slight variations in process and product characteristics may be known by different names according to the region of production or ethnic origin of the beer. For example, sorghum beer is known as *kefir, bantu* or *utshwala* in South Africa (Schwartz, 1956; Novellie, 1968; Novellie and De Schaepdrijver, 1986), *pito* or *burukutu* in Ghana, Togo and Nigeria (Ekundayo, 1969; Demuyakor and Ohta, 1991; Sanni, 1993; Sefa-Dedeh et al., 1999), *dolo*, *doro* or *tchapalo* in Burkina Faso, Mali, Senegal and Côte d'Ivoire (Yao et al., 1995; Konlani et al., 1996; Bougouma, 2002), *tchoukoutou* or *chakpalo* in Benin, Togo, and northern Nigeria (Hounhouigan, 2003), *otika* in Nigeria and Ghana (Faparusi et al., 1973; Sefa-Dedeh and Asante, 1988; Chinyere and Onyekwere, 1996), *bili-bili*, *ambga, red kapsiki,* or *dora-bonga* in Chad, Cameroon and Central Africa (Nanadoum, 2001; Nso et al., 2003; Maoura et al., 2005), *omalovu*, *tombo,* or *epwaka* in Namibia, *ikigage* or *awarwa* in Rwanda (Lyumugabe et al., 2010), *merissa* in Sudan (Dirar, 1978), *talla* in Ethiopia (Steinkraus, 2002; Blandino et al., 2003), *mtama* in Tanzania (Tisekwa, 1989), *munkoyo* in Congo and Zambia (Herbert, 2003), *doro*, *chibuku*, *uthwala* or *chikokivana* in Zimbabwe (Chamunorwa et al., 2002; Togo et al., 2002), *busaa* in Kenya (Nout, 1980). In Burkina Faso, *dolo* is produced throughout the country with slight differences in process or product characteristics depending on the producing ethnic groups. Thus, in Burkina Faso, varieties of *dolo* such as *dolo mossi*, *dagara*, *lobi*, *samo*, *bissa*, *bobo*, *turqua* are produced by different ethnic groups (Bougouma, 2002; Sawadogo-Lingani, 2010). Similarly, in Ghana, the production of *pito* which originates from the northern parts of the country is now widely produced throughout the country. There are different variants of *pito* depending on the method of wort extraction and the fermentation technique practiced by the different ethnic groups in northern Ghana, leading to varieties such as *Nandom pito*, *Kokomba pito* and *Dagarti pito* (Sefa-Dedeh, 1991; Sefa-Dedeh et al., 1999).

Socio-Cultural and Economic Importance of Traditional Sorghum Beer

Traditional sorghum beers play important socio-cultural and economic roles in Africa. Once prepared in family settings and used in ritual ceremonies in honor of ancestors and spirits to establish communication between the visible and invisible world, traditional sorghum beers have now become a common drink among the general population. *Dolo* and *pito* are undoubtedly the most popular ancestral alcoholic drink in West Africa and are widely produced and consumed in both rural and urban communities. In Burkina Faso, about 75% of the sorghum produced is used for the production of *dolo* which is consumed by nearly 60% of the population (Sawadogo-Lingani et al., 2007). Traditional sorghum beer is almost always used for traditional ceremonies and socio-cultural events such as weddings, baptisms, funerals, enthronements, initiations and festivals (Sanni and Lonner, 1993; Sawadogo-Lingani et al., 2007; Aka et al., 2010; Oyewole and Isah, 2012; Coulibaly et al., 2014).

The production of *dolo* in Burkina Faso and similar alcoholic beverages in other West African countries is a female dominated activity that generates substantial income and contributes to the socio-economic development of local populations (Kayode et al., 2005; Maoura et al., 2006; Sawadogo-Lingani, 2010; Pale et al., 2011). West African women have a long and well-documented tradition of entrepreneurial skills (Mandel, 2004), particularly in the food processing microenterprises. In Burkina Faso, women who are engaged in the production and sale of *dolo*, called *dolotières*, represent about 15% of the women population (Herbert, 2003). These women are often organized into associations or cooperatives and licensed to manufacture *dolo* and run a *cabaret* or *dolodrome* (sale joint for *dolo*). Depending on production capacity, a single sorghum malt production unit in Ouagadougou, the city of Burkina Faso, or other lager cities such as Tamale in Ghana employs about 4–6 people, including at least two men for the execution of specific tasks, with employees receiving monthly salaries (Sawadogo-Lingani, 2010). In Ouagadougou, it is estimated that 600 *dolotières* produce 36 million liters of *dolo* per year, which translates to about 9,000 tons of sorghum malt per year. According to Broutin et al. (2003), the production yield is about 4 liters of *dolo* per kilogram of sorghum malt in Burkina Faso, 2 liters of *tchoukoutou* per kilogram of sorghum malt in Benin and 4 liters of *chakpalo* per kilogram of maize malt in Benin. A survey in 2007, showed that the profits from *tchoukoutou* production in Benin, range from 2,365 to 17,212 fcfa per month (1 euro = 656 fcfa) depending on beer yield and quantity of raw grains transformed. The income generated from the production and sale of sorghum beers is often used to support household activities or invested in children's education (Kayodé et al., 2007). In Abidjan (Côte d'Ivoire), there are more than a hundred

tchapalodrômes (places of production and marketing of *tchapalo*) (Yao et al., 1995; Enou, 1997; Aka et al., 2008; Fokou et al., 2016). Thus, a real traditional sorghum beer industry has developed in West Africa, providing livelihoods for several families (Kayode et al., 2005; N'Guessan, 2009; Pale et al., 2011). The African Sorghum beer industry brings together different actors in the value chain including cereal farmers producing, transporters, distributors and traders, producers and traders of sorghum malts and traditional beers, and consumers.

Physico-Chemical, Nutritional, and Organoleptic Characteristics

Physico-chemical and nutritional characteristics of African traditional beers are sparsely reported in literature. The physico-chemical and nutritional parameters usually reported in literature include pH, alcohol content, dry matter, total sugars, proteins, phenolic compounds and antioxidants (**Table 1**). Generally, pH varies from 3.2 to 3.6 for *dolo* and 3.2 to 5.0 for *pito*. These pH values reported for *dolo* and *pito* are similar to the pH of other African traditional beers such as *tchapalo* from Ivory Coast (pH 3.3-3.6), *tchoukoutou* from Benin (pH 3.0–3.8), *bili-bili* from Cameroon and Central Africa Republic (pH 3.5-4.4), *red kapsiki* from Cameroon (pH 2.4–3.3), *ikigage* from Rwanda (pH 3.9) and *merissa* from Sudan (pH 4.0). Alcohol contents also vary from 1.4 to 3.5% (v/v) for *dolo* and from 1.94 to 4.0% (v/v) for *pito* whereas dry matter contents range from 3.8 to 8.2% for *dolo* and from 5 to 7% for *pito*. It thus appears that African opaque beers are acidic alcoholic beverages, and this high acidity is important for the microbiological safety of these drinks. The pH depends on the method of extraction of sorghum malt wort, particularly, conditions such as duration, temperature during the acidification phase, which is carried out by spontaneous uncontrolled lactic acid fermentation. The dry matter contents of *dolo* (3.8–8.2%) and *pito* (5.0–7.0%) are highly variable and lower than that of *tchapalo* (8.4–8.5%), *red kapsiki* (7.0–7.5%) and *tchoukoutou* (15.4–20.2%). Generally, dry matter content of sorghum beers depends on sorghum grain characteristics, malting efficiency and decantation/filtration operation conditions. Traditional sorghum beers contain insoluble substances and yeasts (Rooney and Serna-Saldivar, 1991; Kouame et al., 2015). These insoluble substances are mainly starch fragments and dextrins that have not been degraded during mashing and fermentation (Glennie and Wight, 1986). Similar to dry matter content, the alcohol contents of *dolo* (1.4–3.5%) and *pito* (2.0–5.0%) are very variable but remains within the normal range of alcohol contents of other African traditional sorghum beers which generally vary from 2.0 to 5.2% (v/v) as shown in **Table 1**. The alcohol content of traditional African sorghum beers depends on the duration of the alcoholic fermentation, the concentration of fermentable sugars in the wort and thus on the quality of malt and efficiency of the brewing process. *Dolo* contains phenolic compounds at higher levels than western beer and white wine (Abdoul-latif et al., 2012). In general, traditional sorghum beers contain starch, sugars, proteins, fats, vitamins and minerals (Chitsika and Mudimbu, 1992). They are rich in calories, B-group vitamins (thiamine, folic acid, riboflavin, nicotinic acid) and essential amino acids such as lysine (Chevassus-Agnes et al., 1979; Lyumugabe et al., 2012). According to Holzapfel (2002),

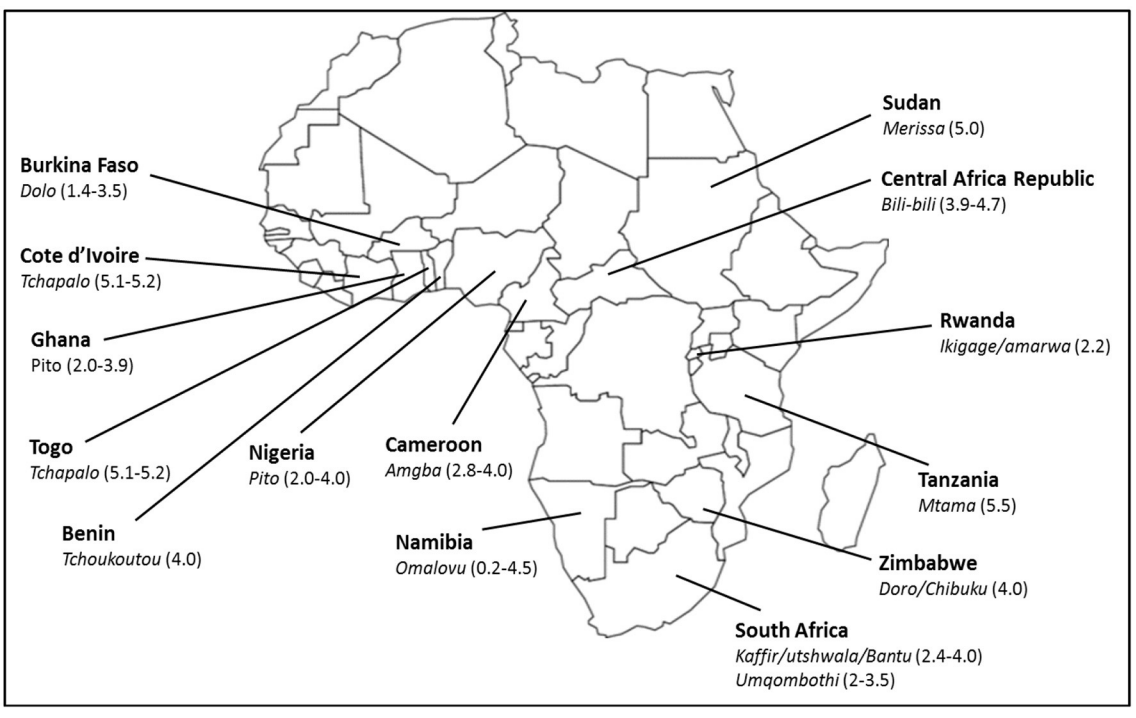

FIGURE 1 | Common traditional sorghum beers (% alcohol [v/v]) produced in Africa.

TABLE 1 | Physico-chemical characteristics and proximate composition of African traditional sorghum beers.

Name	Country	pH	Titratable acidity (%lactic acid)	Dry matter (g/100 ML or %)	Alcohol (% v/v)	Total sugars (g/100 mL)	Proteins (g/100 mL)	Other	References
Dolo	Burkina Faso	3.2–3.6	nr	nr	nr	nr	nr	nr	Sawadogo-Lingani et al., 2007; Glover et al., 2009
		3.40–3.60	nr	3.77–8.16	1.40–3.50	1.1–8.4 mg/100 ml	1.1–6.5 mg/100 ml	Total phenol: 437–578 μg GAE/ml Proanthocyanidins: 38 and 55 μg APE/ml TEAC: 57 and 349 μmol/L	Abdoul-latif et al., 2012, 2013
Pito	Ghana, Nigeria	3.4	nr	nr	nr	nr	nr	nr	Sawadogo-Lingani et al., 2007
		3.4–3.6	0.72–0.96	2.92–4.68	1.40-3.68	0.86–2.35	0.14–0.39	nr	Sefa-Dedeh, 1991
		3.5–5	nr	nr	2-4	nr	nr	nr	Zaukuu et al., 2016
		3.2–3.6	nr	5.0–7.0% (soluble extract)	1.96–3.93	nr	nr	nr	Ayirezang et al., 2016
Tchapalo	Côte d'ivoire	3.33–3.63	0.9–0.99	8.4–8.5 (soluble solids)	5.08–5.22	8.84	nr	nr	Aka et al., 2008
Tchoukoutou/ chakpalo	Benin, Togo	3.0–3.8	0.5–0.8	15.4–20.2	4.0	nr	3.7–7.9%dm	nr	Kayodé et al., 2007, 2011; Osseyi et al., 2011
Amgba/bili bili	Cameroon	3.5–4.4	nr	nr	2.75–4.0	0.13–0.66 (reducing sugars)	0.47–0.85 (soluble proteins) Free amino nitrogen (FAN): 8.69-52.14 mg/L	484.8–540.9	Touwang et al., 2018
Red kapsiki beer	Cameroon	2.40–3.26	0.67–0.81	7.0–7.46% (Soluble matter)	3.85–4.28	41.8–72.9 g/L	nr	843–1150 mg/L total phenol 750-1,300 mg/L Flavanols	Bayoï and Djoulde, 2017
Bili-bili/Dora-bonga	Central African Republic	3.44–3.60	nr	nr	3.94–4.66	0 65–0.67%	2.79–2.90% 3.80–3.82%	nr	Lango-Yaya et al., 2020
Ikigage/amarwa	Rwanda	3.9	nr	nr	2.2	nr	0.92	nr	Lyumugabe et al., 2010
Merissa	Sudan	4.0	nr	nr	5.0	nr	nr	nr	Lyumugabe et al., 2012
Doro or chibuku	Zimbabwe	nr	nr	nr	4.0	nr	nr	nr	Lyumugabe et al., 2012
African traditional sorghum beer	No specific country	3.3–4	0.26	5 –13	2–4.5	nr	nr	nr	Lyumugabe et al., 2012

nr, not reported.

pito contains essential minerals such as zinc (Zn), calcium (Ca), magnesium (Mg) and iron (Fe), which are important micronutrients. Due to its nutritional characteristics, FAO (1995) describes African opaque beer as more of food than a drink. Consumers attribute some therapeutic virtues (laxative, analgesic, anti-malarial, anti-hemorrhoidal, energetic, dietary properties) to their consumption, even though such claims are not scientifically proven (Enou, 1997; Amané et al., 2005; Kayodé et al., 2007; Aka et al., 2010). According to Zaukuu et al. (2016), *pito* is widely consumed as a ceremonial drink in Ghana for its refreshing taste and nutritional characteristics as it provides consumers with a wide range of important polyphenols, micro- and macronutrients that play important roles in the prevention of diseases related to metabolic imbalances such as gastrointestinal disorders, inflammation, obesity and hypertension. In general, however, detailed information on the nutritional and health benefits of West African traditional sorghum beers are scarce and scanty. For a better valorization of these ethnic drinks, it is necessary to undertake research works to highlight their nutritional values and make these data available and accessible in order to be able to evaluate their real contribution to the nutrient intake and well-being of consumers.

African opaque beers are characterized by the variation in their organoleptic characteristics from one production batch to another (Lyumugabe et al., 2012), due to the artisanal nature of the manufacturing process. As far as organoleptic characteristics are concerned, there is no specific description for *dolo* and *pito*. These two drinks, like other African traditional sorghum beers are generally opaque, cloudy, low in alcohol, unfiltered and unstable, and contain insoluble substances and yeasts (Rooney and Serna-Saldivar, 1991; Kouame et al., 2015). African sorghum beers have an acidic or sour taste, which corroborates with their low pH values, have a touch of fruitiness and a characteristic color varying from pale buff to pinkish brown depending on the variety of raw cereal grains used for their production.

PROCESSING OF AFRICAN TRADITIONAL SORGHUM BEER

The traditional processing of African sorghum beers consists of two main phases: malting of sorghum grains and brewing of traditional beer from sorghum malt. The main brewing operations comprises the extraction of wort which includes crushing of sorghum malt, mashing, acidification/saccharification, cooking and cooling. Wort extraction is followed by alcoholic fermentation of the wort using indigenous yeasts. Depending on the ethnic group or local region of production, the brewing process involves either a separate acidification step and an alcoholic fermentation step, or a mixed fermentation combining lactic acid bacteria and yeasts.

Raw Materials

The main raw material for the production of African traditional beer is sorghum grains. Common species of sorghum such as *Sorghum bicolor*, *Sorghum vulgare* and *Sorghum guineese* are generally used alone or in combination with other cereal grains such as maize or millet. However, in a few instaces, maize or millet alone is used for the production of African traditional beer (**Table 2**).

Sorghum grains intended for the production of *dolo* and other similar African beers must have high starch and amylose contents and a high to medium diastatic power after malting (Dicko et al., 2006; Tchuenbou, 2006). Pigmented sorghum varieties (red or brown) are the most commonly used. White varieties of sorghum are usually not used alone for the production of African beers but often used in combination with red or brown sorghum varieties because consumers prefer colored beers which they also perceive to be healthier (Kayode et al., 2005; Sawadogo-Lingani et al., 2010). In an attempt to select appropriate sorghum varieties for the production of *dolo* and *tchoukoutou* in Burkina Faso and Benin, malting properties and brewing characteristics of nineteen varieties of sorghum was assessed (Tchuenbou, 2006). The assessment led to the nineteen varieties belonging to one of three major groups. The first group comprising seven varieties of sorghum (*Sotakaman*, *Zomoaha* 2, *Natisoya* 1, *Kioédré*, *Mewin*, *Chassisoya*, *Chabicoman PR*) had high diastatic power and high β-amylase activity and were rated excellent for the production of *dolo* and *tchoukoutou*. The second group made up of eleven varieties (*Agbanni, Natisoya 2, Chabicoman PN, Soniya, Fissouka, Zoueloure, Banninga, Pisnou, Kapelga, Vrac Cotonou, Gnossiconi*) with medium amylase activity may also be suitable for sorghum beer, while the last group of one variety (*Zomoaha* 1) had very low amylase activity and its use as a brewing malt is of little interest among traditional beer brewers (Tchuenbou, 2006). The advantage of all these sorghum varieties is their relatively high availability, presumably due to some favorable agronomic properties and the ability to germinate even after months of storage. In a survey to identify the types and characteristics of sorghum grains preferred for malting and brewing of *dolo* in Burkina Faso, it was shown that red sorghum is the most preferred grain type because of its consistent ability to sprout well during the malting process (Songre-Ouattara et al., 2016). It is obvious that perception criteria and preferences of the actors (*malteuses* and brewers) are diverse. It will thus be appropriate to scientifically develop a better systematic and qualitative approach to characterizing sorghum quality attributes for African beer production.

Generally, processors use wort properties such as sweetness as an indicator of the quality of the beer. The sweeter the wort, the better or stronger the beer will become due to available fermentable carbohydrates in the wort.

Traditional Malting of Sorghum Grain

Different traditional sorghum malting processes in Africa have been reported in literature (**Table 2**). The main operations that are common to traditional malting of sorghum grains for beer production include steeping of sorghum grains, sprouting and sun drying of sprouted grains. The total duration of the entire traditional malting process varies between 7 and 12 days. Soaking time vary from 5 to 48 h for sorghum malt for *dolo* production, and from 14 to 48 h for malt for *pito* production. These steeping times are comparable to those indicated for

TABLE 2 | Main steps of traditional malting processes of sorghum for the production of African traditional sorghum beers.

Name of final product	Raw material	Steps of the processing	Microorganisms involved	References
Dolo	• Red sorghum • White sorghum	• Cleaning • Steeping (14–24 h) • Draining • Germination (3–4 days) • Sun drying (2–3 days)	*Limosilactobacillus* spp. (*Lactobacillus spp.)* *Pediococcus* spp. *Weissella* spp. *Enteroccocus* spp. *Lactococcus* spp.	Sawadogo-Lingani, 2010
	Red sorghum	• Cleaning • Steeping (24–48 h) • Draining • Germination (2–4 days) • Sun drying (2–4 days)	nr	Broutin et al., 2003
	• Red sorghum • White sorghum Millet	• Cleaning • Steeping (5–25 h) • Draining • Germination (57–96 h) • Short sun drying (30 min) • Maturation in heap (24–96h) • Sun drying (32–82h)	nr	Traoré et al., 2004
	• Red sorghum • White sorghum	• Cleaning • Steeping (12–48 h) • Draining • Germination (2–5 days) • Sun drying (2–3days)	nr	Bougouma et al., 2008
Pito	Red sorghum, white sorghum, maize	• Cleaning • Steeping (14–24 h) • Draining • Germination (3–4 days) • Sun drying (2–3 days)	*Limosilactobacillus fermentum* *Ped. acidilactici Ped. pentosaceus Weissella confusa* *Enteroccocus faecium* *Lactococcus lactis* ssp. *lactis*	Sawadogo-Lingani et al., 2010
	• Red and white sorghum • Maize	• Cleaning • Steeping (24–48 h) • Draining • Germination (4–5 days) • Sun drying	*nr*	Lyumugabe et al., 2012
Tchapalo	Red sorghum	• Cleaning • Steeping (7–12 h) • Germination (3 days) • Sun drying (1–2 days)	nr	Yao et al., 1995; Aka et al., 2008; Coulibaly et al., 2014
Tchoukoutou chakpalo	• Red sorghum • Brown sorghum • Millet • Maize	• Cleaning • Steeping (9–12 h; 12–24 h) • Draining • Germination (3–4 days) • Sun drying (1–2 day)	nr	Lyumugabe et al., 2012; Dossou et al., 2014
Bili bili or Amgba	• Sorghum • Millet	• Steeping (12–72 h) • Washing & Draining (12 h) • Germination (2–4 days) • Maturation in sacks (24–72 h) • Sun drying (1–2 day)	nr	Chevassus-Agnes et al., 1979; Lyumugabe et al., 2012; Touwang et al., 2018
Red kapsiki beer or Te	Sorghum, Maize	• Washing • Steeping • Draining • Germination (2–3 days) • Sun drying (6–10h)	nr	Bayoï and Djoulde, 2017
Bili-Bili	Sorghum	• Washing • Steeping (24–72 h) • Germination in heap (24–36 h) • Germination in thin layers (4 days) • Sun drying	nr	Lango-Yaya et al., 2020

(Continued)

TABLE 2 | Continued

Name of final product	Raw material	Steps of the processing	Microorganisms involved	References
Ikigage or *amarwa*	Red sorghum	• Washing • Steeping (24 h) • Draining • Germination (24 h) • Sun drying (2–3 days)	nr	Lyumugabe et al., 2012
Doro	Red sorghum	• Cleaning • Steeping (24 h) • Germination (sacks, 2–3 days) • Sun drying (3 days)	nr	Lyumugabe et al., 2012

nr, not reported.

the malting of sorghum for the production of *tchoukoutou* (9–24 h), *tchapalo* (7–12 h), *bili-bili* (24–72 h), *ikigage* (24 h) and *doro* (24 h) (**Table 2**). Thus, the shortest steeping times are 5–7 h and the longest steeping times are 24–72 h. Soaking of grains during traditional malting process is generally done at room temperature in cans and jars half buried in the ground for better thermal insulation. Steeping brings the moisture content of the grain to levels that are favorable for respiration and metabolic activities and for the mobilization of primary and secondary metabolites, making germination possible. Steeping has an influence on the quality of malt as it contributes to the elimination of flatulence factors (stachyose, raffinose) and the reduction of phytate content through leaching. The quality of sorghum malt (diastatic power, free amino acids and soluble extract) is also positively correlated with the moisture content of the grain at the end of steeping (Dewar et al., 1997). According to the European Brewery Convention (EBC), a well-soaked barley grain should have a final moisture content of about 43–45 and 33–45% for millet and sorghum grains (Aisien and Ghosh, 1978; Malleshi and Desikachar, 1986; Agu and Palmer, 1998; Ogbonna et al., 2004). In addition, water absorption depends on several factors such as the composition of the steeping water, grain variety, grain storage conditions, soaking time and temperature. Soaking conditions for millet and sorghum generally reported in the literature range from 8 to 51 h at temperatures between 25 and 35°C, with most being between 16 and 24 h at a temperature of 30°C. The overall quality of malt increases with the steeping time. The diastatic power of sorghum malt increases with the steeping temperature with an optimum at 30°C while the malt extract and free amino acids are higher with steeping temperature of 25°C (Dewar et al., 1997; Tchuenbou, 2006). Prolonged soaking period can lead to rapid germination, high losses of nutrients (Pathirana et al., 1983; Bhise et al., 1988), or putrefaction of the grains rather than germination (Shambe et al., 1989). For effect of the composition of water, the steeping of the sorghum grain in alkaline water [0.1% of Ca(OH)2, KOH or NaOH] has been shown to significantly enhance the diastatic powre of sorghum malt and β-amylase activity (Okungbowa et al., 2002). Furthermore, aeration of sorghum grains during steeping has a positive impact on malt quality as it leads to an increase in the percentage of sprouted grains, soluble dry matter of the malt, total nitrogen and free amino acids contents (Dewar et al., 1997). Beta et al. (1995) found that malts from sorghum grains soaked in jars with poor aeration had lower diastase, α and β-amylase activity and protein content compared to malts obtained from sorghum grains soaked in an aerated container.

The duration of sprouting varies from 2 to 5 days for sorghum malt used for the production of *dolo* and from 3 to 5 days for malt used for *pito* production (**Table 2**). These durations are comparable to the germination times reported for the malting of sorghum grains for the production of *tchapalo* (3 days), *tchoukoutou* (3–4 days), *bili-bili* (2–4 days) and *red kapsiki* (2–3 days). Germination of grains in traditional malting takes place at room temperature and away from direct sunlight in cans, baskets, on cemented floors, on tarpaulins or plastic sheets, with watering at variable time intervals to maintain humidity (Fliedel et al., 1996; Bougouma et al., 2008). In air germination, the thickness of the layer can reach 30–50 cm thick. In Burkina Faso, this step may be followed by a maturation phase known as "high germination" where the grains are placed in piles on the floor or in a basket covered with burlap, mats or plastic sheets, with temperatures reaching 60°C at the core of the grain piles (Bougouma et al., 2008). Temperature, germination time and relative air humidity are reported to have significant influence on malt quality. Optimal germination temperatures for sorghum and millet are reported to be between 25 and 30°C. Germination of at 20°C reduces malting losses due to high root growth whereas germination at 30°C causes high losses but increases amylase activity (Agu and Palmer, 1997b). Sorghum malts germinated at 25°C give a better wort quality when compared to sorghum malts germinated at 20°C. Sorghum germination at 30°C for more than 4 days results in a decrease in peptides in the wort (Agu and Palmer, 1999). The α-amylase activity is optimal on the third day of germination and is higher in white sorghum malts than in red sorghum malts, whereas the β-amylase activity is optimal on the third and fourth days of germination (at 30°C) for red and white sorghum respectively (Uvere et al., 2000). The grains are generally germinated at humidities between 85 and 100%. Under uncontrolled moisture conditions such as germination in traditional malting, the grains are reportedly watered at various time intervals i.e., every 8 h (Taylor, 1983), every 6 h (Taylor et al., 1994) or 2–3 times a day

based on visual observation of grain dryness (Pelembe et al., 2004).

Germination or sprouting enriches cereal malts with hydrolytic enzymes, sugars, free amino acids, vitamins and improves technological and nutritional quality (Chavan et al., 1981; Malleshi et al., 1989; Taylor and Robbins, 1993; Taylor and Dewar, 1994; Sripriya et al., 1997; Elmaki et al., 1999; Mbofung and Fombang, 2003; Traoré et al., 2004). According to Wang and Fields (1978), germination increases the lysine, tryptophan and methionine contents with an increase in relative nutrient value from - 55.5 to 66.8% (ungerminated kernels) to 78.3–99.5 (sprouted kernels) for maize and sorghum. Demuyakor and Ohta (1992) reported that maltose, glucose and dextrins are the major sugars produced by starch hydrolysis during germination of sorghum varieties in Ghana, while triose, raffinose and xylose appear in small amounts. Traoré et al. (2004) also found significant levels of fructose, glucose and sucrose in sorghum malt from Burkina Faso. On the other hand, germination promotes the release of cyanide due to high concentrations in young shoots and rootlets of germinated sorghum (Panasiuk and Bills, 1984; Aniche, 1990; Ahmed et al., 1996; Uvere et al., 2000; Traoré et al., 2004). However, the removal of rootlets and further processing are reported to reduce the hydrocyanic acid content of malted grains by over 90% (Dada and Dendy, 1988). During traditional sorghum malting, partial polishing of the dried malt by removing part of the roots and stalks is carried out. Similarly, during the brewing of *dolo*, roots and stalks are also partially removed during the mashing process. According to *dolo* brewers, this is done to reduce bitterness and astringency in *dolo*. In Burkina Faso, Traoré et al. (2004) showed that the degermination of sorghum malt promotes a reduction in cyanide content by about 72% and 74% in millet and sorghum malts, respectively, although the process leads to a decrease in amylase and protein contents.

Following sprouting, the germinated grains are sun dried for periods varying from 2 to 4 days for malt intended for the production of traditional African beers (**Table 2**). However, shorter drying times (6–24 h) are reported for malt intended for the production of *tchapalo*, *bili-bili* and red *kapsiki*. Drying time in traditional malting processes depends on climatic conditions and layer thickness. The green malt is generally spread on the ground, on cemented areas, on mats or plastic sheets, inside concessions, along tared streets, or on the roofs of houses (Bougouma et al., 2008; Aka et al., 2017). Drying times are generally dependent on the intensity of available sunshine and wind speed. During sun-drying, malt is also exposed to bad weather and contaminations from humans, pets, wild birds, city pollution, dust and other impurities carried by the wind.

Drying lowers the water activity of malt thereby favoring the blocking of enzymatic activity, stabilizing and enhacing preservation of the malt (Galzy and Moulin, 1991). The cyanide content of germinated grains decreases during drying (Aniche, 1990; Traoré et al., 2004). The temperature and drying time as well as the relative humidity of the air have an influence on the quality of the malt. Drying temperatures for sorghum and millet malts range from 30 to 65°C with different scales (Aisien, 1982; Lasekan, 1991; Demuyakor and Ohta, 1992). The scale generally chosen is drying at 50°C for 24 h (Beta et al., 1995; Subramanian et al., 1995; Igyor et al., 2001). Amylase activity decreases as the drying temperature increases. Thus, for example, drying sorghum malt at 35, 40, and 45°C reduces its diastatic activity by 7.7, 8.7, and 12.4% respectively (Agu and Palmer, 1996). According to Traoré et al. (2004), sun drying during traditional malting reduces α-amylase activity in millet and sorghum by 16%. Owuama and Ashemo (1994) found low protein and sugar content and thus enzyme inactivation in sorghum malts dried at 65°C compared to those dried at 55°C or at 55°C for 6 h and then 65°C. According to Uvere et al. (2000), drying at 50°C decreases amylase activity and alcohol yield in *burukutu*. For some authors, the best drying conditions are a temperature of 50 to 55°C, a drying time of 24 h and protection against contamination (Morrall et al., 1986; Owuama and Ashemo, 1994; Uriyo and Eigel, 1999; Uvere et al., 2000; Okungbowa et al., 2002). The evaluation of three stabilization modes (freezing at −18°C, freeze-drying, drying at 45°C) on the amylase activity of sorghum malts showed that all these modes lead to a loss of amylase activity, and drying has a greater negative effect than the other two (Tchuenbou, 2006).

Several studies have shown that optimum malting conditions are different for different millet and sorghum varieties, as varieties react differently to extrinsic factors as temperature and aeration that can influence malting and malt quality (Demuyakor and Ohta, 1992; Subramanian et al., 1995). The rate of water absorption and the saturation water content during steeping depend on the varieties. According to Agu and Palmer (1997a), colored sorghum varieties have a higher α and β- amylase activity than white varieties but gives more losses during malting. Similarly, Eneje et al. (2004) concluded that yellow maize has higher enzyme activity and extract yield than white maize. On the other hand, some studies have reached opposite results, which would reflect the impossibility of absolute grading of kernels with respect to their germination ability according to their color. In addition, high-protein grains yield high-protein malts and extracts (Odibo et al., 2002). Under the same malting conditions, some varieties have their β and α-amylasic activity optimal after 3 and 4 days of germination, while for others these activities continue to increase with germination time (Agu and Palmer, 1997b). There are varieties that produce more α-amylase and/or β-amylases (Dufour et al., 1992; Subramanian et al., 1995) or varieties that are more sensitive to anoxic conditions than others (Beta et al., 1995). Malt quality is defined by its intended use. Thus, for brewing malts, quality is determined by the diastatic power (activity of hydrolysis of starch into fermentable sugars through the activity of α and β-amylase, α-glucosidase and limit dextrinase) and the water-soluble extract. The production of hydrolytic enzymes in malted grains is a complex phenomenon, influenced by extrinsic (environmental and agro-climatic conditions) and intrinsic factors (genetics, grain composition, albumen structure and texture, etc) and malting conditions. The water-soluble malt extract (soluble dry matter) is decisive for having a wort containing fermentable sugars (maltose, glucose) and amino acids needed as a source of carbon and nitrogen for beer yeasts. The conditions of sorghum malting and the factors that can influence the quality of the

malt are generally related to an optimum diastatic power, the achievement of a high content of malt extract and free amino acids, and a minimum loss of material during malting. The optimal conditions for the traditional malting of sorghum to obtain a quality malt are soaking to a maximum hydration of 33–36%, germination at 25–30°C for 3–5 days and a drying temperature of the green malt below 65°C (Demuyakor and Ohta, 1992; Agu and Palmer, 1998).

The enzymes generated during malting are essential for brewing operations to obtain a wort rich in soluble matter including fermentable sugars, amino acids and peptides that are also essential for the growth and development of lactic acid bacteria and yeasts. The development of toxinogenic molds and undesirable bacteria in grains during germination can have harmful effects on the quality of malt and finished products, and present health risks for the consumer (Flannigan et al., 1982; Galzy and Moulin, 1991). Malting has a major impact on the composition of sorghum grain, reducing the concentration of anti-nutrients in sprouted grain and improving the bioavailability of certain minerals and overall nutritional quality (Svanberg and Sandberg, 1989; Morero et al., 1991; Mbofung and Fombang, 2003). A reduction in phytates and tannins is generally observed during the malting of sorghum grains and the rate of reduction is a function of soaking and germination times and temperatures (Svanberg and Sandberg, 1989; Mahgoub and Elhag, 1998; Mbofung and Fombang, 2003; Traoré et al., 2004).

Brewing of African Traditional Sorghum Beer

The process of brewing traditional African sorghum beer is artisanal in nature and the equipment used are mainly composed of basic materials such as gourds, jars and canaries made of baked clay, cast iron pots, aluminum, iron or plastic recovery barrels, and woven straw baskets. The main source of energy for the brewing of sorghum beer in Africa is firewood with the use of traditional tripod-stone fireplaces, improved fireplaces built in *banco* and equipped with chimneys (Pale et al., 2010; Sawadogo-Lingani, 2010).

Brewing is the step that produces the fermentable wort for alcoholic fermentation to yield the traditional beer. It is the stage in which starch and proteins are hydrolyzed into fermentable sugars and nitrogen compounds by the enzymes synthesized during malting. Few studies have been carried out on the technological and biochemical aspects related to the traditional brewing of *dolo* and *pito* as well as other similar traditional beers. Majority of existing works on African sorghum beers are limited to a description of the traditional processes from various traditional production units as well as the identification of the microorganisms involved in the process (**Table 3**). Traditional brewing of *dolo* or *pito* is a long and complex process lasting for about 48 h (Sawadogo-Lingani et al., 2007; Sawadogo-Lingani, 2010). **Figure 2** shows the general flow diagram for the brewing of *dolo* in Burkina Faso and *Dagarti pito* in Tamale (Northern Ghana). Red sorghum malt is used exclusively for the brewing of *dolo* in Burkina Faso by of the Mossi women and other ethnic groups where sorghum malt is generally purchased from malt manufacturing hubs (*Malteuses*). In South-Western Burkina Faso (Gaoua) and North Ghana (Tamale), *dolo* or *dagarti pito* is produced by women of the Dagara and Dagarti ethnic groups from a mixture (50:50) of red and white sorghum malt or with white sorghum malt alone, where the beer brewers produce the sorghum malt by themselves (Sawadogo-Lingani, 2010). The brewing operations for *dolo* and *pito* are also similar and generally comprise milling of sorghum malt, mashing, acidification/saccharification, cooking and concentration, cooling, decanting and alcoholic fermentation of the wort by indigenous yeasts (**Table 3**). Mashing as a sub-operation include diluting the malt flour in water, settling and collecting the supernatant, boiling the pellet, mixing the cooked pellet and the supernatant. The duration of the operations and sub-operations vary among different traditional production units. In the traditional brewing of *dolo* and *pito*, the settling time of the aqueous malt flour suspension, which is highly variable (20 min to 12 h), is comparable to the settling times in the brewing processes of Benin's *tchoukoutou* (1 to 2 h), Côte d'Ivoire's *tchapalo* (20 to 30 min), Cameroon's *bili-bili* and *red kapsiki* (1 to 2 h) and Rwanda's *ikigage* (3 h) (**Table 3**).

During the mashing, technological adjuvants are widely used. These adjuvants are extracts of local mucilaginous plants used as flocculating agents to promote settling and clarification of the wort. The plants generally used are yolga (*Grewia bicolor* Juss), okra [*Abelmochus esculentus* (L) Moench], baobab leaves (*Adansonia digitata*), kapok (*Bombax costatum*), taasalogo (*Bridelia micrantha*), boundou (*Ceratotheca cesamoides*) or boulvanka (*Chirococus esculentus*) (Sefa-Dedeh, 1991; Nanadoum, 2001; Bougouma, 2002; Bougouma et al., 2008; Pale et al., 2010). The aqueous extract of these plants (leaves, bark or fruits) has a sticky appearance and is rich in mucilaginous substances. After settling, the supernatant is decanted into a jar and then the remaining pellet (sediment) is heated to boiling point to gelatinize the residual starch and then mixed with the supernatant. For *dolo* and *pito*, the residual starch is heated to a temperature of about 59–68°C. Similar temperatures (65–70°C) have been reported for *tchoukoutou*, *ambga* and *bili-bili* (Lyumugabe et al., 2012; Lango-Yaya et al., 2020). Sorghum starch gelatinization temperatures range between 67 and 81°C (Akingbala et al., 1982). The hydrolytic enzymes in the sorghum malt extract (supernatant) hydrolyze the gelatinized starch and the concentration of fermentable sugars in the must increases. Analogous with modern beer brewing, the principle of mashing is based on infusion and/or decoction to extract fermentable wort by the solubilization of soluble substances in water and the enzymatic hydrolysis of starch and other macromolecules. While barley wort is reported to contain more maltose than glucose (Dufour et al., 1992) sorghum malt worts is reported to contain similar levels of glucose and maltose (Taylor, 1992; Byrne et al., 1993), and the difference has been attributed to the low levels of β-amylase in sorghum malt (Palmer, 1989).

During African sorghum beer brewing, another important process following starch gelatinization and hydrolysis is acidification/saccharification. The mixture of the gelatinized residual startch and the malt wort supernatant is acidified by spontaneous lactic fermentation at ambiet temperature for

TABLE 3 | Mains steps of brewing processes of African traditional beers and microorganisms involved.

Name of product	Country	Raw material	Steps of the processing	Microorganisms involved	References
Dolo and *Dagarti pito*	Burkina Faso, Ghana	Malted sorghum, malted millet or maize	• Grinding • Mixing malted flour with water (1vol flour for 5–10 vol water) • Decantation (20 min−12 h) • Heating the sediment until boiling (97–98°C) • Mixing with the supernatant (59–68°C) • Acidification (12–16 h) • Boiling (3–5 h) • Cooling and decantation (4–5 h) • Alcoholic fermentation (9–14 h) with traditional starter yeast	• *Limosilactobacillus fermentum (lactobacillus fermentum)* • *Lactobacillus delbrueckii* subsp *jakobsenii (Lactobacillus delbrueckii* subsp *delbrueckii)* • *Lactobacillus delbrueckii* subsp. *bulgaricus* • *Pediococcus acidilactici* • *Lactobacillus lactis* • *Lactococcus lactis* • *Saccharomyces cerevisiae (*dominant at 45–90%)	Konlani et al., 1996; van der Aa Kühle et al., 2001; Broutin et al., 2003; Sawadogo-Lingani et al., 2007; Glover et al., 2009; Lyumugabe et al., 2012
Pito	Ghana, Nigeria	Malted sorghum, millet or maize	• Grinding • Mixing with water • Boiling (3–4 h) • Acidification (12 h) • Dilution • Cooking (3 h) • Cooling • Alcoholic fermentation (12–24 h) with local starter yeast	• *Limosilactobacillus fermentum (lactobacillus fermentum)* • *Lactobacillus delbrueckii,* • *Lactobacillus* spp. • *Leuconostoc* spp. • *Saccharomyces cerevisiae* dominant at 45% • *Candida tropicalis* • *Kloeckera apiculata* • *Hansenula anomala* • *Torulaspora delbrueckii* • *Schizosaccharomyces pombe* • *Kluyveromyces africanus* • *Saccharomyces cerevisiae, Debaryomyces hansenii* • *Pichia anomala* • *Aspergillus clavatus,* • *Mucor hiemalis, Cladosporium sphaerospemum, Cladosporium herbarum* • *Bacillus subtillis,* • *Candida spp.,* • *Geotrichum candidum*	Sefa-Dedeh et al., 1999; van der Aa Kühle et al., 2001; Kolawole et al., 2007; Lyumugabe et al., 2012; Zaukuu et al., 2016
Tchoukoutou/chakpalo	Benin	Malted sorghum, millet or maize	• Grinding (fine flour) • Mixing with water • Decantation (1–2 h) • Boiling of the sediment (2h) • Mixing with the supernatant (65–70°C) • Acidification (13–14 h) • Filtration • Boiling (6–9 h) • Cooling • Alcoholic fermentation (13-14 h) with traditional leaven	• *Limosilactobacillus fermentum (lactobacillus fermentum)* • *Lactobacillus divergens* • *Lactobacillus fructivorans* • *Lactobacillus* sp. • *Saccharomyces. cerevisiae (dominant 68%)* • *Candida albicans (11%)* • *Torulaspora delbrueckii Saccharomyces pastorianus* • *Candida kunwiensis* • *Dekkera anomala* • *Candida etchellsii* • *Clavispora lusitaniae* • *Candida krusei*	Kayode et al., 2005; Kayodé et al., 2007, 2011; Lyumugabe et al., 2012; Greppi et al., 2013; Dossou et al., 2014

(Continued)

TABLE 3 | Continued

Name of product	Country	Raw material	Steps of the processing	Microorganisms involved	References
				• *Hanseniaspora guillermondii Debaryomyces nepalensis* • *Candida glabrata* • *Kluyveromyces marxianus Hanseniaspora uvaru*	
Tchapalo	Côte d'ivoire	Malted sorghum, millet or maize	• Grinding • Mixing with water • Decantation (20–30 min) • Boiling of the sediment (100°C, 2–2 h 30) • Mixing with the supernatant • Acidification (9–12 h) • Filtration • Cooking (100°C, 4–5 h) • Cooling (5–6 h) • Alcoholic fermentation with traditional levean (9–12 h)	• *Limosilactobacillus fermentum (lactobacillus fermentum)* • *Lactobacillus cellobiosus Lactobacillus brevis* • *Lactobacillus coprophilus Lactobacillus plantarum Lactobacillus hilgardii* • *Enterococcus* sp., • *Pediococcus* sp. • *Leuconostoc* sp. • *Saccharomyces cerevisiae* • *Candida tropicalis* • *Pichia kudriavzevii* • *Pichia kluyveri* • *Kodamaea ohmeri* • *Meyerozyma caribbica*	Aka et al., 2008, 2017; Djè et al., 2009; N'Guessan et al., 2011; Coulibaly et al., 2014
Burkutu/Burukutu	Nigeria	Malted sorghum, millet or maize	nr	• *Lactobacillus plantarum Lactobacillus brevis* • *Limosilactobacillus fermentum (lactobacillus fermentum)* • *Lactobacillus pentosus* • *Lactococcus lactis* • *Leuconostoc mesenteroides Pediococccus damnosus Pediococcus pentosaceus Streptococcus thermophilus* • *Saccharomyces cerevisiae Saccharomyces chavelieri* • *Saccharomyces* sp. • *Candida acetobacter* • *Candida utilis* • *Candida spherica* • *Candida pelliculosa* • *Rhodotorula glutinis* • *Rhodotorula mucilaginosa Cryptococcus albidu*	van der Aa Kühle et al., 2001; Blandino et al., 2003; Jimoh et al., 2012; Lyumugabe et al., 2012; Adewara and Ogunbanwo, 2013)
Otika alcoholic	Nigeria	Malted sorghum	nr	• *Lactobacillus brevis,* • *Limosilactobacillus fermentum (lactobacillus fermentum)* • *Lactobacillus plantarum, Leuconostoc mesenteroides, Enterobacter clocae,* • *Saccharomyces cerevisiae* • *Candida krusei,*	Oriola et al., 2017; Oluwafemi, 2020

(Continued)

TABLE 3 | Continued

Name of product	Country	Raw material	Steps of the processing	Microorganisms involved	References
				• *Candida tropicalis* • *Bacillus cereus* • *Bacillus subtilis*	
Doro or *Chibuku*	Zimbabwe	Malted red sorghum	• Milling • Mixing with water (7 kg/24 l) • Boiling (3–5 h) • Cooling • Lactic fermentation (48 h) • Alcoholic fermentation (5–7days)	• *Lactobacillus plantarum* • *Lactobacillus delbrueckii* • *Lactobacillus* sp • *Lactococcus lactis* • *Lactococcus raffinolactis* • *Lactococcus lactic subsp. lactis* • *Leuconostoc mesenteroides* subsp. *mesenteroides* • *Streptococcus* sp. • *Enterococcus* sp • *Saccharomyces cerevisiae*	Chamunorwa et al., 2002; Togo et al., 2002; Jespersen, 2003; Lyumugabe et al., 2012
Bili bili or Amgba *Bili bili (Tchad)*	Cameroon Tchad	Malted sorghum or millet	• Grinding (fine flour) • Mixing with water • Decantation (1–3h) • Cooking of the sediment • Mixing with the supernatant (65–70°C) • Acidification (9–18h) • Cooking (100°C) • Cooling (30°C) • Alcoholic fermentation (12–24 h) with traditional leaven	• Lactic acid bacteria • *Saccharomyces cerevisiae* • *Kluyveromyces marxianus* • *Cryptococcus albidius* • *Debaryomyces hansenii* • *Candida melibiosica* • *Dekkera bruxellensis* • *Rhodotorula mucilaginosa* • *Torulaspora delbrueckii*	Maoura et al., 2005; Lyumugabe et al., 2012; Touwang et al., 2018
Red kapsiki beer or Te	Cameroon	Malted Sorghum or maize	• Grinding • Mixing with water (1:9, w/v) • Decantation (1–3 h) • Cooking of the sediment (3–5h) • Cooling • Mixing with the supernatant • Decantation & Acidification (overnight) • Cooking of the sour wort (5–10 h) • Cooling • Alcoholic fermentation (12 h at least) with artisanal starter	• Spore forming bacteria • Coliforms • Streptococcus • *Salmonella* • *Shigella* • Sulfite-reducing clostridia	Bayoï and Djoulde, 2017
Bili-bili	Central African Republic	Malted sorghum	• Grinding (crude) • Mixing with water • Decantation (1–2 h) • Heating the sediment until boiling • Mixing with the supernatant (65–70°C) • Cooking • Decantation & cooling • Alcoholic fermentation (10 h) with traditional leaven	• *Lactobacillus* • Lactic acid bacteria • Enterococci • Streptococci • Coliforms • Yeasts	Lango-Yaya et al., 2020
Ikigage or *amarwa*	Rwanda	Malted red sorghum	• Grinding • Mixing with water (infusion, 63–71°C) • Cooling • Decantation (3 h) • Fermentation with addition of leaven (30°C, 12–24 h)	• *Limosilactobacillus fermentum (lactobacillus fermentum)* • *Lactobacillus buchneri* • *Lactobacillus* sp • *Saccharomyces cerevisiae* • *Candida inconspicua*	Lyumugabe et al., 2010, 2012

(Continued)

TABLE 3 | Continued

Name of product	Country	Raw material	Steps of the processing	Microorganisms involved	References
				• *Issatchenkia orientalis* • *Candida magnolia* • *Candida humilis*	
Merissa	Sudan	Malted red sorghum or millet, Sorghum grains	• Grinding • Mixing with water • Lactic fermentation (36 h) • Alcoholic fermentation (8–10 h)	• Lactic acid bacteria • Acetic acid bacteria • *Saccharomyces* spp.	Dirar, 1994
Kaffir beer/bantu beer/utshwala	South Africa	Sorghum	nr	• *Limosilactobacillus fermentum (lactobacillus fermentum)* • *Lactobacillus plantarum* • *Lactobacillus brevis* • *Lactococcus dextranicum* • *Saccharomyces cerevisiae* • *Candida krusei* • *Kloeckera apiculata*	van der Walt, 1956; Lyumugabe et al., 2012

nr, not reported.

between 12 and 16 h (Sawadogo-Lingani et al., 2007). This duration is similar to those of the acidification of *tchoukoutou* wort (13–14 h), *tchapalo* (9–12 h) and *bili-bili* (9-18 h), but shorter than the acidification times of *chibuku* (48 h) and *merissa* (36 h). This acidification/saccharification is a spontaneous lactic fermentation characterized by a drop in pH and growth of lactic acid bacterial counts reaching 8.8–9.9 log cfu/ml at the end of acidification (Sawadogo-Lingani et al., 2007). The predominance of lactic acid bacteria as the original flora in raw sorghum grains and their proliferation during steeping perhaps give LAB a competitive advantage in the brewing of *dolo* or *pito*, leading to their dominance in acidification of sorghum wort (Sawadogo-Lingani et al., 2007, 2010; Sawadogo-Lingani, 2010). The common LAB species involved in the acidification of *dolo* wort include *Limosilactobacillus fermentum* (Basonym: *Lactobacillus fermentum)*, *Lactobacillus delbrueckii* subsp. *jakobsenii* (Basonym: *Lactobacillus delbrueckii* subsp. *delbrueckii*), *Pediococcus acidilactici*, *Lactobacillus lactis* and *Leuconostoc* spp., with a predominance of *Limosilactobacillus fermentum* (Sawadogo-Lingani et al., 2007; Adimpong et al., 2013; Zheng et al., 2020). Various other species of *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Leuconostoc*, *Enterococcus* and *Streptococcus* genera have also reportedly been associated to the spontaneous fermentation of *dolo* and *pito* wort and others similar traditional beers such as *Tchoukoutou*, *Tchapalo*, *Burkutu*, *Bili-bili*, *Chibuku*, *Otika*, *Red kapsiki,* and *Ikigage* (**Table 3**). The spontaneous and uncontrolled lactic acid fermentation found during the steeping of sorghum grains for malting has also been dominated by lactic acid bacteria with *Limosilactobacillus fermentum* as the dominant species (Sawadogo-Lingani et al., 2010), perhaps giving a competitive advantage to this species during acidification of *dolo* and *pito* wort in the brewing phase. Following acidification, the sour supernatant/wort is collected and undergoes boiled for long hours during which water is lost through evaporation and concentrates the wort. In the production of *dolo* and *pito*, this boiling can take between 3 and 5 h. This duration of wort boiling is similar to those reported for other African traditional sorghum beers such as *tchapalo* wort (4–5 h) and *chibuku* wort (3–5 h), but shorter than the boiling times of the *tchoukoutou* (6–9 h) and *red kapsiki* (5–10 h). The boiled concentrated wort is then cooled to room temperature and undergoes alcoholic fermentation.

Alcoholic fermentation of sorghum wort for the production of African traditional beers is generally achieved by the back-slopping technique where part of a previous fermented beer (usually collected from the bottom sediments of previous production) or indigenous dried yeast leaven obtained from previous production is used to inoculate the new batch. The yeast biomass from the previous production is collected, sun-dried and then stored to be used as a local starter culture for subsequent productions. In certain localities, the fresh yeasts are fixed on wooden or fiber supports, or woven belt and these supports are introduced into the canaries containing the wort for fermentation (Sefa-Dedeh, 1991). In most case of African traditional sorghum beers, sorghum wort is inoculated with traditional yeasts and fermentation duration varies between 8 and 24 h at ambient temperature. The duration of fermentation varies from 9 to 14 h for *dolo* and *pito*, similar to that of *tchapalo* (9–12 h) and *merissa* (8–10 h), but shorter than the fermentation times for *tchoukoutou* (12–24 h) and *bili-bili* (12–24 h) while *chibuku* has the longest fermentation time of about 5–7 days. In Western breweries, the fermentation is ensured by selected yeast strains (*S. cerevisae* or *S. carlsbergensis*) and the fermentation time ranges between 8 and 15 days at 10–16°C (Waites et al., 2001). Fermentation is the important step by which yeast converts the sugars of the wort into ethyl alcohol. Interest in the characterization and identification of the yeasts responsible for the alcoholic fermentation of African traditional beers including *dolo* and *pito* has been demonstrated in several studies (**Table 3**). It is found that *Saccharomyces cerevisiae* is the dominant species responsible for the alcoholic

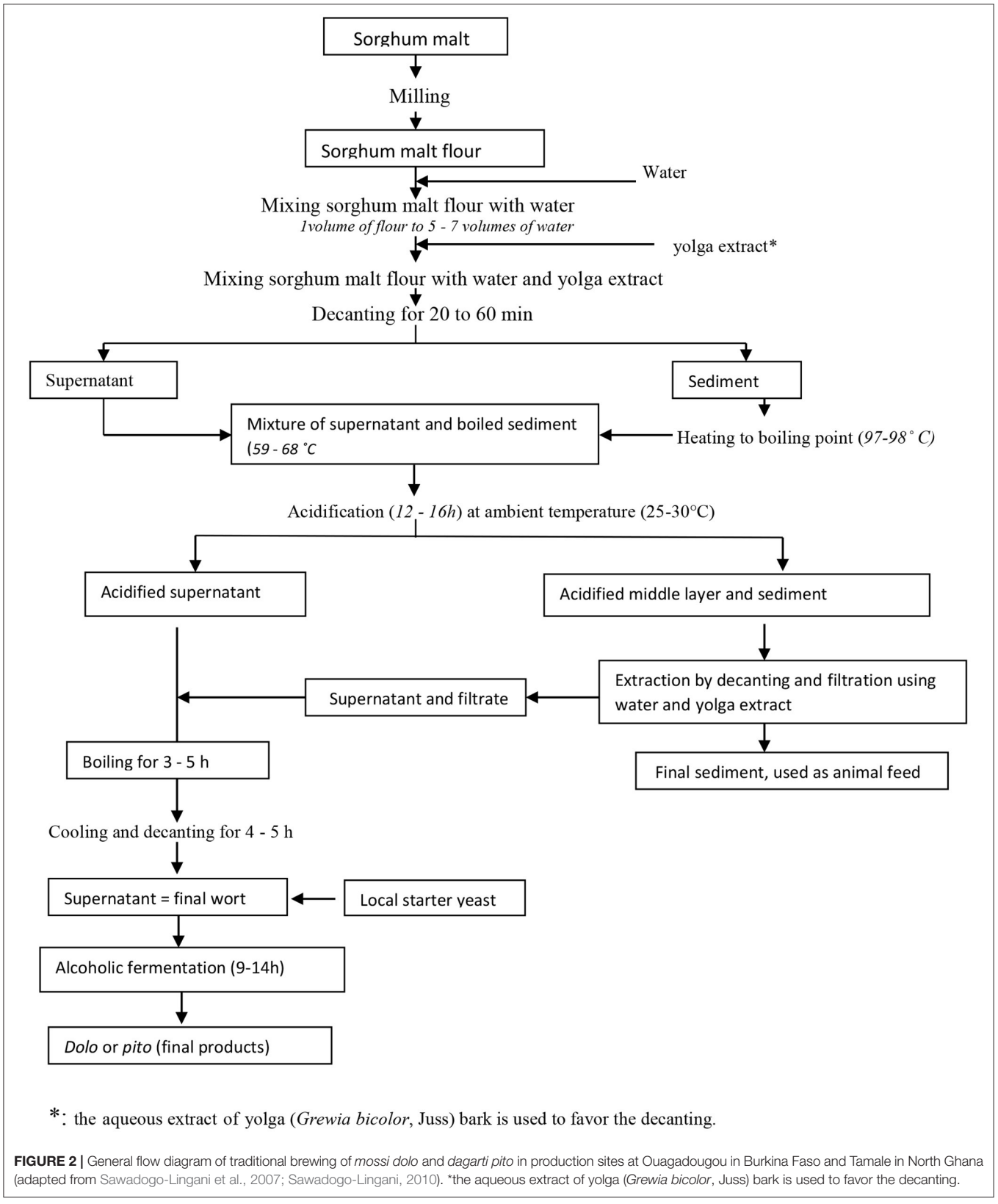

FIGURE 2 | General flow diagram of traditional brewing of *mossi dolo* and *dagarti pito* in production sites at Ouagadougou in Burkina Faso and Tamale in North Ghana (adapted from Sawadogo-Lingani et al., 2007; Sawadogo-Lingani, 2010). *the aqueous extract of yolga (*Grewia bicolor*, Juss) bark is used to favor the decanting.

fermentation of *dolo* and *pito* as well as other African traditional beers. Other yeast species identified during the fermentation of African sorghum beers include *S. pastorianu*, *S. chavelier*, *Candida* spp., *Kloeckera* spp., *Hansenula* spp., *Torulaspora* spp., *Schizosaccharomyces* spp., *Kluyveromyces* spp., *Debaryomyces* spp., *Pichia* spp., *Dekkera* spp., *Clavispora* spp., *Hanseniaspora* spp., *Rhodotorula* spp., *Cryptococcus* spp. (**Table 3**). However, most of these yeasts are considered contaminants not taken part in the fermentation; some may even be pathogenic and affect the safety of the traditional sorghum beer.

Packaging, Storage, and Shelf Life

At the end of alcoholic fermentation, *dolo* or *pito* is well frothy and sparkling, and ready for consumption. Traditional sorghum beers such as *dolo* and *pito* do not undergo filtration or stabilization and are consumed in the active fermentation state. The *dolo* or *pito* is packaged in canaries, jars, plastic buckets or barrels of 20 or 5 L cans for sale to consumers in cabarets or to *dolo* retailers. At the cabarets, *dolo* is served and drunk in gourds. African traditional sorghum beers have a poor keeping quality with a shelf-life of about 24–72 h at ambient temperature (Novellie and De Schaepdrijver, 1986; Tisekwa, 1989; Broutin et al., 2003; Maoura and Pourquie, 2009; Lyumugabe et al., 2010; Aka et al., 2017). A few investigations (Osseyi et al., 2011; Dahouenon-Ahoussi et al., 2012; Ayirezang et al., 2016) have been conducted in an attempt to improve the shelf-life of *dolo* and *pito* or similar beers. Recent investigations show that the shelf-life of *pito* can be extended through pasteurization and/or the addition of *Moringa oleifera* leaf extract for up to 28 days. However, *pito* samples that contained the *moringa* extract were less favored by consumers (Ayirezang et al., 2016). According to Dahouenon-Ahoussi et al. (2012), the use of essential oil of Citronella (*Cymbopogon citratus*) at 1 ml/L was effective in stabilizing African sorghum beer against the spoilage effects of continued fermentation. Rodrigue Christian et al. (2014) also evaluated the influence of *Hemizygia bracteosa* (Benth) leaf powder on the quality of *chakpalo* produced in Benin and reported that the use of the powdered leaves during mashing had an effect on the physico-chemical parameters, providing a slightly sweet drink, less acidic, with low alcohol content.

SAFETY OF AFRICAN SORGHUM BEERS

The microbiological and sanitary quality of sorghum malt, as well as the resulting risks, are highly dependent on the malting conditions. The conditions necessary for soaking and germination (temperature of 30°C, humidity above 85%) are also ideal for the proliferation of the inherent microflora of the raw cereal grains, generally consisting of yeasts, molds, enterobacteria, lactic acid bacteria and spore-forming aerobic bacteria. Although lactic acid bacteria have dominated in the spontaneous fermentation during sorghum grain steeping for malt production, the frequent association of other undesirable microorganisms compromises the quality and safety of sorghum malts. In general, bacterial species of safety concern such as *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Stapylococcus aureus*, *Klesiella aerogenes*, *Sarcina* spp., and molds such as *Botryodiplodia theobromae*, *Aspergillus flavus*, *Aspergillus niger, Rhizopus stolonifer*, *Penicillium* spp., *Fusarium* spp., have been identified in sorghum grains and malts (Ilori et al., 1991; Ogundiwin et al., 1991). However, like many other traditional African fermented foods and beverages, sorghum beers are generally considered to be microbiologically safe due to the antimicrobial effects exerted during lactic acid fermentation, the alcohol content as well as the long cooking hours of sorghum malt wort prior to acoholic fermentation which potetially eliminates pathogenic microorganisms. This notwithstanding, the spontaneous nature of the fermentation processes (without properly defined starter cultures), poor control measures (including time-temperature) during fermentation and poor post-processing handling including packaging predisposes these products to contamination by pathogenic microorganisms.

Another safety concern for African sorghum beers is related to handling and storage conditions of the raw materials (cereal grains) which predispose them to mycotoxin contamination due to growth of toxigenic molds. Mycotoxins isolated from sorghum and millet grains include *patulin*, *trichothecene*, *zearalenone* and *aflatoxins* (McFarlane et al., 1995). In addition, Dufour et al. (1992) in a survey showed that about 80% of sorghum varieties from Taiwan and Africa (South Africa, Cameroon, Burundi, Ghana, Kenya, Nigeria, Rwanda, and Sudan) were contaminated with aflatoxins; 19% of which were contaminated before malting, and 52% after malting. Moreover, Matumba et al. (2011) indicated the presence of aflatoxins at levels of 6.6 to 54.6 μg/kg in a sorghum malt from Malawi. However, red varieties of sorghum which are commonly used to produce African sorghum beer are less susceptible to the development of aflatoxins than white varieties (Ratnavathi and Sashidhar, 2000), because of their richness in tannins that provide a protective role against grain mold. Additionally, the production process of African sorghum beer has been shown to decrease mycotoxin levels although the process does not completely eliminate these toxins (Trinder, 1988; Dufour et al., 1992). There is therefore the need to incorporate safety standards throughout the production process of African sorghum beers, beginning with supply of quality raw materials through fermentation to post-processing handling, packaging and distribution.

USE AND VALORIZATION OF BY-PRODUCTS FROM *DOLO* PRODUCTION

The valorization of by-products contributes to improving the competitiveness and sustainability of the African sorghum beer sector. Currently, valorization undertaken by actors are related to yeast biomass and *dolo* dreche. In *dolo* production units, the yeast biomass settling at the bottom of packaging containers such as canaries, jars, buckets, barrels, cans and gourds is collected, washed with water and then sun dried. The dried yeast called *rabilé* (Burkina Faso) or *dambeli* (Northern Ghana) is packaged in plastic bags and marketed as food yeast. In

addition to its use as a local fermenter or starter for the alcoholic fermentation of sorghum malt wort, dried yeast is used as a food condiment in the preparation of sauces, dishes and grilled chickens by local population and in some restaurants. An important source of protein, amino acids and B-group vitamins, dry yeast gives flavor to dishes and improves their nutritional value, contributing to the improvement of the diet of consuming populations. Yeast biomass is well-known for its richness in proteins, essential amino acids, fatty acids and B-group vitamins. A good valorization strategy could allow to better valorize this by-product of traditional breweries for its use as a food additive in human and animal food, as unicellular proteins and a source of vitamins.

Dolo dreche or spent grain, the solid residue obtained after rinsing and filtering the mash after acidification by spontaneous lactic fermentation, is recovered for animal feed. It is marketed to pig farmers. Indeed, in Burkina Faso, pig production, mainly managed by women who own 60% of the country's farms, is one of the fastest growing livestock sectors. It is gaining importance in societies where there is a shift from ruminant to monogastric livestock production, with higher rates of return and advantageous feed efficiency. The majority of farmers use locally abundant raw materials such as artisanal sorghum (*dolo*) brewers' spent grains. For the supply of dolo *dreche*, breeders establish a tacit contract of supply with the *dolotières* for an exclusivity on all its production or then for a given quantity of baskets of *dolo dreche* which are sold between 200 and 500 FCFA per basket of ∼10 liters (FAO, 2012). This residue, used as animal feed, still contains nutrients in quantity and quality such as sugars, dietary fiber, proteins, fats, minerals, vitamins etc. Even though research and innovation are still missing in an African context, it is obvious that it can be better valorized and used in human food and thus contribute to the achievement of food and nutritional security of local populations. Collected under appropriate conditions followed by adequate pre-treatment (drying, grinding, sieving, etc.), it can be used in human food. The residues from sieving could still be used as animal feed.

As traditional breweries in Africa are heavy consumers of firewood, and they generate large quantities of incandescent embers, some of which are often extinguished to provide charcoal. This charcoal is sold to households and contributes to increasing the income of traditional brewers. Similarly, large quantities of wood ash are also generated, which are sold to traditional potash manufacturer. The potash is sold and used as ingredient for local soap preparation, as food ingredient to neutralize acidity, make alkaline or softener in the preparation of dishes, and in the processing of fermented seeds (*maari*, *mandchoua*, *bikalga*, etc) used as food condiments (Thorsen et al., 2015; Kere-Kando et al., 2020).

CONSTRAINTS AND STRATEGIES FOR SUSTAINABLE PRODUCTION OF SORGHUM BEER

The African traditional sorghum beer is an ethnic beverage that is facing constraints for its development and expansion outside the production regions. Some of these constraints, their implications and prospects for sustainable production are summarized in **Table 4**. Various research works have been undertaken to bring innovations and value-addition to African traditional beer processes. Initial approaches for sustainable production of African sorghum beers were aimed at optimizing and controlling fermentation processes through the development of starter cultures (Sefa-Dedeh et al., 1999; Orji et al., 2003; Glover et al., 2005, 2009; Maoura et al., 2005; Sawadogo-Lingani et al., 2008a,b; Yao et al., 2009; N'Guessan et al., 2010, 2011, 2016; Adewara and Ogunbanwo, 2013; Lyumugabe et al., 2014). **Table 5** summarizes the results of works geared toward developing starter cultures for controlled fermentation of African sorghum beers. These studies have shown, for the most part, that the selected starters lead to a reduction in fermentation time and an improvement in the beer production process. Moreover, these selected starters lead to sorghum beers with physico-chemical and sensory characteristics (pH, color, titratable acidity, alcohol content, specific gravity, taste, flavor) comparable to traditionally produced beers (Sefa-Dedeh et al., 1999; Orji et al., 2003; Glover et al., 2005, 2009; Sawadogo-Lingani et al., 2008b). However, these experiments were carried out on laboratory and pilot scales, and the processes have not been scaled-up or replicated in *dolo* and *pito* production units. The production of yeasts starter cultures with optimal technological properties in dried form can be promoted for that purpose. Collaborative projects such as the GreenGrowth project (DFC No 13-04KU) has established culture collections (Biobank) of microorganisms involved in the processes of West African fermented products including traditional sorghum beers, with the aim of promoting sustainable use of beneficial indigenous microorganisms.

Other innovative work aimed at improving the process of African traditional sorghum beer has been carried out. For example, a fractional factorial plan has been developed and applied to optimize the artisanal process of *tchapalo* (Amané et al., 2012), a traditional beer similar to *dolo* and *pito*. The authors, through the screening of factors, highlighted the importance of some of the processing operations (drying, soaking, germination, type of ferment, fermentation, decanting, cooking, pre-cooking) and critical points for improvements. The establishment and resolution of a mathematical model enabled the proposal of optimum conditions for the production of *tchapalo*. The proposed optimized processing conditions including 15 h of soaking, 72 h of germination, 10 h of drying, 30 min of decantation, 1 h of pre-cooking and 2 h of cooking resulted in a time saving of over 3 days and enabled the production of *tchapalo* with consistent quality. These results constitute the basis for the industrialization of *tchapalo* and may be applicable to other African traditional sorghum beers such as *dolo* and *pito*. In an attempt to improve the sensory characteristics of sorghum beers, local bitter plants such as *Vernonia amygdalina* and *Nauclea diderrichii* were used in the brewing of sorghum beer under laboratory-controlled conditions (Desobgo Zangué et al., 2013). The two bitter plants, commonly used for their medicinal properties, could adequately bitterize sorghum beers and therefore, should be explored to enhance the sensory qualities of African sorghum beers as occurs

TABLE 4 | Challenges and perspectives for sustainable production of African traditional sorghum beers.

Challenge/constraints	Implications	Perspectives for sustainability
Decrease in productivity and availability of quality raw material (sorghum) due to climate variability and change	Inconsistent supply of no quality and no standardized cereal (sorghum) leads to high cost of production variable product characteristics including physico-chemical, nutritional and organoleptic properties from one production batch to another	Ensuring a supply of adapted and quality raw materials through the dissemination and extension of improved varieties of cereal (sorghum) suitable for traditional sorghum beer production
Artisanal nature of the processing	Time consuming and labour-intensive process. Long cooking/concentration time of the wort with a high consumption of firewood used as the main source of energy. Complex and non-standardized process leads to inconsistent quality and variability in products	Optimize and standardize the process Introduce technological innovations in the process Modernize the production process
High consumption of firewood used as the main source of energy	Strong contribution to deforestation with a negative impact on the environment	Develop and implement strategies for the dissemination and adoption of improved stoves
Spontaneous fermentations or the used of no properly defined starter cultures	These practices coupled with poor time/temperature controls expose fermented products to undesirable and pathogenic microorganisms, compromising the sanitary quality of sorghum beer and thus the safety of the consumers.	Promote the implementation of good hygiene and manufacturing practices in production units to improve the quality of traditional sorghum beer while maintaining the biodiversity of microorganisms with technological potential.
Poor hygiene of the production and sales environment	Contamination from the environment with negative impact on the quality and safety of the final products, and on the safety of the consumers	Regulate the production and selling environment Promote the application of good hygiene practice guides to improve the hygiene of the production and sales environment
Unpackaged, poor packaging or inappropriate packaging material used, unstabilized drinks store at ambient conditions for selling	Contamination of the product by the environment in addition to the continuation of fermentation which induces a short shelf-life (1–3 days)	Search for an appropriate stabilization and packaging process that preserves the original quality of sorghum beer in order to extend its shelf life for wide distribution and consumption.
Lack of control of technological and sanitary parameters sanitary quality of the products (raw materials, intermediates products, finished products) during the processing	Variability of the physico-chemical, nutritional and organoleptic characteristics of the products from one production to another;	Promote the regulation of the products of the traditional sorghum beer value chain Develop, disseminate and promote the adoption of technical specifications and products quality standards
Weak technological and scientific knowledge of the processing and on the nutritional and therapeutic properties of the finished products.	Process always artisanal and non-standardized Low value added of traditional sorghum beer Distribution market always internal and local	Conduct research to provide in-depth and detailed scientific results on sorghum beer technology and its nutritional and therapeutic characteristics.

in the use of *Humulus lupulus* in Western beer production These plants also proved to be excellent sources of free amino acids, thus improving the characteristics of the must before fermentation.

The production of beers from raw agricultural materials in many regions of Sub-Saharan Africa consumes a significant proportion of total wood-fuel. Thus, the cooking/concentration of sorghum beer wort is a high fuel-intensive operation using firewood as the main source of fuel. Being a high energy consuming process with firewood as the main source of fuel in production, the direct environmental impacts are deforestation and greenhouse gas emissions, with potential negative consequences for the climate. Yaméogo et al. (2013) reported that the people of Vipalogo (a village in Burkina Faso) use 1,422 m^3 of wood per year for *dolo* production and their needs per year for timber to build huts, attics and sheds were estimated at 25 m^3. Today, around 2.7 billion people in developing countries rely on the traditional use of biomass, mostly firewood or charcoal, for cooking. This contributes to deforestation and severe health problems as the related smoke emissions cause respiratory diseases (World Health Organization, 2009).

TABLE 5 | Development of starter cultures for controlled fermentation of different African traditional beers.

Traditional beer	Types of fermentation	Selected strains & characteristic	Results	References
Dolo	Lactic fermentation and acoholic fermentation Mixed or co-fermentation (lactic + alcoholic)	• *Lactobacillus fermentum* • *Saccharomyces cerevisiae* • *Lactobacillus fermentum* + *Saccharomyces cerevisiae* • Used as fresh cells suspension	Four strains of *Lb. fermentum* and one strain of *S. cerevisiae* were tested in a series of three trials productions: (i) a production of *dolo* by double lactic fermentation with *Lb fermentum*, and alcoholic fermentation with *S. cerevisiae; (ii)* a production of *dolo* by lactic and alcoholic mixed or co-fermentation (*Lb. fermentum* + *S. cerevisiae*); (iii) a production of *dolo* in a real environment by a double lactic and then alcoholic fermentation with the same strains. The results showed that the starter cultures reduced the duration of lactic fermentation (9 h instead of 12-16 h) and of lactic + alcoholic fermentation (12 h instead of 21-48 h) in the case of co-fermentation. Furthermore, sensory analysis revealed that the *dolos* produced with the selected starters had organoleptic and physico-chemical characteristics comparable to those of the traditional *dolo* and were considered acceptable by the tasters.	Sawadogo-Lingani et al., 2008b; Glover et al., 2009
Tchapalo	Alcoholic fermentation	• *Saccharomyces cerevisiae* • *Candida tropicalis* • *Saccharomyces cerevisiae* + *Candida tropicalis* • Formulation of freeze-dried cells for alcoholic fermentation	Strains *Saccharomyces cerevisiae* F12–7 and *Candida tropicalis* C0–7 isolated from sorghum beer were used in a mixed culture at a ratio of 2:1 (*C. tropicalis*/*S. cerevisiae*).and freeze-dried using as protective agents (sucrose, glucose, glycerol) and support materials (millet, maize, sorghum and cassava flours) at 1:1 ratio (v/v). The freeze-dried yeasts viabilities were between 4.0% and 10. 6%. sucrose was found to be the best protectant giving cell viabilities of 8.4–10.6%. Millet flour was the best support material after drying. When the freeze-dried yeast powders were stored at 4°C and room temperature (25–28°C) for up to 3 months, the survival rates were the highest with cassava flour as the support material.	N'Guessan et al., 2010, 2011, 2016
Tchapalo	• Lactic fermentation • Alcoholic fermentation by commercial yeast	• *Lactobacillus fermentum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus, Lactobacillus plantarum* • Used to evaluate as single starter cultures for controlled lactic fermentations of the wort • Commercial *Saccharomyces cerevisiae (*used at 1%) for controlled *alcoholic* fermentation during 12 h	LAB strains used as single starter cultures in the sorghum wort for lactic fermentation, grew increasing organic acids and titratable acid and dropping pH. But *L. fermentum* (strains S6, S42, S45), *P. acidilactici* (strains S7, S52), and *P. pentosaceus (*strain S5) acidified quickly the sorghum wort. The *tchapalo from that worts were* similar to those of obtained by spontaneous fermentation. These starter cultures will be used for sweet wort and *tchapalo* commercial production and thereby to improve their safety and consumer acceptability of these products	Aka et al., 2020
Pito	Alcoholic fermentation	*Saccharomyces cerevisiae*	Reduction of fermentation time Better control of the production process Acceptable organoleptic characteristics.	Sefa-Dedeh et al., 1999; Glover et al., 2005

(Continued)

TABLE 5 | Continued

Traditional beer	Types of fermentation	Selected strains & characteristic	Results	References
Pito	• Lactic acid fermentation and alcoholic fermentation • Mixed or co-fermentation (lactic+ alcoholic)	• *Lactobacillus plantarum* • *Saccharomyces cerevisiae* • *Lactobacillus plantarum* +*Saccharomyces cerevisiae* • *Pediococcus halophilus* + *Candida tropicalis* • Used as fresh cells suspension	Reduction of fermentation time Better control of the production process Acceptable organoleptic characteristics.	Orji et al., 2003; Yao et al., 2009
Burukutu	• Lactic acid fermentation • Alcoholic fermentation • Mixed or co-fermentation (lactic+alcoholic)	• *Lactobacillus fermentum* • *Saccharomyces cerevisiae* • *Lactobacillus fermentum* + *Saccharomyces cerevisiae*	Reduction of fermentation time Better control of the production process Acceptable organoleptic characteristics.	Adewara and Ogunbanwo, 2013
Burukutu	Alcoholic fermentation	*Saccharomyces cerevisiae*	Reduction of fermentation time Better control of the production process Acceptable organoleptic characteristics	Maoura et al., 2005
Bili bili	Alcoholic fermentation	*Saccharomyces cerevisiae*	Reduction of fermentation time Better control of the production process Acceptable organoleptic characteristics,	Maoura et al., 2005

In order to reduce wood consumption and preserve the environment, improved wood or gas fireplaces have been designed and promoted among a few women *dolo* producers in Ouagadougou, Burkina Faso. Improved cook-stoves potentially help to alleviate the negative implications of wood-fuel usage since they increase the efficiency of the cooking process thereby reducing the wood-fuel consumption. The saving rate of the improved cook-stoves is about 25% as compared to a traditional tripod stone stove. This is remarkable, but still below the potential energy savings of about 40% achieved in controlled cooking tests, showing the need to test the effectiveness of new technologies under real world conditions and based on a sufficiently large and representative usage. A program like FAFASO (Foyer Amélioré au Burkina Faso) has been implemented with the objective to evaluate the productive use and to promote the improved cook-stoves for local beer breweries in Burkina Faso. The improved cook-stoves made for breweries (Roumdé stoves) are much larger than the household cook-stoves and are made of clay and bricks rather than metal and saves at least 60 to 70% of the firewood needed with a traditional stove for brewing process (Grimm and Jörg, 2013). However, it seems that the saving rate goes rapidly down if the improved stove is badly maintained. In one field test conducted by Sanogo et al. (2011), a damaged improved cook-stove even needed more firewood per liter of *dolo* than a traditional stove, confirming that a rigorous assessment of the effectiveness of such stoves requires a test under real world conditions which includes the status of the stove and how these stoves are maintained. In 2012, about 50% of all breweries in Ouagadougou and Bobo-Dioulasso city adopted another new technology aimed at reducing firewood consumption during brewing of *dolo*. However, since then, the trend of adoption has declined. The exact reasons for this decline are not known and therefore need to be investigated. The improved fireplace designed for *dolo*, seem to be a fixed fireplace, made of refractory bricks with an opening for the fuel inlet and one or two chimneys for the smoke outlet. It is a multi-purpose firebox that can be built for 2, 3, or 4 pots made of aluminum or fired clay. The selected fireplaces consume 60 and 90% less wood than traditional *dolo* fireplaces. In addition to significantly reducing greenhouse gas (GHG) emissions, the improved fireplaces offer benefits to its users in the form of money savings, reduced carbon monoxide and fine particle emissions that are harmful to health, protection against heat emanating from the fireplace, faster cooking (thus saving time), better cleanliness and convenience (Grimm and Jörg, 2013). In any case, reflection and research should continue to find other sources of energy as a substitute for firewood in a perspective of climate sustainability.

Regarding availability of the raw material, sorghum is one of the main cereals grown in Burkina Faso, as well as in other West African countries. A varietal diversity of this cereal is managed by farmers and national agricultural research centers for various production objectives. Despite various efforts, yields have remained low and the increase in production is mainly due to the expansion of cultivated areas. This situation is aggravated by climate variability and change. Research programs have therefore focused on maintaining sorghum biodiversity and increasing productivity through the participatory development of improved variety lines adapted to local climatic conditions and farmers' needs and preferences (vom Brocke et al., 2014). The same is true for other cereals such as millet, maize and rice. Thus, in the Regional Catalog of Plant Species and Varieties (ECOWAS-UEMOA-CILSS, 2016) there are 1,496 varieties of 11 priority crops including 413 rice varieties, 279 maize varieties, 96 millet varieties and 171 sorghum varieties, all of which are released at the national level. These cereals used as raw materials in the processing of traditional beers are nowadays experiencing significant drop in productivity due to climate variability and change. Breeders in agricultural research centers have developed

better adapted hybrid varieties. In Burkina Faso for example, improved basic and certified seeds are produced by agricultural research centers. basic seeds are also made available to seeds producers for the production of certified improved seed. Certified seeds are subsidized by the State for farmers' organizations at affordable prices. To ensure sustainable production of traditional sorghum beer, strategies should be developed to promote the adoption of these varieties among famers for a sustainable supply of raw materials.

In terms of innovation in the processing of sorghum beers, private businesses have set up mini breweries that produce small quantities of stabilized *dolo* and *pito*, packaged in bottles and appropriately labeled. Thus, a mini brewery with modern stainless-steel equipment has been designed since 2003 in Ghana in Kwabenya (northern suburb of Accra). This company produces sorghum beer with a capacity of 200 liters of beer per day, three times a week. The local beer industry provides a living for nearly 6,000 people in the northern parts of Ghana where the sorghum is produced farmers as a major crop (Gamba, 2019). In Benin, a small enterprise has been set up to produce traditional *tchapalo* (corn or sorghum beer) packaged in properly labeled brewery bottles in a factory and are sold at competitive price while maintaining its special traditional taste (CTA, 2002). Between the years 2003 and 2004, Zambian Breweries (ZB) launched a clear beer made from locally grown sorghum and has since then been producing African sorghum beer. The "new Eagle" beer which uses locally produced sorghum as its raw materials has opened opens up new market channel for many smallholder farmers who, for the first time, have a sustainable commercial outlet for their sorghum production (CTA, 2006). In Burkina Faso, Unité de Malterie de Ouidtinga (UMAO) has been producing malt from local cereals under controlled conditions for both modern and traditional beers since 1999, and from 2003 has been producing traditional beer (*dolo* or *ram*) and sweet wort (*ran noodo*) packaged in bottles in modern plants with optimized malting and brewing processes. UMAO also offers *dolo* packaged under pressure for festive ceremonies on order. A manual of Good Hygiene Practices for sorghum malting and *dolo* brewing has been developed (Bougouma et al., 2008) for the benefit of women producing sorghum malt and *dolo* brewers in Ouagadougou. The implementation of this manual would improve the quality of *dolo* through the improvement of some technological practices and hygienic environments during malting, brewing and sale of the *dolo*.

Today, new types of consumers, especially young people and urban dwellers, are entering the traditional beverage value chain. These young people, faced with unemployment, idleness and altered lifestyles, indulge in the consumption of alcoholic beverages, prompting *dolo* producers to adopt various bad practices in order to increase the alcoholic strength of *dolo* by adding adulterated hard liquor to satisfy this category of consumers. For a sustainable production of the natural sorghum beer, it is important to properly characterize and conserve the microflora of technological interest to develop them into starter cultures for controlled fermentation, to regulate the production through the establishment of quality standards in order to preserve their natural and original characteristics and to better valorize by-products and waste to increase the competitiveness of the value chain. The standardization of the process and regulation could lead to a new trend in the production of a range of fermented beverages with different degrees of alcohol content in order to satisfy different categories of consumers without compromising the originality of the beverage. However, the emphasis should be on low-alcohol beer, because there is currently a strong trend for this type of beer in other parts of the world including Europe. Appropriate packaging and stabilization process should be developed to extend the shelf life and diversify the channels of distribution.

CONCLUSIONS

This review presents currently available information on African traditional sorghum beers. It emerges that despite its popularity and its socio-cultural and economic importance, the manufacturing process of this ancestral drink is still artisanal although some innovations have been introduced in recent years. The traditional sorghum beer sector is a promising sector that brings together a diversity of actors. Yet, African sorghum beer production faces sustainabilty challenges, particularly related to the development of efficient and environmentally friendly processing technologies, raw material supply, variability in product quality and safety, high energy consumption and its associated impact on the environment, poor packaging, and short shelf-life. The development and emergence of this sector will require the development and implementation of strong strategies and actions at all levels of the value chain by the different actors. Thus, for sustainable production African traditional sorghum beers, strategies must be geared toward addressing sustainability challenges by improving quality and availability of raw material supply, processing technology (starter culture adoption), safety, packaging, recycling and waste treatment, as well as methods of improving energy consumption. Actions must be pursued to better valorize traditional sorghum beer and its by-products, assure a sustainable supply of adapted and quality raw materials, optimize and standardize processing technologies for malting, brewing and packaging of sorghum beer. There is also the need to control fermentation systems while preserving the biodiversity of the fermenting microorganisms associated with African sorghum beers.

AUTHOR CONTRIBUTIONS

HS-L designed the manuscript. All authors contributed to writing the manuscript. JO-K, HS-L, and LJ critically revised the manuscript. HS-L, JO-K, RG, BD, MJ, and LJ read and corrected the manuscript. HS-L, JO-K, LJ, and MJ validated the submitted version of the manuscript.

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Sustainable Ethanol Production From Sugarcane Molasses by *Saccharomyces cerevisiae* Immobilized on Chitosan-Coated Manganese Ferrite

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The interaction between nanostructures and yeast cells, as well as the description of the effect of nanoparticles in ethanol production are open questions in the development of this nanobiotechnological process. The objective of the present study was to evaluate the ethanol production by *Saccharomyces cerevisiae* in the free and immobilized state on chitosan-coated manganese ferrite, using cane molasses as a carbon source. To obtain the chitosan-coated manganese ferrite, the one-step coprecipitation method was used. The nanoparticles were characterized by X-ray diffraction obtaining the typical diffraction pattern. The crystal size was calculated by the Scherrer equation as 15.2 nm. The kinetics of sugar consumption and ethanol production were evaluated by HPLC. With the immobilized system, it was possible to obtain an ethanol concentration of 56.15 g/L, as well as the total sugar consumption at 24 h of fermentation. Productivity and yield in this case were 2.3 ± 0.2 g/(L * h) and 0.28 ± 0.03, respectively. However, at the same time in the fermentation with free yeast, 39.1 g/L were obtained. The total consumption of fermentable sugar was observed only after 42 h, reaching an ethanol titer of 50.7 ± 3.1, productivity and yield of 1.4 ± 0.3 g/(L * h) and 0.25 ± 0.4, respectively. Therefore, a reduction in fermentation time, higher ethanol titer and productivity were demonstrated in the presence of nanoparticles. The application of manganese ferrite nanoparticles shows a beneficial effect on ethanol production. Research focused on the task of defining the mechanism of their action and evaluation of the reuse of biomass immobilized on manganese ferrite in the ethanol production process should be carried out in the future.

Keywords: ethanol production, manganese ferrite, Saccharomyces, sugarcane molasses, immobilization

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INTRODUCTION

The possibility of obtaining a renewable source of energy that is easy to access, safe and effective is one of the goals that humanity must achieve. Ethanol obtained by biotechnological methods is an important alternative to fossil fuels. The ethanol production from sugarcane shows the lowest production costs. Mainly, three types of raw materials are being used for this purpose, such as: sugar juice, starchy crops, and lignocellulosic materials (Mohd et al., 2017). The most common microorganisms used in fermentation from are the yeasts, especially, *S. cerevisiae*, though the bacterial species *Zymomonas mobilis* is also potentially used nowadays for this purpose (Arshad et al., 2018).

The biocatalysts immobilization has been the subject of research in recent decades, demonstrating that the immobilization of cells presents certain technical and economic advantages compared to a free-cell system (Elakkiya et al., 2016). In an attempt to improve ethanol fermentation, cell immobilization techniques have been developed to increase the rate of ethanol production, keep cells in a viable state, and achieve their normal function (Zapata and Peláez, 2010). Currently, in the immobilization of fungal spores and yeast cells, magnetic nanoparticles were used (Palacios et al., 2017). Nanoparticles have the ability to bind to the cell surface helping in the biomass separation and the biocatalysts reuse, since under the application of an external magnetic field, the particles are attracted and precipitated keeping the immobilized cells (Palacios et al., 2017).

Recently, many studies have focused on the synthesis of nanomaterials due to their excellent properties: a large surface-volume ratio, high specific surface area, easy separation under external magnetic fields and strong adsorption capacity (Vlazan et al., 2015). Manganese ferrite is an important member of ferrite family. Superparamagnetic iron oxide nanoparticles show a variety of applications in the modern era of science and engineering, for example, in medicine, mainly for magnetic hyperthermia (Mazarío et al., 2016), drug administration (Wahajuddin and Arora, 2012), as contrast agents in diagnosis by magnetic resonance imaging (MRI) (Mazarío et al., 2016), biosensors (Haun et al., 2010), ferrofluids (Moussaoui et al., 2016), they also can be used successfully in water at low temperature in thermochemical cycles (Prieto et al., 2007).

Various fabrication methods have been reported to prepare spinel manganese ferrite nanocrystals, including the sol-gel method (Banalata et al., 2014), coprecipitation (Irfán et al., 2012), synthesis in reverse micelles (Das et al., 2021) and the hydrothermal method (Mebdir and Sadiyha, 2019). Each method of ferrite obtaining has its advantages and disadvantages. The coprecipitation method offers many advantages over other methods, such as absence of contamination, more homogeneous mixing of the components and control of the particle size of the powders (Amighian et al., 2006), simplicity without the need for postcalcination. In the coprecipitation technique, several parameters, such as the relationship between ions, ionic strength, pH and precipitation temperature, can affect the structure and magnetic properties of ferrites (Goodarz et al., 2011). Coprecipitation from aqueous solutions is a relatively simple method and is therefore suitable for mass production. However, it provides limited control over stoichiometry, particle size, and size distribution (Amighian et al., 2006).

Although the methods for manganese ferrite obtaining are well-known, its functionalization with chitosan in one step was not previously described. However, Gregorio et al. (2012) and Osuna et al. (2012) described the method of magnetite nanoparticles synthesis coated with chitosan in one step coprecipitation, showing that the increase of chitosan concentration in the system does not lead to an increase in active amino groups. The optimal chitosan concentration was 0.125% (W/V).

The goals the present study were: (1) to obtain and characterize chitosan-coated manganese ferrite nanoparticles by one step coprecipitation method; (2) to evaluate the ethanol production from blackstrap molasses by free *S. cerevisiae* and yeast immobilized on chitosan-coated manganese ferrite nanoparticles.

MATERIALS AND METHODS

Materials

The salts hydrated manganous sulfate (Jalmek), hexa-hydrated ferric chloride (Jalmek) and sodium hydroxide (Jalmek) were of analytical grade and were used as reagents. Chitosan with low molecular weight and 75% deacetylation degree from Aldrich was used.

Preparation of Chitosan-Coated $MnFe_2O_4$ Nanoparticles

A nano-sized manganese magnetic ferrite powder was prepared by the one-step coprecipitation method (Osuna et al., 2012) using hydrated manganese sulfate, ferric chloride hexahydrate, sodium hydroxide, and chitosan as starting materials. Ferric chloride hexahydrate was dissolved together with the required amount of hydrated manganese sulfate in a distilled water at 70°C. The ratio between the Fe and Mn cations was kept at 2: 1, respectively. Upon reaching this temperature, chitosan was added at 0.125% (w/v). The mixture was stirred until chitosan was solubilized. To 100 mL of the mixture, 20 mL of the sodium hydroxide solution (8 M) were added dropwise, the pH was set at 14. Unlike the methodology proposed by Osuna et al. (2012), the mixture was subjected to hydrothermal treatment in an autoclave for 5 h.

After the reaction, a dark brown precipitate was formed which was separated using the magnet. It was washed with water several times to remove impurities until the pH of the residual solution reached 7. This process took about 4 h. Next, the sample was dried in an oven keeping the temperature at 40°C for 3 h. The powder was treated in the mortar. Manganese ferrite was prepared in a similar manner without the addition of chitosan.

The chitosan-coated ferrite was kept at room temperature in a desiccator before being used in the fermentation.

Characterization of Manganese Ferrite Coated With Chitosan

X-ray Diffraction and Particle Size

X-ray powder patterns were recorded using a Siemens D-5000 diffractometer operating with Cu-Kα radiation ($\lambda = 1.54056$ Å)

at 35 kV and 25 mA in the range of 2 to 80°C with a scanning speed of 0.02°C/s. The average crystallite size of the samples was determined from the total width to half the maximum using the Scherrer's formula:

$$D = \frac{0.9\lambda}{\beta \cos\theta}$$

where D is the average size of the crystallite, λ is the wavelength of the X rays, θ is the corresponding Bragg diffraction angle (in radians), and β is the total width in the maximum half (in radians) after the instrumental error correction. The X-ray diffraction patterns were also subjected to a structural analysis by the Rietveld method by means of the material analysis using diffraction (MAUD) program version 2.80.

Vibrating Sample Magnetometer

The magnetic properties of the synthesized chitosan-coated nanoparticles were measured in an alternating gradient magnetometer (AGM) Micromag 2900 manufactured by Princeton Measurements. The powders were measured in triplicate at room temperature. The magnetic field was applied from 12 to −12 kOe.

Fourier Transform Infrared Spectroscopy

Nanoparticles were characterized by Fourier transform infrared spectroscopy (FTIR). The FTIR spectra of both samples were analyzed through a Thermo Nicolet Nexus Fourier FT-IR spectrometer in the frequency range of 4,000–900cm^{-1} using a KBr disc method.

Scanning Electron Microscopy

Chitosan-coated magnetic nanoparticles (C-MNP), yeast cells, and their complexes with C-MNP were lyophilized and subjected to analysis on environmental scanning electron microscope (SEM) Brand Philips model XL-30 conditioned with a spectrometer Apollo Brand EDAX (USA). Each sample dispersed in ethanol was fixed on a polished bronze mirror holder and then sputtered with copper (99.97% purity). Sample drying was performed in a high vacuum evaporator JEOL model JEE400 at 0.00002 mbar, 18 mA for 15 s. Micrographs were taken under the following conditions: working distance at 39 mm, spot size at 4.5, and energy at 20 or 40 keV.

Strain Preservation and Inoculum Preparation

A yeast strain *S. cerevisiae*-150 donated by the Technological Institute of Durango (Durango, Mexico), was used. The strain was stored at 4°C in solid medium containing 20 g/L of glucose, 10 g/L of peptone, 10 g/L of yeast extract, and 15 g/L of agar.

To obtain the inoculum, the sample taken with a microbiological loop was transferred to 30 mL of synthetic medium (glucose 20 g/L, peptone 10 g/L, yeast extract 10 g/L). Fermentation was carried out for 18 h, at 150 rpm and 32°C until obtaining a concentration of 10^7 cells/mL.

Alcoholic Fermentation

In assay molasses from the Tamaulipas sugar industry was used. This molasses is characterized by pH 5.23 ± 0.01, 82.16 ± 1.16° Brix, ashes at 9.17 ± 0.09%, percentage of reducing sugars and nitrogen 66.34 ± 1.14% and 0.21 ± 0.06%, respectively. Anaerobic fermentation was carried out in 500 mL Erlenmeyer flasks using 300 mL of medium with cane molasses adjusted to 20° Brix using the solution with 1.0 g/L of KH_2PO_4, 1.59 g/L of $(NH_4)_2SO_4$, and 0.5 g/L of $MgSO_4 \times 7H_2O$. The medium was autoclaved in reactors prior to fermentation. The reactors were equipped with rubber caps, glass capillaries, and a CO_2 trap. The inoculum was applied at 10% (v/v) achieving a concentration of 10^6 cells/mL. Nanoparticles (C-MNP) were added to the inoculum 30 min before their addition to the fermentation medium, achieving the final concentration at 460 mg/mL. The yeast immobilization was confirmed by quantification of cells in the supernatant, using a Neubauer chamber and an optical microscope (Olympus), after the application of the magnetic field to the reactor. Fermentation was carried out for 42 h, at 150 rpm and 32°C. Sampling was done every 6 h.

Sugars and Ethanol Quantification

Sugars (glucose and fructose) and ethanol were quantified by HPLC (Waters) containing a quaternary pump (Waters, model 600E), a refractive index detector (Waters, model 410), an automatic injector (Waters, model 717), aminex software HPX column 87-P (Bio-Rad, Cambridge, MA) and Empower 3 software. The 5 mM H_2SO_4 solution was used as the mobile phase. The injection volume was 20 μL, the column temperature was 60°C, the flow rate was 0.65 mL/min, and the run time was in the range of 30 to 40 min. To perform the quantification, an 8-point calibration curve was constructed for the glucose, fructose and ethanol standards (Sigma, St. Louis, MO), injected in triplicate. All reagents were HPLC grade. The chromatographic peaks of the samples were identified by comparison with the retention times of the standards. The total sugar concentration was calculated by summing the individual sugar concentrations.

Statistical Analysis

ANOVA tests with a significance level at $p < 0.01$ were performed using software Statistica 7.0 (Stat Soft, Tulsa, OK, USA). Kinetic data were analyzed by ANOVA test and plotted in Excel with corresponding standard deviations. Subsequently, the nanoparticles coated with chitosan were applied in the alcoholic fermentation of molasses.

RESULTS AND DISCUSSION

Applying the one-step coprecipitation procedure proposed by Osuna et al. (2012) with modification by hydrothermal treatment in an autoclave, the dark brown materials susceptible to the effect of external magnetic field were obtained, which were characterized by different methods to verify that it is magnetic manganese ferrite nanoparticles with and without chitosan.

X-ray Diffraction and Particle Size

The XRD diffraction patterns of the manganese ferrite nanoparticles (Crystallography Open Database, Waerenborgh) are shown in **Figure 1**. The patterns show the reflection planes (111), (220), (311), (400), (511), (440) which confirm the

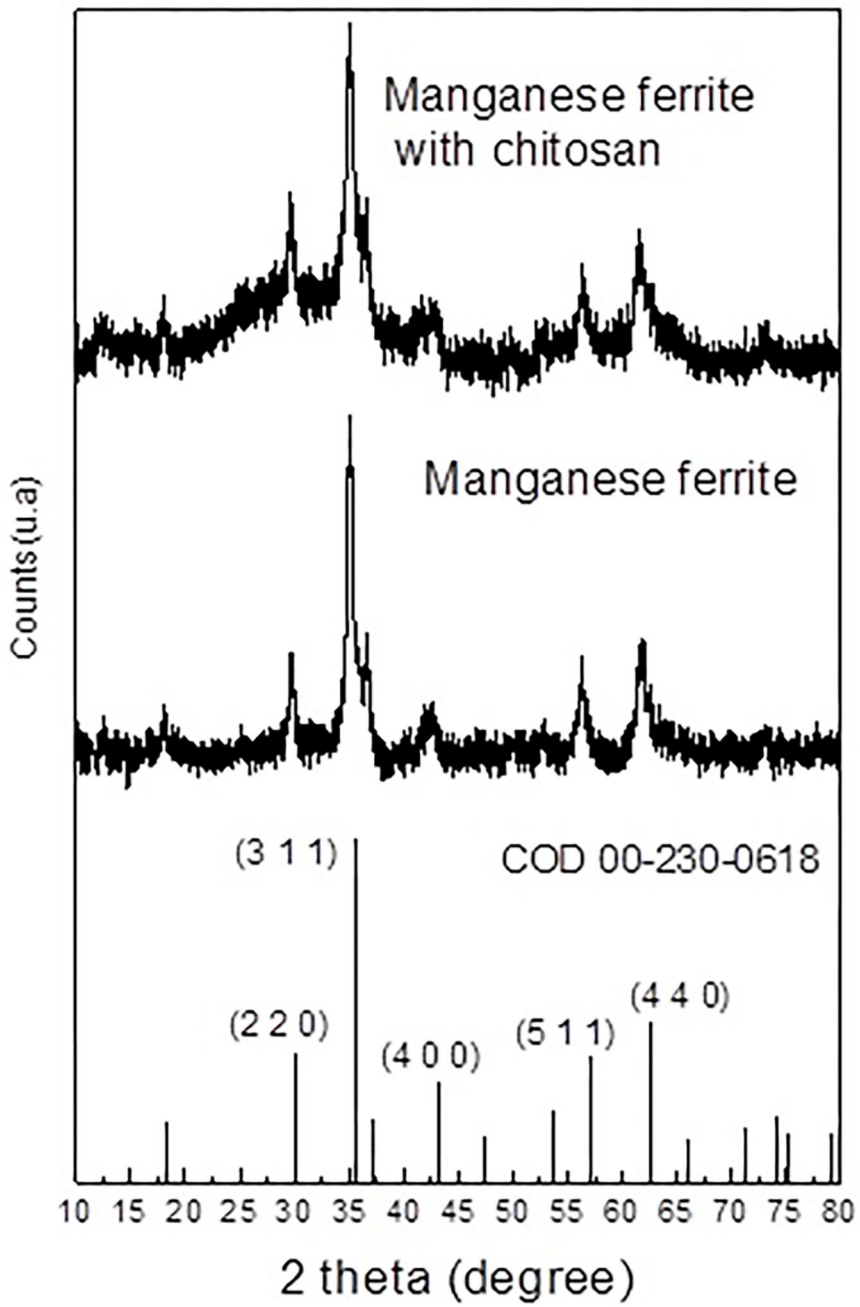

FIGURE 1 | X-ray patterns of magnetic nanoparticles prepared by one step coprecipitation method.

presence of single-phase $MnFe_2O_4$ with a face-centered cubic structure in nanoparticles with and without chitosan at 2⊖ (Goodarz et al., 2011; Moussaoui et al., 2016).

The average particle size was determined from Scherrer's equation. **Table 1** shows that the presence of chitosan during the synthesis of the nanoparticles favored the formation smaller particles in comparison with the ferrite synthesized in the absence of chitosan.

Goodarz et al. (2011) synthesized manganese ferrite nanoparticles using a heat treatment method followed by calcination. The crystallite diameter of the nanoparticles obtained was calculated as 12 nm. This value is less than quantified for manganese ferrite obtained in the present study (15.2 nm) and greater than that detected for nanoparticles coated with chitosan (11 nm). According to different authors (Fontijn et al., 1999; Colombo et al., 2012; He et al., 2013), the size and

TABLE 1 | Comparison of crystallite diameters quantified by Scherrer's equation for manganese ferrite obtained with and without chitosan.

Sample	Crystallite diameter quantified by Scherrer's equation
Chitosan-coated manganese ferrite	11.1 nm
Manganese ferrite	15.2 nm

properties of the nanoparticles strongly depend on the control of the operational parameters of the synthesis, such as the molar concentration of the solutions, the reaction temperature, the reaction rate, and the pH of the solution.

According to our knowledge, the method applied in the present study for the synthesis of chitosan-coated manganese ferrite nanoparticles was not previously reported. However, it was verified that the obtained material contains the nanoparticles of the compound of interest, as well as their crystallite size are comparable with the values reported in literature for other methods of their synthesis.

Magnetic Properties of Chitosan-Coated Manganese Ferrite

Figure 2 shows the hysteresis curves for chitosan-coated manganese ferrite. These curves were prepared using the averages of the magnetization values for the analyzed material and their replicas. A ferromagnetic behavior is seen, with a coercivity field at 185.52 ± 1.74 Oe, a saturation magnetization at 12.78 ± 0.43 emu/g, and a remanence at 5.6 emu/g. Saturation is a characteristic of magnetic materials. Magnetic saturation is the state reached in a sample when increases in the applied external magnetic field cannot further increase the magnetization of the material. At saturation, the total magnetic flux density does not increase with increases in applied external field. The field of coercivity refers to the intensity of the magnetic field necessary to bring the magnetization of the sample to zero once saturation is reached. The detected value of the coercivity field is lower than that reported in other publications (Goodarz et al., 2011, Irfán et al., 2012, Yang et al., 2010).

The value of magnetic saturation is 15.78 emu/g reported by Goodarz et al. (2011), 16.3 emu/g reported by Irfán et al. (2012), 21.2 emu/g reported by Yang et al. (2010). It is worth mentioning that the values reported in the literature correspond to nanoparticles without chitosan. The presence of chitosan increases the percentage of non-magnetic matter in the system obtained. This is the cause of lower magnetic saturation value. Considering that the magnetization in magnetic nanoparticles depends directly on the crystallinity (Osuna et al., 2012), the higher magnetization values could be improved with an improvement in the crystallinity of the magnetic nanoparticles.

Then, the obtained results demonstrate the ferromagnetic behavior, which means that after removing the external magnetic field, the system maintains a certain amount of magnification. Furthermore, the magnetic properties of chitosan-coated manganese ferrite are similar to those reported in literature, although a different method was applied for its synthesis.

C-MNP Characterization by Fourier Transform Infrared Spectroscopy

FTIR analysis was performed to identify the presence of chitosan functional groups. In **Figure 3**, the comparison of spectra of chitosan and chitosan-coated nanoparticles is presented. The broad band around 3,365 cm^{-1} (A) is assigned to the stretching of the O-H group of the macromolecular association (Osuna et al., 2012). The band at 2,925 cm^{-1} (B) is assigned to the CH_2-bond portion of the methylene groups, and the weak band at 2,856 cm^{-1} (C) is assigned to the -CH- bond of the methylene group that can be considered to be the characteristic peak of the chitosan structure. The characteristic bands at 1,709 cm^{-1} (D) and 1,633 cm^{-1} (E) can be assigned to the C = O groups of chitosan. The characteristic bands appearing at 1,570 cm^{-1} (F) correspond to the N-H bending vibration. The band at 1,321 cm^{-1} (G) is related to the stretching vibration C-N. Furthermore, the stretching vibration of C-O can also be found at 1,065 cm^{-1} (H) (Pavia et al., 1996). Thus, the FTIR results verify the presence of chitosan in the nanostructured material.

Scanning Electron Microscopy Characterization (SEM)

SEM micrographs of manganese ferrite (**Figure 4A**). **Figure 4B** shows *S. cerevisiae*-150 after 24 h of culture in cane molasses. The micrograph shows an approximate cell diameter of 2 to 4 μm that coincides with the literature data (López et al., 2016). **Figure 4C** shows the *S. cerevisiae* cells immobilized on the chitosan-coated manganese ferrite obtained after 24 h of culture in molasses.

The obtained micrographs are the evidence of the microorganism interaction with the nanoparticles. SEM analysis shows that the adsorption mechanism was not affected by the fermentation process. The micrographs are similar to those reported by Palacios et al. (2017), who immobilized *Kluyveromyces marxianus* in chitosan-coated magnetite. In both cases the nanoparticles are placed on the cell surface. However, unlike the SEM micrographs reported by Palacios et al. (2017) where the cells were completely covered with nanoparticles, in this study only a part of the cell surface interacted with the magnetic carrier, probably due to a lower C-MNP: cells ratio, due to increased cell concentrations during fermentation.

The nanostructured system can be described as an agglomerate of chitosan-coated nanoparticles, probably due to magnetic remanence (**Figure 2**). According to **Figure 4C**, this system covers the surface of the cells. Yeast cells with their almost spherical shape (**Figure 4B**) are partially covered with magnetic nanosystem (**Figure 4C**). Yeast interaction with C-MNP is evidenced by SEM micrographs.

Peng et al. (2010) reported SEM micrographs for *Saccharomyces cerevisiae* chemically immobilized on magnetite nanoparticles, which were functionalized with chitosan in the presence of glutaraldehyde in two-step methodology before interaction with yeast cells. Similarity is observed between the SEM micrographs presented in the present study and in the case of the report by Peng et al. (2010). The nanostructured system appears as agglomerates of many nanoparticles embedded in the chitosan matrix that were found around the cells. The surface of

the adsorbent had many minute structures between spaces. The author attributed them to reactions that occur on the surface of the particle. It is worth mentioning that in the present study the immobilization was carried out without glutaraldehyde, which leads to define the interaction between support and yeast, as adsorption without chemical bond only due to weak bonds between the support and the microorganism.

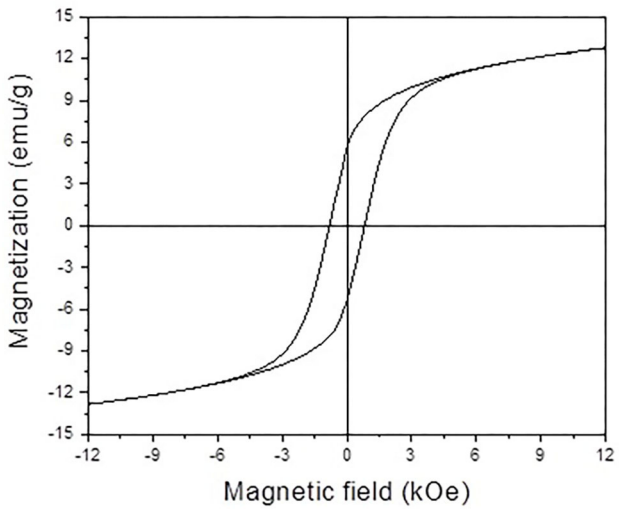

FIGURE 2 | Magnetization curves determined at room temperature for chitosan-coated manganese ferrite nanoparticles.

Alcoholic Fermentation

A concentration of C-MNP (460 mg/mL) was applied to carry out the alcoholic fermentation of cane molasses by immobilized *S. cerevisiae*, giving the ration 2×10^3 cells/mg. This concentration was selected because the count of not immobilized cells in supernatant after 30 min of incubation and mixing with culture medium, using the Neubauer chamber, showed total adsorption of cells on magnetic support, after it removal by the external magnetic field.

In fermentation, the support without chitosan was not applied because it did not show interaction with the cells, monitoring by cell count. The incubation time was selected based on results reported by Palacios et al. (2017), who show the pseudo-steady state after this period of time.

Figure 5 shows kinetics of sugar consumption in the presence of free and immobilized yeast. In the presence of C-MNP, total sugar consumption was observed at 24 h, while in the presence of free yeast at 42 h. This result agrees with those observed in the kinetics of ethanol production (**Figure 6**). This result agrees with those observed in the ethanol production kinetics (**Figure 6**). With the immobilized system, it was possible to obtain an ethanol concentration of 56.15 g/L. Productivity and yield in this case were 2.3 ± 0.2 g/(L * h) and 0.28 ± 0.03, respectively. However, at the same time in the fermentation with free yeast 39.1 g/L of ethanol were obtained. After 42 h, an ethanol titer was 50.7 ± 3.1, productivity and yield of 1.4 ± 0.3 g/(L * h) and 0.25

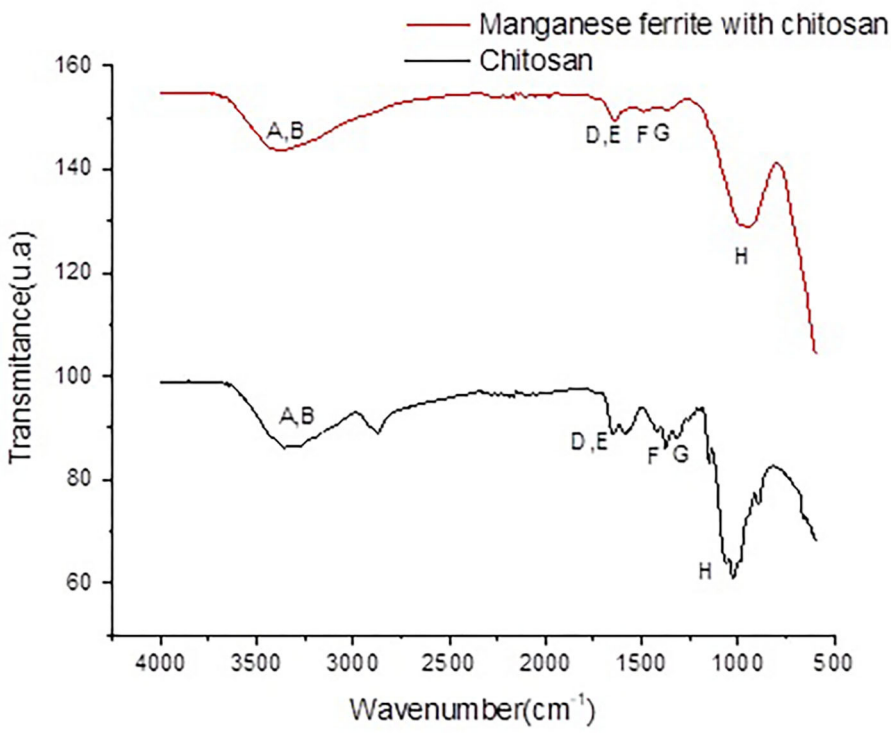

FIGURE 3 | FT-IR spectra of chitosan and chitosan-coated magnetic nanoparticles in the region between 900 and 4,000 cm^{-1}.

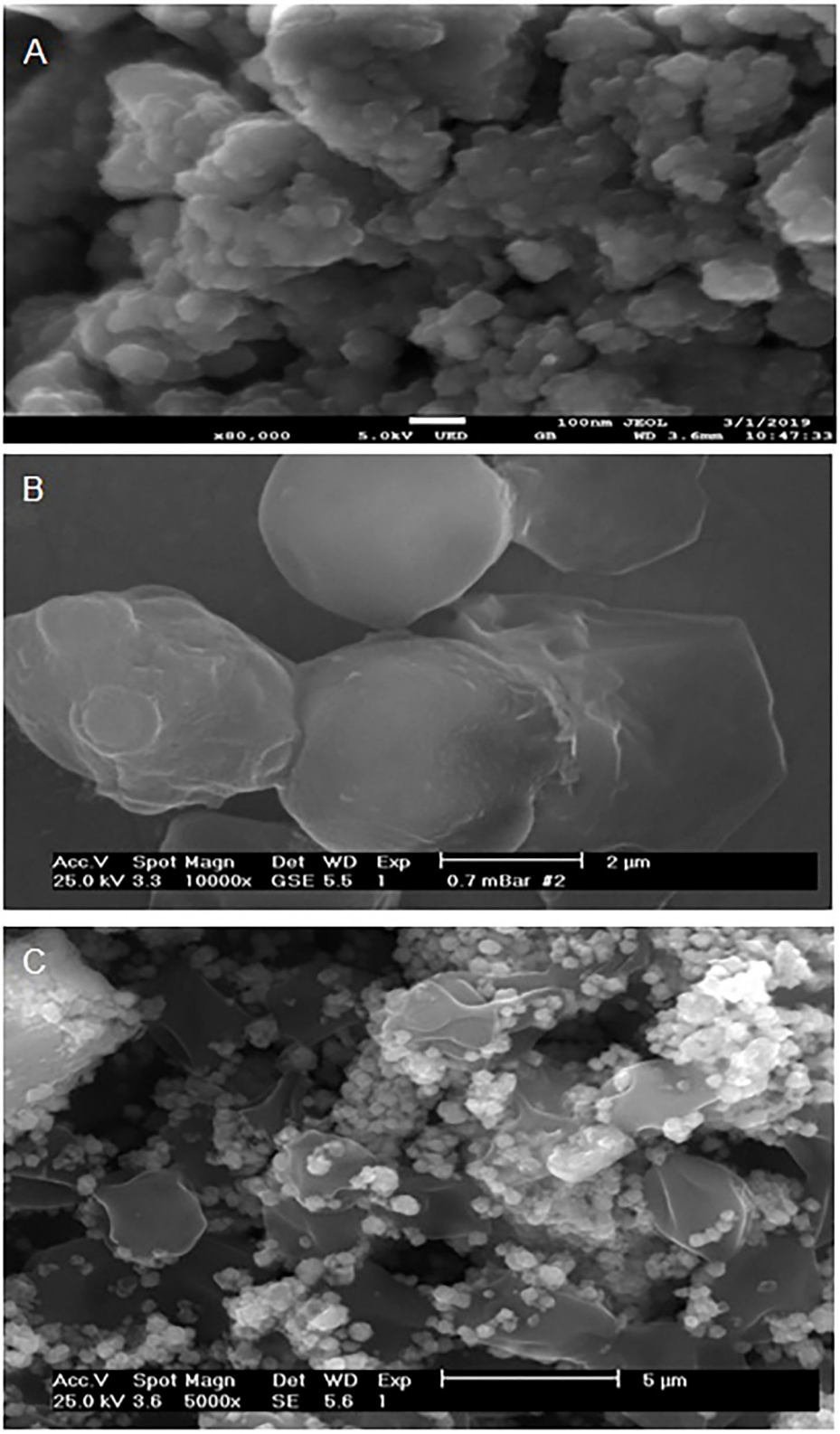

FIGURE 4 | SEM micrographs of manganese ferrite **(A)**, *S. cerevisiae* 150 **(B)**, *S. cerevisiae* 150 immobilized on C-MNP **(C)**.

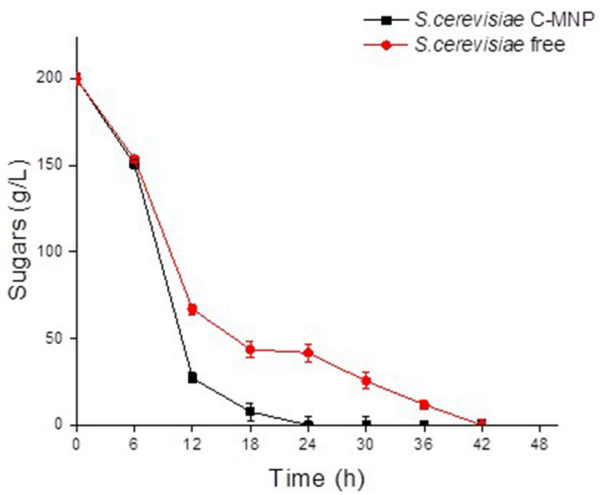

FIGURE 5 | Kinetics of sugar consumption during cane molasses fermentation by free and immobilized on C-MNP *S. cerevisiae*-150 (molasses at 20° Brix, at 150 rpm and 32°C).

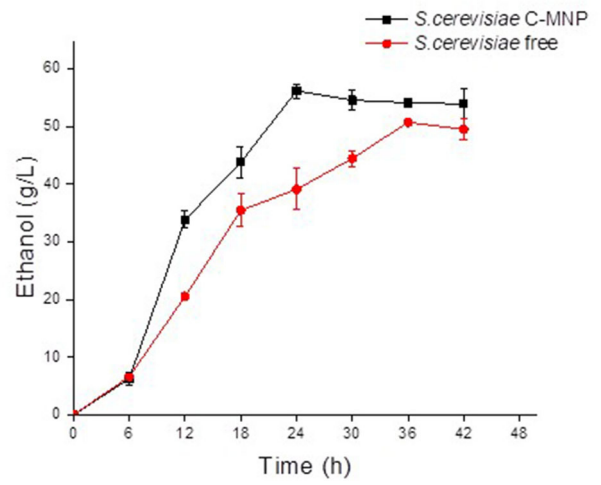

FIGURE 6 | Kinetics of ethanol production during cane molasses fermentation by free and immobilized on C-MNP *S. cerevisiae*-150 (molasses at 20° Brix, at 150 rpm and 32°C).

± 0.4, respectively. Therefore, a reduction in fermentation time, higher ethanol titer and productivity were demonstrated in the presence of nanoparticles. It is likely, that in the system with the agglomerated yeast cells coated with nanoparticles, an ethanol concentration gradient is achieved, preventing the inhibition of fermentation.

Nuanpeng et al. (2018) studied the influence of immobilization of *Saccharomyces* by entrapment in 2% sodium alginate gel on ethanol production, observing that immobilization favored ethanol production. Zheng et al. (2012) using *S. cerevisiae* immobilized in an alginate-based MCM-41 mesoporous zeolite to ferment sugar molasses, obtained 78.6 g/L as the highest ethanol concentration. The method commonly used for yeast immobilization is gel trapping because the cells are protected from inhibition by alcohol, the yeast can be separated from the fermentation medium and reused in several cycles (Bai et al., 2008). In this case, the gel pore size must be sufficient so as not to limit the diffusion of enzymes responsible for the hydrolysis of oligo- and polysaccharides.

Rattanapan et al. (2011) used immobilized yeast cells on thin-layer silk cocoons for fermentations of blackstrap molasses by the adsorption method. The initial concentration of sugars was 240 g/L at the beginning of the fermentation process, 80.6 g/L of ethanol were obtained at 33°C after 48 h of fermentation. Vučurović and Razmovski (2012) used sugar beet pulp as a support for the *S. cerevisiae* immobilization in the bioethanol production from 120 g/L of sugars at the beginning; 52.3 g/L of alcohol was obtained after 48 h of fermentation. The immobilized cells demonstrate several advantages in ethanol production, such as high cell density, easy separation from the medium, high substrate conversion, lower inhibition, short reaction time, and cell recycling. The immobilized system shows faster substrate consumption compared to the free system.

In the present study, the highest ethanol concentration reached was 57 g/L when yeast immobilized in C-MNP was used. The observed differences can be related to the characteristics of the strain applied in the fermentation, with a higher concentration of fermentable sugars. The characteristics of the immobilized systems, for example, a greater protection of the strain against inhibitory effects, for example, ethanol, may be another factor that influences the production of ethanol.

It is worth mentioning that the optimization of the ethanol production process with *S. cerevisiae* immobilized in C-MNP can be optimized in future studies by selecting the support concentration, as well as the operational conditions of the process.

CONCLUSIONS

By applying the one-step coprecipitation procedure with the use of hydrothermal treatment in an autoclave, the dark brown chitosan-coated manganese ferrite nanoparticles were obtained. The nature of the material was verified by XRD, showing a particle size of around 15.2 nm. The magnetic properties were confirmed on a vibrating sample magnetometer. The presence of chitosan was verified by the FT-IR assay. The obtained the manganese ferrites nanoparticles were applied in the fermentation of cane molasses. Immobilization was confirmed by SEM micrographs and cell free counting. In the presence of immobilized yeast, a reduction in fermentation time, higher ethanol titer, and productivity were demonstrated when compared with free yeast. The application of manganese ferrite nanoparticles shows a beneficial effect on ethanol production. In the future, research should be carried out focused on the task of defining the mechanism of C-MNP action, optimization of their use, and evaluating the reuse of immobilized biomass on manganese ferrite in the ethanol production process.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AI, RR, CA, and JM-H: conceptualization and supervision. AN and RR: methodology. AN, AI, RR, and JM-H: software. AI and RR: validation and data curation. AI, RR, and JM-H: formal analysis. AN and JG: investigation. AI, RR, MA-G, NS-C, and JM-H: resources. AN: writing—original draft preparation. AI, RR, CA, AF, JS-C, and JM-H: writing-review and editing. JS-C: visualization. JM-H: project administration. AI and JM-H: funding acquisition.

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Physicochemical Characteristics and Lactic Acid Bacterial Diversity of an Ethnic Rice Fermented Mild Alcoholic Beverage, *Haria*

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Haria, a rice fermented alcoholic beverage, is prepared and consumed by the vast number of Indian tribal people as a staple drink. Lactic acid bacteria are the dominant microbial community in this beverage. Participating lactic acid bacterial diversity in this beverage were determined by using PCR denaturing gradient gel electrophoresis (PCR-DGGE) as *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lysinibacillus* sp., *Lysinibacillus fusiformis*, and a group of uncultured *Bacillus* sp. The beverage was enriched with a significant amount of lactic acid (17.63 mg/g), acetic acid (0.18 mg/g), folic acid, thiamine, pyridoxine, ascorbic acid, linolenic acid, linoleic acid, palmitic acid, and oleic acid. The phytase activity in this beverage was shown highest (18.93 U/g) at the fourth day of fermentation. The beverage was also augmented with essential minerals like calcium, ferrous, magnesium, and sodium, whereas the quantity of chromium, lead, cobalt, and nickel were gradually decreased during the course of fermentation. Gas chromatography–mass spectrometry (GC-MS) analysis clearly revealed that three types of esters were produced during fermentation. This study clearly demonstrated that a group of lactic acid bacteria along with other microorganism provide a wide array of bioactive substances make this beverage more nutritious.

Keywords: lactic acid bacteria, DGGE, phytase, lactic acid, esters

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INTRODUCTION

Fermentation is one of the oldest technical skill of human for food preservation and processing. Ethnic fermented foods or beverage are defined as food products from locally available raw materials including grains, legumes, vegetables, milk, fish, and meat prepared by native people following their ancestral knowledge using very simple utensils. Cereal is the most common dietary substance preferred by all human community because of its high productivity (over 60%) and caloric value (Das et al., 2012). However, its nutritive value and the sensory properties are somehow poor due to presence of natural contaminants, antinutrients, inadequacy of essential amino acids, and fatty acids (Blandino et al., 2003). Microbial (either naturally or starter based) interaction during the course of fermentation transformed the cereal-based food stuffs biochemically and organoleptically into enriched one with adequate micronutrients (vitamins, minerals, amino acids, etc.), health beneficial edible microbes (i.e., probiotics), fermentable sugars (i.e., prebiotic),

dietary fibers, phytochemicals, digestive enzymes, and exerted many health promoting attributes. Considering these beneficial effects, the grain-based probiotic functional foods are becoming more popular than conventional dairy-based products in Japan and Europe (Nout, 2009).

Rice-based fermented beverages (generally regarded as rice beer) are very popular in the Asia-Pacific region in contrast to alcoholic beer from barley or other grains in Western countries (Steinkraus, 1998). During the rice fermentation, starch matrix supports the growth of specific group of microbes such as lactic acid bacteria (LAB), mold, and yeasts, and these enzymatically almost decomposed the complex substrate into assimilable form, and therefore, fermented beverage is more utilizable and has more health beneficial effects than any solid/semisolid-based fermented counterpart (Ray et al., 2016).

Haria is an inexpensive mild-alcoholic traditional rice fermented beverage, consumed as a staple food by the tribal and low-income groups of lateritic West Bengal, Orissa, Jharkhand, Bihar, Assam, and in many states of Central India (Ghosh et al., 2014, 2020). It has ethnomedicinal importance as a remedial agent for many degenerative and infectious diseases particularly against dysentery, diarrhea, amoebiasis, acidity, and vomiting (Ghosh et al., 2014). During its preparation, parboiled rice is mixed with a traditional starter tablet, popularly called as Bakhar (in Bengali), and kept in heat-sterilized earthen pot for 3–5 days for fermentation (Ghosh et al., 2014). After fermentation, the cream-colored butter-milk-like filtrate is consumed by the people. The starter tablet is unique, which consists of specific part of plants (without any old ferment) that blended with rice dust and shaped in a ball-like structure (∼10 g dry weight). The herbal constitute of the starter not only fortifies the rice with different nutraceuticals (phytochemicals) but also provides active microbes that participate in the fermentation. These endophytic microbes are generally safe for human consumption, and their quantitative involvement during the course of fermentation is almost constant, as ethnic people added a specific quantity of starter with the substrate (2–3 g of starter/200 g of rice).

In our earlier studies, it was observed that the dominant microbes in *haria* are lactic acid bacteria, bifidobacteria, molds, and yeasts (Ghosh et al., 2014, 2015a,b; Ray et al., 2016). However, the detection of the uncultured bacteria and the nutritional enrichment has not been studied so far. Considering the popularity of this beverage among the tribal groups of West Bengal, a complete portrait of *haria* should be evaluated. The present research focused on the application of culture-independent techniques to explore the total community structure of LAB during *haria* preparation. Simultaneously, reactive metabolites were also examined in relation to the course of fermentation.

MATERIALS AND METHODS

Sample Collection

Eighteen (three samples of each day and Bakhar) samples were collected from different villages of West Midnapore and Bankura districts of West Bengal, India. For PCR denaturing gradient gel electrophoresis (PCR-DGGE) analysis, one batch samples was collected randomly and then kept in sterilized container and transported into the laboratory in an ice box and stored in the laboratory at −20°C for further analysis.

Isolation of Total Genomic DNA Pool From *Haria*

Haria was initially diluted with sterile distilled water (1:10, w/v), then centrifuged at 1,000 rpm for 10 min to remove the solid particles, and the supernatant was taken. The supernatant was again centrifuged at 5,000 rpm, and the pellet was washed with 2 ml of buffer (pH 8.0) [100 mM Tris–HCl, 100 mM sodium ethylenediaminetetraacetic acid (EDTA), 100 mM sodium phosphate, 1.5 M NaCl]. The pellet was then suspended in 1,000 μl of buffer (pH 8.0) (100 mM Tris–HCl, 100 mM sodium EDTA, 100 mM sodium phosphate, 1.5 M NaCl). Thereafter, 50 μl proteinase K (10 mg/mL, Sigma) and 25 μl lysozyme (40 mg/ml, Sigma), 1 ml of 1% cetyl trimethylammonium bromide (CTAB) was added and incubated at 37°C for 30 min with inversion of content at 10-min interval. Then, 50 μl of sodium dodecyl sulfate (SDS) (10%) was added, and the mixture was incubated at 65°C for 1 h with gentle shaking in every 20-min interval following centrifugation at 8,000 rpm. The supernatant was collected, and equal volumes of phenol, chloroform, and isoamyl alcohol (25:24:1, v/v) were added. The aqueous phase was recovered after centrifugation at 10,000 rpm for 10 min, and DNA was precipitated with 0.6 volume of 2-isopropanol at 4°C for minimum of 30 min. It was centrifuged again at 10,000 rpm for 5 min, and the pellet was washed with 70% ice-cold ethanol and dissolved in 200 μl of TE buffer. The RNA was digested by adding 2 μl of RNase solution [10 mg of RNase (Sigma) dissolved in 1 ml of Milli-Q water] followed by a 30-min incubation at 37°C. The presence of DNA was verified on a 1% agarose gel.

PCR-DGGE Fingerprinting of LAB

Extracted DNA was used as a template for PCR amplification. A ∼340 bp of the 16S ribosomal RNA (rRNA) gene was amplified using LAB-specific bacterial primers Lact-F (5′-AGCAGTAGGGAATCTTCCA-3′) and Lact-R (5′-ATTYCACCGCTACACATG-3′) (Ritchie et al., 2010). GC clamp was anchored in the 5′ end of the reverse sequence. The PCR reaction mixture of 25 μl volume was prepared by using 2× PCR master mix for the required number of reactions. In brief, each reaction mixture contained 12.5 μl of PCR master mix, 1 μl of each primer (20 pmol), 1 μl of DNA template (50 ng/μl), and 9.5 μl of sterile Milli-Q water. Samples were amplified in a thermocycler using the following protocol: initial denaturation at 95°C for 5 min; 20 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min with an increase in temperature of 0.5°C in each cycle, extension at 72°C for 1 min; 10 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, extension at 72°C for 1 min; and final extension at 72°C for 7 min.

DGGE was performed with a DCode electrophoresis system (Bio-Rad) with gel dimension of 16 cm × 16 cm × 1 mm. PCR products were loaded onto a 38–53% gradient of urea and formamide in a polyacrylamide gel (8%) and electrophoresed at a

constant temperature of 60°C and a constant voltage of 60 V for 16 h. Gels were stained with ethidium bromide (0.5 mg/L) in TAE buffer for 20 min, destained in sterile deionized water for 10 min, and viewed by UV transillumination.

The visualized DGGE bands were excised from the original gel and incubated in 100 μl sterile distilled water at 4°C overnight. A 1-μl aliquot of elution was subjected to the PCR amplification using corresponding primers, without GC clamp. PCR products were excised from 1% agarose gel and purified with a HiPurA Quick Gel Purification Kit (HiMedia, India) and then subjected to sequencing. Forward and reverse DNA sequencing reaction of PCR amplicons were carried out with respective primers using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Texture Analysis

The tests were performed by a texture analyzer (TA-XT Plus, UK). A back extrusion test can provide the means to contain a volume of semisolid material to enable its testing. The vessel containing the sample was centrally located beneath a disk plunger, which then moved down into the sample and extrudes it up and around the edge of the disk. The diameter of the disk was 35 mm, and 75% of the sample holder was filled with test sample. When a 5-g surface triggered was attained (i.e., the point at which the disks lower surface was in full contact with the product), the disk proceeds to penetrate to a depth of 25 mm. At this point, the probe was allowed to return to its original position. The force area of the curve up to this point was taken as a measurement of consistency, and force at this point was the indication of firmness. The maximum negative force was recorded to indicate the cohesiveness.

Viscosity

Solid free liquid was extracted by centrifugation of fermented materials at 5,000 rpm for 5 min. The apparent viscosity was measured on a Bohlin CVO rheometer (Malvern Instrument, Malvern, UK) with a cone and plate geometry (CP 2°/20 mm diameter) maintaining a gap of 70 μm. Viscosity measurements were carried out on beverages previously adapted at 25°C for 1 h.

Analysis of Organic Acids Content

Water/salt-soluble extract from *haria* was prepared following the method of Ghosh et al. (2015a). Briefly, 10 g of *haria* was diluted with 30 ml of 50 mM Tris–HCl (pH 8.8), kept at 4°C for 1 h, vortexing at 15-min intervals, and centrifuged at 20,000 × g for 20 min. The supernatant, containing the water/salt-soluble fraction, was filtered through a Millex-HA 0.22-mm pore size filter (Millipore Co., Bedford, MA) and used for analyses. The extracts were analyzed by high-performance liquid chromatography (HPLC) using an Agilent HPLC system (Agilent Technology, 1200 infinity series). Organic acids were determined by using Zorbax SB C18 column, and elution was carried out at 60°C, with a flowrate of 0.6 ml/min, using 10 mM H_2SO_4 as mobile phase (Coda et al., 2011).

Determination of Hydrosoluble Vitamins

Hydrosoluble vitamins were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) as mentioned by Ghosh et al. (2015b).

Fatty Acid Analysis

Approximately, 20 mg of oven-dried samples (added 100 μg heptadecanoic acid as internal standard) was suspended in 1 ml of 5% methanolic solution of potassium hydroxide and saponified at 70°C for 1 h. Then, the pH of the mixture was adjusted to 2 with HCl. A mixture of 1 ml water and 1 ml chloroform was added, and the samples were shaken vigorously. The chloroform phase was collected after the centrifugation (4,000 rpm, 4°C, 15 min), and the extraction was repeated again from the upper phase with 1 ml chloroform. The collected chloroform phases were pooled and evaporated under stream of nitrogen. One milliliter of 14% methanolic solution of boron-trifluoride was added to the saponified samples and incubated at 70°C for 1.5 h. The fatty acid methyl-esters (FAMEs) were partitioned with hexane, which was evaporated to dryness under nitrogen. Finally, the derivatized samples were reconstituted in 100 μl hexane before chromatographic analysis.

The extracted samples were analyzed by gas chromatography (Agilent Technology, 6890N GC), equipped with a flame ionization detector (FID). A capillary column, HP-Innowax (60 m × 0.25 mm × 0.5 μm) was used. The temperature of the injector and detector was both set to 250°C. The oven temperature was held at 50°C, for 2 min, then programmed to rise from 50 to 200°C, at 20°C/min, and from 200 to 240°C at 3°C/min, and finally held at 50 min at 240°C. Nitrogen was used as carrier gas.

GC-MS Analysis

The ripened fermented material (fourth day) was extracted with dichloromethane and then analyzed by following the method of Ghosh et al. (2015a) in a gas chromatography–mass spectrometry (GC–MS) using a PerkinElmer Clarus 600C mass spectrometer equipped with a split/splitless injector and a flame ionization detector (FID). The compounds were identified by Perkin-Elmer inbuilt NIST mass spectral library.

Analysis of Organic Acids Content

Water/salt-soluble extract from *haria* was prepared following the method of Ghosh et al. (2015a). Briefly, 10 g of *haria* was diluted with 30 ml of 50 mM Tris–HCl (pH 8.8), kept at 4°C for 1 h, vortexing at 15-min intervals, and centrifuged at 20,000 × g for 20 min. The supernatant, containing the water/salt-soluble fraction, was filtered through a Millex-HA 0.22-mm pore size filter (Millipore Co., Bedford, MA) and used for analyses. The extracts were analyzed by high-performance liquid chromatography (HPLC) using an Agilent HPLC system (Agilent Technology, 1200 infinity series). Organic acids were determined by using Zorbax SB C18 column, and elution was carried out at 60°C, with a flowrate of 0.6 ml/min, using 10 mM H_2SO_4 as mobile phase (Ghosh et al., 2015b).

Phytase Activity

Phytase activity in the ferment was measured according to the method of Shimizu (1992) with slight modification. The reaction mixture, containing 150 μl of extract and 600 μl of substrate (3 mM Na-phytate in 0.2 M Na-acetate, pH 4.0), was incubated at 45°C. The reaction was stopped by adding 750 μl of 5% trichloroacetic acid. The released inorganic phosphate was measured spectrophotometrically at 700 nm by adding 750 μl of freshly prepared color reagent. The coloring reagent composed of four volumes of 1.5% (w/v) ammonium molybdate in 5.5% (v/v) sulfuric acid solution and one volume of 2.7% (w/v) ferrous sulfate solution. One unit (U) of phytase activity was defined as the amount of enzyme required to liberate 1 nmol of phosphate per minute under the assay condition.

Determination of Free Minerals

The contents of free minerals including heavy metals in fermented and unfermented rice were determined by using an atomic absorption spectrophotometer (AAS) [Shimadzu Analytical (India) Pvt. Ltd] following the method described by Ghosh et al. (2015b). Briefly, rice products (5 g) were dissolved in 25 ml of deionized water and homogenized. Then, it was centrifuged at 12,000 rpm for 10 min, and the supernatant was used for analysis.

Statistical Analysis

All the laboratory experiments were carried out for five times, and the values were represented as the mean ± standard deviation (SD). Data were statistically analyzed using one-way ANOVA and the Duncan's multiple range test in Sigma Plot 11.0 (USA) to determine the significant relationship between the means.

RESULTS

PCR-DGGE Fingerprinting of Lab

PCR-DGGE fingerprinting of LAB in *haria* is shown in **Figure 1**. Using the NCBI homology search, the sequenced DGGE bands were analyzed to identify the corresponding LAB with higher than 97% nucleotide sequence identities (**Table 1**). Four different genus were observed in *haria*: *Lactobacillus* sp. (bands a, c, h), *Lactiplantibacillus* sp. (band b), *Lysinibacillus* sp. (bands d–f), and the uncultured *Bacillus* sp. (g, i). The starter tablet, Bakhar, contained six types of bacteria (a–f). The "g" and "i" bands reflected the uncultured *Bacillus* sp., and the "a" and "c" were *Lactobacillus brevis*. The "d," "e," and "f" bands were *Lysinibacillus fusiformis*, *Lysinibacillus* sp., and uncultured *Lysinibacillus* sp., respectively. It is intriguing to note that the *Lysinibacillus* sp. was not survived in rice during fermentation as evidenced by the disappearance of "e" band after the first day. On the other hand, *Lactiplantibacillus argentoratensis*, uncultured *Lactobacillus* sp., and uncultured *Bacillus* sp. were gradually mounted during the course of fermentation as evidenced by the dominancy of "b," "g," and "i" (**Figure 1**).

Texture Analysis

Textural properties of the residual rice granules before and after fermentation were measured by a texture analyzer (TA-XT

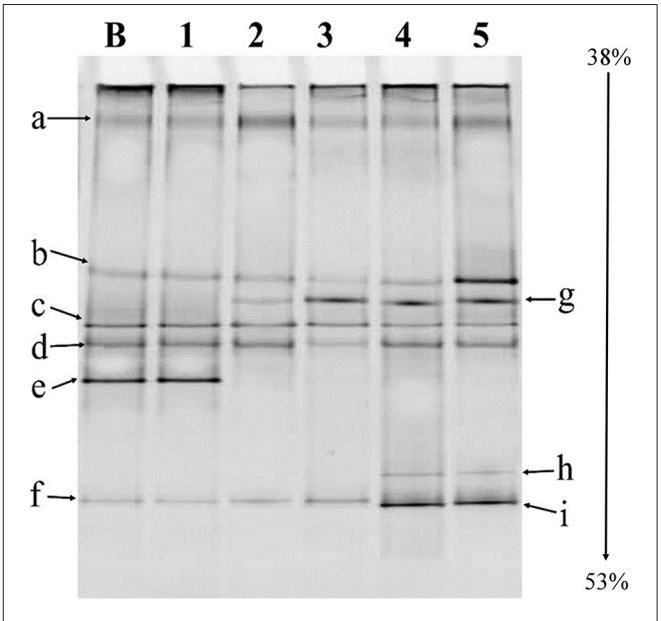

FIGURE 1 | PCR denaturing gradient gel electrophoresis (PCR-DGGE) fingerprinting of lactic acid bacteria (LAB). B, Bakhar; 1, first day fermented material (FM); 2, second day FM; 3, third day FM; 4, fourth day FM; 5, fifth day FM.

TABLE 1 | Species-identification of the fragments excised from PCR-DGGE shown in **Figure 1**.

Band	Closest relative	No. of nucleotides sequenced	% Identity	Accession no.
a	*Lactobacillus brevis* (MT613460.1)	333	99.70 %	KU248768
b	*Lactiplantibacillus argentoratensis* (LC620497.1)	321	100.0 %	KU248769
c	*Lactobacillus brevis* (MT640328.1)	335	99.40 %	KU248770
d	*Lysinibacillus fusiformis* (MT605500.1)	345	98.82 %	KU248771
e	*Lysinibacillus* sp. (MT541001.1)	330	100.00 %	KU248772
f	Uncultured *Lysinibacillus* sp. (KR140152.1)	364	97.32 %	KU248773
g	Uncultured bacterium clone (FJ930351.1)	356	99.14 %	KU248774
h	Uncultured *Lactobacillus* sp. (KU991825.1)	345	99.71 %	KU248775
i	Uncultured *Bacillus* sp. (HM152600.1)	334	99.40 %	KU248776

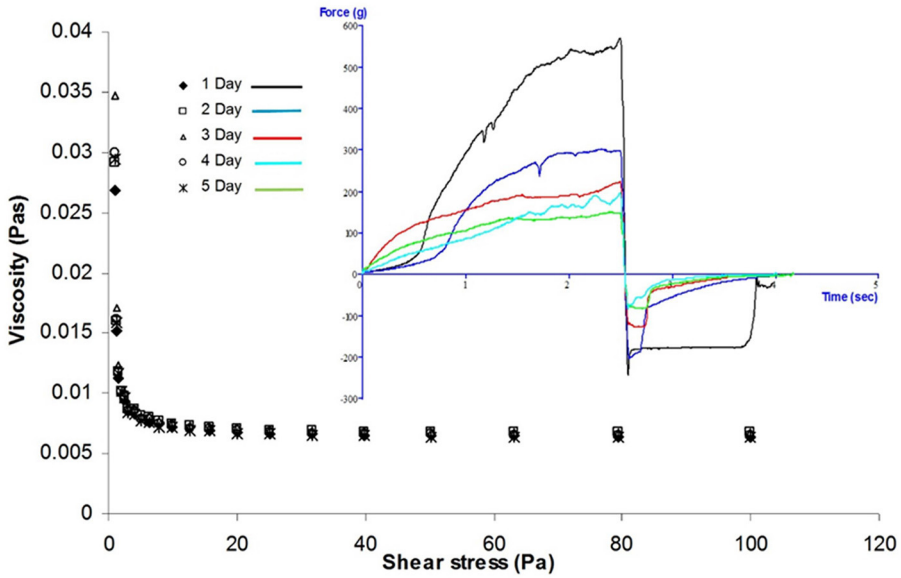

FIGURE 2 | Changes of texture and apparent viscosity during *haria* preparation.

TABLE 2 | Lactic acid, acetic acid, and hydrosoluble vitamins content of haria.

Fermentation time (day)	Organic acid content (mg/g)		Vitamin content/100 g				
	Lactic acid	Acetic acid	Folic acid (μg)	Thiamine (mg)	Riboflavin (μg)	Pyridoxine (μg)	Ascorbic acid (μg)
0	1.11 ± 0.12^{f}	0.02 ± 0.01^{e}	32.14 ± 1.21^{e}	0.43 ± 0.04^{e}	72.03 ± 0.81^{a}	3.23 ± 0.09^{f}	0^{f}
1	4.63 ± 0.58^{e}	0.06 ± 0.01^{d}	180.83 ± 3.03^{c}	0.68 ± 0.18^{e}	39.18 ± 0.39^{b}	93.65 ± 0.92^{e}	93.46 ± 1.26^{e}
2	8.11 ± 0.61^{d}	0.09 ± 0.02^{cd}	268.33 ± 5.28^{a}	1.09 ± 0.26^{d}	28.81 ± 0.24^{c}	211.32 ± 2.36^{d}	252.31 ± 3.33^{d}
3	11.55 ± 0.80^{c}	0.11 ± 0.03^{bc}	201.28 ± 4.91^{b}	2.35 ± 0.33^{c}	20.31 ± 0.21^{d}	346.35 ± 4.08^{c}	428.62 ± 4.39^{c}
4	13.12 ± 0.83^{b}	0.13 ± 0.03^{b}	179.43 ± 4.48^{c}	2.93 ± 0.33^{b}	18.44 ± 0.21^{e}	432.81 ± 4.69^{b}	512.45 ± 6.02^{b}
5	17.63 ± 0.83^{a}	0.18 ± 0.04^{a}	155.83 ± 4.01^{d}	3.41 ± 0.38^{a}	16.31 ± 0.19^{f}	481.62 ± 5.02^{a}	538.24 ± 6.51^{a}

Values are the mean (±SD) of lactic acid ($F = 392.581$; $df = 5, 24$; $P < 0.001$), acetic acid ($F = 23.225$; $df = 5, 24$; $P < 0.001$), folic acid ($F = 1825.768$; $df = 5, 24$; $P < 0.001$), thiamine ($F = 100.977$; $df = 5, 24$; $P < 0.001$), riboflavin ($F = 13{,}509.267$; $df = 5, 24$; $P < 0.001$), pyridoxine ($F = 15{,}574.409$; $df = 5, 24$; $P < 0.001$), ascorbic acid ($F = 13{,}743.334$; $df = 5, 24$; $P < 0.001$). Values within a column followed by different superscripts are significantly different according to ANOVA (Duncan's multiple range tests).

Plus, UK). From the force vs. time graph, firmness, consistency, and cohesiveness were calculated. Firmness, consistency, and cohesiveness were measured from maximum positive force, mean positive area, and maximum negative force of the curve, respectively. The firmness of *haria* was gradually decreased from 570.1 g force (first day) to 147.9 g force (fifth day) (**Figure 2**). Consistency and cohesiveness were also gradually decreased during fermentation, and their value was 272.745 and 84.5 g force, respectively, at the end of the fermentation.

Viscosity

Viscosity of the fermented soup after separation of the rice granule was measured on a Bohlin CVO rheometer. From the graph, it was observed that viscosity of the fermented liquid was gradually increased up to third day (0.0347 Pa s) and then decreased (0.0294 Pa s at fifth day) (**Figure 2**). The decrease in the apparent viscosity of the *haria* with the increase in shear rate at 25°C temperatures evaluated that the fluid has shear thinning characteristics. Hence, this is non-Newtonian shear thinning fluid.

Organic Acid Content

The organic acids content of the fermented material was quantified by HPLC, and it was observed that the concentration of lactic acid (17.63 mg/g) and acetic acid (0.18 mg/g) was accumulated in highest level on the fifth day of fermentation (**Table 2**).

Vitamin Content

Haria contains different types of hydrosoluble vitamins. Folic acid, thiamine, pyridoxine, and ascorbic acid contents were relatively higher in the ripened material than the unfermented one. Folic acid content was highest on the second day (268.33 μg/100 g) and then declined. Besides, thiamine (3.41 mg/100 g), pyridoxine (481.62 μg/100 g), and ascorbic acid (538.24 μg/100 g) content gradually increased during the course

of fermentation (**Table 2**). On the other hand, riboflavin content gradually decreased during fermentation.

Fatty Acid Analysis

Dietary fatty acids play a significant role in the daily caloric intake of the human population in India. Health professionals worldwide recommend a reduction in the overall consumption of trans-fatty acids (TAs) and cholesterol while emphasizing the need to increase intake of n-3 polyunsaturated fats (Daley et al., 2010). The fatty acids profile of *haria* has been tabulated in **Table 3**. It was found that palmitic acid (C16:0) was the most abundant saturated fatty acid present in *haria*. The amount was gradually increased up to the third day (18.84 μg/ mg) and then declined. Oleic acid (C18:1) content reached the highest level of 14.11 μg/mg on the second day. *Haria* also contained two essential fatty acids: linolenic acid (13.51 μg/mg) and linoleic acid (0.39 μg/mg).

Phytase Activity and Determination of Minerals

Phytase activity in *haria* reached the highest level at fourth day (18.93 U/g) and then declined (**Figure 3**). Simultaneously, it was observed that the quantities of Ca^{++} (0.77 ppm on second day), Fe^{++} (0.43 ppm on fifth day), Mg^{++} (5.42 ppm on third day), Mn^{++} (0.81 ppm on first day), and Na^{+} (0.99 ppm on third day) were profoundly higher than the unfermented rice (**Table 3**). On the other hand, Cu^{++} and Cr^{+++} contents gradually declined during the fermentation, and their quantities were 0.21 and 0.04 ppm, respectively, in the consumable product (**Table 3**). Pb^{++}, Co^{++}, and Ni^{++} were not detected in the mature product.

GC-MS Analysis

The type and occurrence of volatile compounds were analyzed in GC-MS, which was equipped with Perkin-Elmer inbuilt NIST mass spectral library. Eleven types of volatile compounds were detected in *haria* by GC-MS (**Table 4**). These are dichloroacetic acid heptadecyl ester; bicyclo (3.3.1) non-2-ene; ethanone,1-(2,4,6-trihydrophenyl); chloroacetic acid, dodecyl ester; 2h-indene3,3a,4,5,6,7-hexahydro; mucoinisitol, hexaacetate; chloroacetic acid, tetradecyl ester; 1-acetyl-3-(6-methyl-3-pyridyl) pyrazoline; epi-inositol, hexaacetate; N-(3-methyl-4-propionylphenyl) acetamide; and 1h-indene,2,3,4,5,6,7-hexahydro.

DISCUSSION

PCR-DGGE technique for the first time applied by ben Omar and Ampe (2000) to study the community structure of LAB in Mexican fermented maize dough Pozol. In this study, a LAB-specific primer was used to amplify the specific gene of LAB community only in *haria*, and thereafter, DGGE fingerprint has been constructed (**Figure 1**). It was observed that *L. brevis*, *L. argentoratensis*, and *L. fusiformis* present throughout the fermentation in *haria*. *Lysinibacillus* sp. is an uncommon group originated from starter, and this may be associated with the rice dust. Other participating microbes in ferment are endophytic in origin and derived from the starter.

TABLE 3 | Fatty acid and mineral content in haria.

Fermentation time (day)	Fatty acid concentration (μg/mg of dry sample)				Mineral content (ppm)									
	Palmitic acid	Oleic acid	Linolenic acid	Linoleic acid	Calcium	Ferrous	Magnesium	Manganese	Sodium	Copper	Chromium	Lead	Cobalt	Nickel
0	3.46 ± 0.28^{d}	4.82 ± 0.59^{e}	4.36 ± 0.22^{e}	0.33 ± 0.04^{b}	0.45 ± 0.03^{d}	0.07 ± 0.01^{e}	4.15 ± 0.23^{d}	0.06 ± 0.01^{e}	0.52 ± 0.02^{e}	0.33 ± 0.03^{a}	0.43 ± 0.03^{a}	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
1	5.70 ± 0.54^{c}	5.07 ± 0.64^{e}	4.90 ± 0.39^{e}	0.35 ± 0.04^{ab}	0.64 ± 0.04^{b}	0.19 ± 0.02^{d}	4.92 ± 0.30^{c}	0.81 ± 0.04^{a}	0.83 ± 0.05^{c}	0.23 ± 0.02^{c}	0.11 ± 0.01^{b}	ND	ND	0.01 ± 0.01
2	5.84 ± 0.62^{c}	14.11 ± 1.21^{a}	13.51 ± 1.36^{a}	0.39 ± 0.04^{a}	0.77 ± 0.07^{a}	0.23 ± 0.02^{c}	5.34 ± 0.32^{ab}	0.51 ± 0.03^{b}	0.92 ± 0.06^{b}	0.27 ± 0.03^{b}	0.06 ± 0.04^{cd}	ND	ND	ND
3	18.84 ± 1.48^{a}	11.96 ± 1.15^{b}	11.09 ± 1.54^{b}	0.35 ± 0.04^{ab}	0.69 ± 0.05^{b}	0.38 ± 0.03^{b}	5.42 ± 0.31^{a}	0.32 ± 0.02^{c}	0.99 ± 0.06^{a}	0.26 ± 0.03^{b}	0.08 ± 0.03^{c}	ND	ND	ND
4	16.73 ± 1.29^{b}	9.51 ± 1.02^{c}	9.05 ± 1.39^{c}	0.28 ± 0.03^{bc}	0.53 ± 0.04^{c}	0.41 ± 0.03^{ab}	5.02 ± 0.30^{b}	0.01 ± 0.01^{d}	0.81 ± 0.05^{c}	0.21 ± 0.02^{c}	0.06 ± 0.03^{cd}	ND	ND	ND
5	15.72 ± 1.24^{b}	6.63 ± 0.68^{d}	6.51 ± 0.33^{d}	0.26 ± 0.03^{c}	0.51 ± 0.04^{cd}	0.43 ± 0.04^{a}	4.64 ± 0.21^{c}	ND	0.75 ± 0.06^{d}	0.21 ± 0.02^{c}	0.04 ± 0.02^{d}	ND	ND	ND

Values are the mean (±SD) of palmitic acid ($F = 222.693$; $df = 5, 24$; $P < 0.001$), oleic acid ($F = 86.861$; $df = 5, 24$; $P < 0.001$), linolenic acid ($F = 61.047$; $df = 5, 24$; $P < 0.001$), linoleic acid ($F = 8.585$; $df = 5, 24$; $P < 0.001$), calcium ($F = 33.931$; $df = 5, 24$; $P < 0.001$), ferrous ($F = 145.047$; $df = 5, 24$; $P < 0.001$), magnesium ($F = 13.975$; $df = 5, 24$; $P < 0.001$), manganese ($F = 882.016$; $df = 5, 24$; $P < 0.001$), sodium ($F = 49.012$; $df = 5, 24$; $P < 0.001$), copper ($F = 16.128$; $df = 5, 24$; $P < 0.001$), and chromium ($F = 138.500$; $df = 5, 24$; $P < 0.001$). Values within a column followed by different superscripts are significantly different according to ANOVA (Duncan's multiple range tests). ND, not detected.

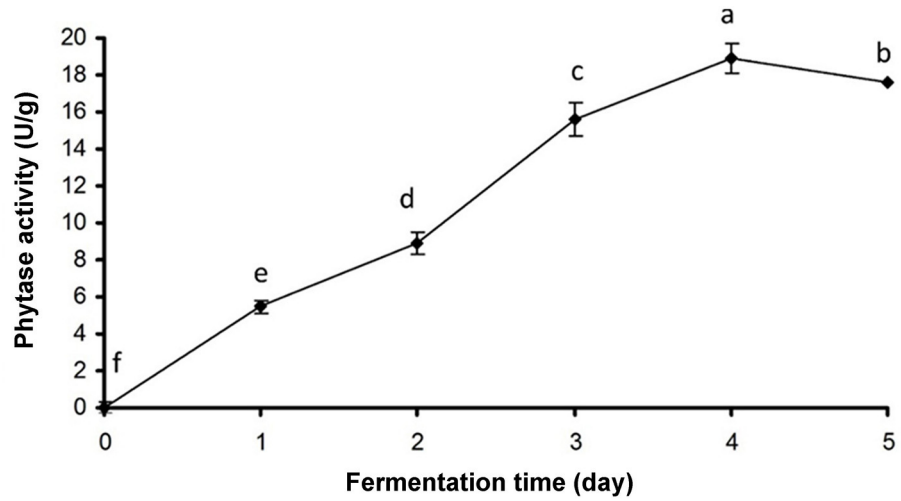

FIGURE 3 | Phytase activity of *haria*. Bars represent SD values of replicates. Values are the mean (±SD) of phytase activity ($F = 851.341$; df = 5, 24; $P < 0.001$). Different letters on the bars are significantly different ($P < 0.001$) in Duncan's multiple range test.

LAB are considered as health beneficial probiotic organism and are emerging for present day's biotherapeutics. The multistrain and multispecies lactic acid bacterial composition makes this beverage more health beneficial. Kim et al. (2010) identified *L. plantarum*, *Leuconostoc pseudomesenteroides*, *L. paracasei*, *L. harhibinensis*, and *L. parabuchneri* in a Korean rice wine "takju" analyzed by using PCR-DGGE technique. Osimani et al. (2015) found different types of LAB by using PCR-DGGE analysis, such as *Weissella confusa*, *Weissella oryzae*, *Leuconostoc citreum*, *Lactococcus lactis*, *Pediococcus parvulus*, *Pediococcus ethanolidurans*, and *L. casei* in boza, in a cereal-based fermented beverage of Bulgaria. Bhaati Jaanr, a similar type of rice beverage in India, also contain *L. bifermentans* (Tamang and Thapa, 2006). Marsh et al. (2014) stated that the room temperature at initial stage of rice fermentation favors the growth of mesophilic bacteria such as *Lactococcus* and *Leuconostoc*, whereas in the later stage, when the temperature of the fermented material increased due to microbial interaction/metabolism, thermophilic bacteria such as *Lactobacillus* and *Streptococcus* are augmented (Marsh et al., 2014). This may be one of the reasons for phasic occurrence of diverse groups of LAB in the *haria* fermentation.

The decrease in firmness of *haria* was observed as the fermentation proceeded (**Figure 2**). The gel firmness might be caused by retrogradation of starch gels, which could be associated with the syneresis of water and crystallization of amylopectin leading to harder gels (Miles et al., 1985). Similarly, the value of the consistency and cohesiveness were decreased during fermentation; hence, it can be stated that the starch gel became thinner either the action of amylolytic enzymes or the granules took up the moisture during fermentation (Hor et al., 2021). It has already reported that *haria* contains amylolytic enzymes such as α-amylase and gluco-amylase (Ghosh et al., 2015a). The participating microbes secreted sufficient amylase, which can degrade the starch granule and thus reduce the consistency and cohesiveness of the fermented material.

The extent release of starch from rice granule depends on acidity of the medium, composition (amylose/amylopectin ratio), structure of the granule, degree of gelatinization, and swelling power. Lower pH and enzyme (amylase) produced by the microorganisms during fermentation (Ghosh et al., 2014, 2015a) facilitated the release of starch fraction from rice granule, and hence, viscosity was gradually increased up to the third day (**Figure 2**). However, the large starch granule degradation and sugar consumption rate (by alcohol producing yeast and other microorganisms) was higher than the amylose release rate; therefore, viscosity of the material was decreased gradually after the third day.

Most LAB are able to metabolize glucose or starch by homolactic or heterolactic fermentation to produce lactic acid, which further creates the unfavorable condition for the growth of pathogenic organisms (Lv et al., 2012). In our earlier study, it was observed that the pH of the fermented material was gradually decreased, and at the end of the fermentation (fifth day), the pH was 3.55 (Ghosh et al., 2014). The decrease in pH during fermentation is likely due to utilization of free sugars (Efiuvwevwere and Akona, 1995; Mugochi et al., 2001) and accumulation of organic acids (e.g., lactic acid and other) during the course of microbial growth (Nyanga et al., 2008). It was observed that the concentration of lactic acid and acetic acid increased concurrently and reached the highest level on the fifth day (**Table 2**). Puerari et al. (2015) found 1.4 g/L of lactic acid in chicha, a rice-based fermented beverage produced by Umutina Brazilian Amerindians. Lactic acid has several health beneficial effects like immune stimulatory, cholesterol lowering, inducer for endocrine secretion, stress remover, and brain stimulating and show protective roles for intestinal mucosa, flora, and overall intestinal functions (Ghosh et al., 2014).

TABLE 4 | GC-MS analysis of sugar derivatives produced during the course of *Haria* fermentation.

Retention time (min)	Compound name	Formula	Expected MW (kDa)
8.44	Dichloroacetic acid heptadecyl ester	$C_{19}H_{36}O_2Cl_2$	366
8.854	Bicyclo(3.3.1)non-2-ene	C_9H_{14}	122
11.14	Ethanone,1-(2,4,6-trihydrophenyl)	$C_8H_8O_4$	168
13.74	Chloroacetic acid, dodecyl ester	$C_{14}H_{27}O_2Cl$	262
14.46	2H-indene3,3a,4,5,6,7-hexahydro	C_9H_{14}	122
16.16	Mucoinisitol, hexaacetate	$C_{18}H_{24}O_{12}$	432
19.68	Chloroacetic acid, tetradecyl ester	$C_{16}H_{31}O_2Cl$	290
19.817	1-Acetyl-3-(6-methyl-3-pyridyl)pyrazoline	$C_{11}H_{130}N_3$	203
22.08	Epi-inositol, hexaacetate	$C_{18}H_{24}O_{12}$	432
27.01	N-(3-methyl-4-propionylphenyl)acetamide	$C_{12}H_{15}O_2N$	205
28.21	1H-indene,2,3,4,5,6,7-hexahydro	C_9H_{14}	122

The best fitted compounds were selected according to Perkin-elmer inbuilt NIST mass spectral library and analyses by GC-MS TurboMass software detected in the GC-MS.

Rice contains different types of vitamins, but these vitamins are easily destroyed during processing such as milling, cooking, etc. During fermentation of cereals, the participating microbes, mainly LAB, are able to produce vitamins specifically the B vitamins (riboflavin, folic acid, etc.) (Capozzi et al., 2012). *Haria* contains different types of hydrosoluble vitamins (folic acid, thiamine, pyridoxine, and ascorbic acid), and their amounts were comparatively higher than the unfermented rice (**Table 2**). Therefore, it can be argued that microbial interplay might fortify the rice with adequate quantity of vitamins, which are very essential for human health. It was already proved that the amount of folic acid, niacin, riboflavin, and vitamins B12 and B6 were increased when fermentation is led by LAB (Shahani and Chandan, 1979; Alm, 1982). On the other hand, riboflavin content was gradually decreased during fermentation; probably, the participating microbes could not synthesize riboflavin or the microbes utilized for their growth. Boza contained thiamine, riboflavin, pyridoxine, and nicotinamid (Arici and Daglioglu, 2002). Basappa (2002) reported about the production of vitamin cyanocobalamin during finger millet fermentation by the participating microbes. In addition, the presence of different fatty acids in *haria* (**Table 3**) might enrich it with different health beneficial effects. Palmitic acid (C16:0) could alter the central nervous system for insulin secretion and suppress the body's natural appetite-suppressing signals from leptin and insulin in a rat model (Benoit et al., 2009). Moreover, linolenic acid (13.51 μg/mg) and linoleic acid (0.39 μg/mg) (**Table 3**) had antiobesitic, anticarcinogenic, antiatherogenic, antidiabetagenic, immunomodulatory, apoptotic, and osteosynthetic effects (Benjamin and Spener, 2009). Clearly, the presence of vitamins and fatty acids enriched the beverage.

Phytate is a natural antinutrient mostly present in cereals (Lopez et al., 2000). Its negatively charged phosphate groups form very stable complex with minerals (particularly divalent cations) causing unavailability of ions for intestinal absorption (Lopez et al., 2002). Phytase (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) degrades phytic acid (dephytinization) into myo-inositol and inorganic phosphate and helps in the unfasten of the minerals (De Angelis et al., 2003). It is now considered as an industrially important value-aided enzyme for quality improvement of phytate-rich feed and fodders (Singh and Satyanarayana, 2008). De Angelis et al. (2003) reported about the production of phytase by *Lactobacillus* sp. Due to dephytinization by the phytase enzyme, *haria* contain high amount of free minerals such as Ca^{++}, Fe^{++}, Mg^{++}, Mn^{++}, and Na^{+} (**Table 3**) probably due to the production of phytase enzyme (**Figure 3**). These minerals may contribute significant impact on mineral balance as well as physiological homeostatis of consumers. Chakrabarty et al. (2014) observed that the mineral contents were increased during judima (a glutinous fermented rice in Northeast India) fermentation. The level of Ca, Fe, Na, K, and P were also improved in Bhaati jaanr (Tamang and Thapa, 2006). Besides, Cu and Cr contents were gradually decreased during the fermentation of *haria*. Huang et al. (2015) demonstrated that foodborne *Lactobacillus paracase* has chromate reduction ability. Moreover, specific probiotic bacteria have the capacity to remove different types of heavy metals by adsorbing on their surface (Halttunen et al., 2008).

The aroma and taste of alcoholic beverages are critical to their acceptance by consumers, and the primary determinants of aroma and taste are volatile compounds (Arellano et al., 2012). Eleven types of volatile compounds were detected in *haria* by GC-MS (**Table 4**). Among these volatile compounds, three esters, namely, dichloroacetic acid heptadecyl ester, chloroacetic acid dodecyl ester, and chloroacetic acid tetradecyl ester, were detected. These esters give a unique flavor and aroma to *haria*. Production of these esters are generally controlled by the enzyme, acylcoenzyme A, which may be produced by the participating microbes, mainly yeast (Miller et al., 2007; Arellano et al., 2012). Chen and Xu (2010) also found different types of esters in Chinese wine, which was mainly fermented by yeast. Inositol (myo- and epi-inositol) has been found to be therapeutically effective in depression, panic disorder, and obsessive–compulsive disorder in double-blind controlled trials (Bersudsky et al., 1999).

CONCLUSION

The present study for the first time demonstrated that a group of lactic acid bacteria interplayed during the

fermentation of *haria*, and these bacteria originated from herbal residues. The application of PCR-DGGE-based metagenomic approach has been facilitated to explore the occurrence and dynamics of specific group of LAB at each stage of fermentation. The synergistic action of lactic acid bacterial consortia and other microorganisms (*Bacillus* sp. and yeast) fortified the rice by decreasing antinutrient phytic acid and improving the bioavailability of minerals, lactic acid, and acetic acid. All these credible scientific evidence supported its traditional use and putative health beneficial attributes. Proper scientific intervention by standardizing the process parameters and the adaptation of newer technologies will be helpful for the exploitation this beverage as functional and healthy drink for the world community.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, KU248768 https://www.ncbi.nlm.nih.gov/genbank/, KU248769.

AUTHOR CONTRIBUTIONS

KG and KM conceived and designed the experiments. KG and AA performed the experiments. KG wrote the paper. KM and SH critically revised the manuscript. KG, AA, SH, and KM approved the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Linking the Diversity of Yeasts Inherent in Starter Cultures to Quorum Sensing Mechanism in Ethnic Fermented Alcoholic Beverages of Northeast India

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In this review, the relevance of diversity of yeasts and their interactive association in household ethnic fermentation are discussed. The longstanding traditional household fermentation practice involves preparation of fermented product such as alcoholic beverages from various indigenous agricultural products with the help of microorganisms cultivated from local environment and perpetuated for hundreds of years through generations indoctrinating an indigenous knowledge system. Northeast India is known for its rich physiographic and geo-demographic diversity and is home to several ethnicities who follow unique practices of household traditional fermentation. The diversity of yeasts present within the microbial inoculum used for fermentation by different indigenous communities has been keenly studied and reported to be unique in spite of their common source for starter substrates. *Saccharomyces* yeasts are primarily involved in alcoholic fermentation, whereas non-*Saccharomyces* yeasts, which are reportedly confined to a particular geographical region, have been reported to contribute toward the final outcome of fermentation produce. During fermentation, interaction among these large microbial communities and their resulting physiological expression within the fermentation micro-environment is believed to affect the final quality of the product. Mechanism of quorum sensing plays an important role in these interactions in order to maintain proportionality of different yeast populations wherein the quorum sensing molecules not only regulate population density but also effectively aid in enhancement of alcoholic fermentation. Additionally, various secondary metabolites, which are secreted as a result of inter-species interactions, have been found to affect the quality of beverages produced. This review concludes that diverse species of yeasts and their interaction within the fermentation micro-environment influence the sustainability and productivity of household ethnic fermentation.

Keywords: ethnic fermentation, yeast, diversity, quorum sensing, alcoholic beverage

INTRODUCTION

Yeasts are single-celled eukaryotic microorganisms known to be the key players in the age-old practice of alcoholic fermentation for thousands of years. The practice of fermentation involving yeasts is one of the oldest human technologies dating back to the Neolithic period when spontaneous fermentation was carried out from plant sources such as grape must (Compagno et al., 2014). Due to their ability to ferment sugary substances into ethanol, yeasts are incorporated during formulation of inoculum or starter materials, which are used for preparation of fermented beverages in household fermentation worldwide (Hesseltine et al., 1988). This ancient practice of household ethnic fermentation in Southeast Asia involving yeasts is believed to have been originated from China, which spread across most of the neighboring countries with time (Limtong et al., 2002). Apart from the Asiatic region, the tradition of fermented product preparation is widespread among most of the indigenous communities belonging to different regions all over the world (Kabak and Dobson, 2011). These longstanding systems of alcoholic beverage preparation from various agricultural products with the aid of locally perpetuated fermentative agents have been in practice for generations indoctrinating indigenous knowledge system (Parasar et al., 2017; Nath et al., 2020a).

Methods of preparation of starter culture cakes among various communities is almost similar with minor variations observed in the methods followed and plant condiments added (Anupama et al., 2018). Household fermentation is recognized to be a popular practice among home brewers of indigenous communities in the entire Southeast Asia (Tsuyoshi et al., 2005) that reflect the ethnic heritage of existing communities, and northeast India is not an exception. Interestingly, in spite of a common source of ancestry of the indigenous communities of northeast India and usage of similar methods for ethnic fermentation, the diversity of yeasts perpetuated through those natural and spontaneous fermentation processes and maintained by the communities have been found to be unique (Buragohain et al., 2013; Tiwari et al., 2014; Parasar et al., 2017). Furthermore, since each community produces fermented beverage of distinct quality, the role of these genotypically diversified yeasts in both intra- and intercommunication is a question to be inquired, and their precise role in attributing quality characteristics in the final products of the fermented beverages needs to be understood. In addition to the interaction among yeasts in a consortia, the role of added plant condiments during starter material preparation may also play an important role in generating quality attributes as well as resuscitation of microbes within the starter cake/material propagated generation after generation (Nath et al., 2019).

In a fermentative micro-environment, intra- and interspecies interactions occur between co-existing yeasts and other microbes (Fleet, 2003) where the final quality of fermented beverage is believed to be affected due to the interaction among these large microbial communities and their resulting physiological expression (Ciani et al., 2016). Apart from the formation of ethanol, certain yeast species produce secondary metabolites such as fatty acids and killer toxins that act as growth inhibitors for other yeast species that eventually kill vulnerable yeasts (Pérez-Nevado et al., 2006). Direct physical interaction among different yeasts or with other microbes was also believed to be the limiting factor for the growth of certain non-*Saccharomyces* yeasts that spawn and proliferate during initial stages of fermentation (Nissen and Arneborg, 2003; Nissen et al., 2003; Renault et al., 2013). Noticeably, death of desirable yeast cells is triggered when unwanted yeast population reaches high cell density threshold during progressive fermentation (Nissen and Arneborg, 2003; Pérez-Nevado et al., 2006). These findings therefore suggest the importance of yeast interactions during both spontaneous and induced fermentation.

Apart from the above, it has been confirmed that quorum sensing molecules (QSMs) expressed by yeasts also play a key role in the expression of various population density-dependent characteristics that can affect the quality of a fermented product. QSMs are hormone-like molecules that accumulate in the external environment in proportionality to cell density population (Sprague and Winans, 2006) and either activates or represses certain genes when a particular threshold level of the molecules is obtained (Fuqua et al., 1994). The two fungal QSMs, viz., tryptophol and phenylethyl alcohol, were first recognized in *Candida albicans* (Lingappa et al., 1969) followed by the discovery of three additional QSMs, viz., farnesol, tyrosol, and farnesoic acid (Hornby et al., 2001; Oh et al., 2001; Chen et al., 2004; Hornby and Nickerson, 2004). These QSMs have further been examined by several researchers and their multifarious role in controlling morphogenic transition of yeast to filamentous form and vice versa has been established in the preceding years. Moreover, the QSM tyrosol has been reported to induce the upregulation of genes associated with DNA replication (Chen et al., 2004), thereby implying the probable enhancing effect of the molecule on cellular growth. This assumption was found to be true as was noted in our recent investigation where tyrosol significantly increased the population density of *Candida tropicalis*, *Wickerhamomyces anomalus*, and *Saccharomyces cerevisiae* (Nath et al., 2020b) during alcoholic fermentation.

Due to the involvement of diverse yeast species in the production of fermented goods, it is necessary to explore the link between their interactions and product outcome. There has been extensive research on determining the diversity of yeast conglomerates in fermentative cakes from northeast India, but unfortunately no clear explanation of yeast–yeast interferences or intercommunication within these starter cakes as well as fermented products could be inferred until the preparation of this report. As mentioned earlier, each indigenous community of northeast India produces fermented beverages with distinct quality attributed by specific yeast lineages tamed and cultivated by the individuals of these communities who exclusively preserve and perpetuate starter materials that are unique to each community type. It therefore becomes important to document the diversity of yeasts persisting within the starter materials, and it is pertinent that subtle differences in genotypes and phenotypes of diverse yeasts and the subsequent interactions among them are key factors that determine the oenological properties of final output in fermented beverages (Granchi et al., 2002). Keeping in view about the importance of cell–cell interactions during

fermentations, we extrapolate that the diversity of yeast species in starter cultures of northeast India should be explored and the inherent oenological properties be evaluated to understand the complexity of traits unique to the region. The volatile profiles of higher alcohols, esters, carbonyls, and sulfur compounds have not been studied and the prospects of delineating yeasts that produce secondary alcohols, acetic acid, acetaldehyde, ethyl acetate, acetoin, and glycerol have never been accessed (Steward, 2017). We assume that the quality traits need appreciation so that the prospective yeasts can be commercialized for large-scale production of chemicals and metabolites. Earlier, we had examined the factors that determine the persistence of yeast communities within the fermentation micro-environment but a larger view of the entire repertoire of yeasts from the region is still lacking. Moreover, the role of QSMs during fermentation does interfere and alter the quality of final products (Nath et al., 2020b), and henceforth, the array of QSMs expressed by the native yeasts of the region has to be deciphered to validate unique combination of yeasts with profound benefits.

BIBLIOMETRIC INFORMATION

The importance of traditional and sustainable food has received tremendous attention in recent years due to several reasons including their benefits to health, environment friendly methods of preparation, natural origin, absence of toxic effects, and prospects of commercialization. The exploration of indigenous knowledge systems inherent in cultures and traditions practiced through ages have helped in the conservation of innumerable diversity of both conventional and non-conventional food that have not been fully explored in the context of the Indian subcontinent. India is home to 550 ethnic communities (Schedule Tribes in India, jagranjosh.com) with a major proportion residing in the northeastern part of the country. Traditional brewing and household preparation of fermented food and drinks has been a major source of activity inculcated in their customs and traditions. The diversity of yeasts and other microbes inherent in these traditional brewing methods have been studied only recently, and we could find literature from 1994 onwards. In this review, all bibliographic literature available in PubMed, Web of Science, and Scopus indexed journals were consulted (1994–2020). A good number of research publications on the diversity and identity of yeasts during fermentation have been published since 1990 to date (PubMed 1,008 papers). To get a clear view of the extent of work conducted on the subject, we searched various scientific engines and found that the approximate number of papers published for the last 10 years stands at 1,432, which were retrieved from PubMed using various keywords/phrases [(1) Yeasts in traditional fermentation, (2) Traditional fermentation from northeast India, (3) Yeasts from northeast India, (4) Quorum sensing in yeasts, (5) Quorum sensing in traditional fermented yeasts, (6) Quorum sensing of *Saccharomyces* and non-*Saccharomyces* yeasts, (7) Quorum sensing in non-conventional yeasts, etc.], which reflected that the extent of research is not very pronounced. The search query found that the maximum number of documents (1,008) were related to the general term "yeasts in traditional fermentation" where a lot of literature on a global scale was available. This was followed by the term "quorum sensing in yeasts" where an appreciable number of publications (382) could be retrieved and 80% of the publications were related to *Candida* and *Cryptococcus* spp. that cause fungal infections in human. We could retrieve only nine publications related to the term "quorum sensing of *Saccharomyces* and non-*Saccharomyces* yeasts," which suggests that the area remains unexplored and very little attention is drawn toward quorum sensing among yeasts and their subsequent impact on fermentation/traditional fermentation, which therefore remains as a major gap. Bibliometric analysis enabled us to understand the global context of the subject, themes that have not been addressed yet and also the major drawbacks of the extent of research impact (Ashraf et al., 2021). The data, when analyzed, showed that the available knowledge on the subject is still limited and the milestones achieved are yet to be acknowledged, which has hindered the prospects of commercialization of ethnic foods on a scientific basis.

DIVERSITY OF YEASTS IN STARTER CULTURES OF NORTHEAST INDIA

The northeastern part of India includes a biogeographic realm with a characteristic biome and encompasses unique features in terms of rich biodiversity and multiple ethnicities of its people. The indigenous communities follow their own practice of household traditional fermentation. Each community harbors unique distribution of yeast species, which have been perpetuated throughout generations (Buragohain et al., 2013; Tiwari et al., 2014; Parasar et al., 2017) and this has resulted in the variety of alcoholic beverages assorted and produced by the communities through traditional fermentation practice. In household fermentation practiced by the communities of northeast India, solid-state starter cultures in the form of "flat cakes" or "spherical balls" are prepared from starchy substances such as rice, millets, maize, or wheat that are powdered and mixed with microbial inoculum (Nath et al., 2020a). These "cakes" supplement carbon constituents for the growth of yeasts that initiate fermentation. In the Rabha community, fermentative starter cake (known as *bakhaor*) is prepared in the form of "flat globules" by mixing soaked rice granules (*Oryza sativa* L., land variety sali and ahu) with considerable amount of old starter cakes (containing fermentation yeasts) and various plant condiments (Deka and Sarma, 2010). In order to prepare a consumable beverage, rice is tightly cooked and air cooled over bamboo mats, which act as substrate for rice beer. Starter cake is powdered (inoculum) and mixed with boiled rice. Later, this mixture is placed inside earthen pots while a special cylindrical structure made of bamboo (*janthi*) is placed inside and the mouth of the pots are tightly closed with banana leaves. After a few days (4–5 days in summer, 7–8 days in winter), fermented rice beer accumulates inside the *janthi*. This beer is further fermented using water and starter cakes, followed by distillation using series of earthen or metallic pots. Finally, a strong, consumable fermented beverage is produced (Deka and Sarma, 2010).

The presence of non-*Saccharomyces* species along with chief fermentative yeasts in starter cakes of various indigenous communities has been observed universally. The microbial inoculum in starter materials has been maintained for generations. However, in spite of extensive perpetuation for hundreds of years, the quality and consistency of the fermented produce have not varied much and appear to be constant across continued generations without any noticeable change in their fermentation efficiency. This consistency in fermentation outcome indicates the steady maintenance of yeasts and their stable genetic features. Amylolytic yeasts presumably play a crucial role in fermentation by enabling the breakdown of high carbon materials such as starch into simpler glucose molecules while fermentative yeasts thrive and carry out fermentation on those ready-made energy sources. Competition over common resources arises when two or more species of yeasts compete to thrive within a specified fermentation environment. However, cooperation between amylolytic and non-amylolytic yeasts always prove to be a beneficial trait when the population has to rely on starchy carbon source (Nath et al., 2020a). To note, non-*Saccharomyces* yeasts such as *W. anomalus* produces volatile compounds that enhance the aroma characteristics of wines, thereby enabling better quality yield of fermented beverage (Ye et al., 2014).

Fermented products are categorized into solid or semi-solid fermented food and fermented beverages. The diversity of yeasts in fermentative sources has been well-documented from Southeast Asia (Boekhout and Robert, 2003). The documentation of diversified yeasts from fermentative foods of northeast India have also been done from time to time. Buragohain et al. (2013) had reported the presence of *S. cerevisiae*, *Debaryomyces hansenii, W. anomalous*, and *Candida glabrata* in starter cakes collected from the tea ethnic community, Ahom, Nepali, Bodo, Adivasi, Karbi, and Dimasa communities of Assam, Meitei community of Manipur, Angami community of Nagaland, Apatani community of Arunachal Pradesh, and the Khasi community of Meghalaya. The presence of *Candida parapsilosis* and *Geotrichum candidum* in *kinema* fermented food of the Gorkha and Nepali communities was reported from the state of Sikkim (Sarkar et al., 1994). Dewan and Tamang (2006) had reported about the presence of *Saccharomycopsis fibuligera* and *Candida* spp. from *chhu* or *sheden* ethnic fermented milk products of the Bhutias, the Lepchas, the Monpas, the Sherdukpen, the Khambas, the Membas, and the Tibetan people living in the higher altitudes of the eastern Himalayas. The persistence of the yeasts representing *Saccharomycopsis* and *Candida* have also been reported from *dahi* (curd) from the entire northeast India (Dewan and Tamang, 2007); *Candida* spp. from *goyang* fermentation of the Sherpa community in Sikkim (Tamang and Tamang, 2007); *S. cerevisiae, Pichia burtonii*, and *Candida castellii* from *maseura* or *masyaura* fermentations (Chettri and Tamang, 2008); and *S. cerevisiae, Saccharomyces kluyveri, D. hansenii, P. burtonii*, as well as *Zygosaccharomyces rouxii* from *selroti* fermented product of the Gorkha community (Yonzan and Tamang, 2010). Chettri (2012) had described about the presence of *S. cerevisiae*, *D. hansenii*, and *P. burtonii* in *tungrymbai* fermented food of the Khasi community from Meghalaya as well as from *bekang* fermentation of Mizoram. Tamang et al. (2016) reportedly marked about the presence of *Saccharomyces* and *Torulopsis* yeasts in *soibum* fermentation of the Meitei community from Manipur. On the contrary, Thapa et al. (2004) reported the predominance of *Candida* and *Saccharomycopsis* yeasts in *ngari* and *hentak* fermented drinks of Manipur, *Candida chiropterorum, Candida bombicola*, and *Saccharomycopsis* spp. from *gnuchi* of the Lepcha community of Sikkim while the presence of same yeasts had been reported from *suka ko maccha* and *sidra* fermentations prepared by the Gorkha community from Sikkim (Thapa et al., 2006).

Since solid-state starter cultures are primarily used as inoculum for fermented beverage production, these have a special mention in most of the published reports from the region, such as starter cake *marcha/murcha*, which is used to produce the alcoholic drink called *jaanr* in Sikkim and Himalayan terrains (Shrestha et al., 2002; Tamang, 2005, Thapa and Tamang, 2020). Incidentally, the persistence of the yeasts *S. fibuligera*, *Saccharomycopsis capsularis*, *Pichia anomala*, *P. burtonii*, *S. cerevisiae*, *Saccharomyces bayanus, W. anomalus*, and *C. glabrata* seem to be ubiquitously present along the eastern Himalayan landscape (Tamang and Sarkar, 1995; Thapa, 2001; Tsuyoshi et al., 2005; Sha et al., 2018). The ecology and distribution of yeasts in fermented products from the rest of the seven northeastern states have also been reviewed by several researchers. The supremacy of *S. cerevisiae* over other yeasts in consortia was observed in a starter material called *piazu*, which is used to prepare alcoholic brew called *zutho* by the Angami community of Nagaland (Teramoto et al., 2002). Similar observations were noted for the alcoholic brew called *litchumsu*, which is prepared by the Ao community of Nagaland (Das et al., 2012). *Zutho* in the state of Nagaland is also produced from another starter culture named *Khekhrii*, wherein the dominance of *W. anomalus* and *P. anomala* was observed (Sha et al., 2018). Similarly, existence of *S. cerevisiae*, *P. anomala*, *Trichosporon* spp., *C. tropicalis*, *Pichia guilliermondi*, *C. parapsilosis*, *Torulaspora delbrueckii*, *Pichia fabianii*, *Pichia kudriavzevi*, *Candida montana*, and *C. glabrata* was reported from *hamei* starter culture, which is employed in the production of *atingba* and *yu* alcoholic beverages by Meitei and Kabui community of the state of Manipur (Jeyaram et al., 2008, Sha et al., 2018, Wahengbam et al., 2020). In Meghalaya, starter cultures called *wanti* and *thiat* are used in preparation of alcoholic beverages (Anupama et al., 2018; Mishra et al., 2018). The starters of *wanti* are primarily composed of the yeasts *S. cerevisiae*, *Rhodotorula mucilaginosa*, and *W. anomalus*, which is used to prepare *chubitchi* beverage by the Garo community, *kyiad* beverage by the Khasi community, and *sadhiar* beverage by the Jaintia communitiy of Meghalaya (Mishra et al., 2018). The starters of *thiat* contain yeasts belonging to *S. fibuligera, W. anomalus*, and *Pichia terricola*, which is used to prepare alcoholic beverages by the Khasi community (Sha et al., 2018). Earlier investigations revealed the presence of *W. anomalus* in *epo* starter culture perpetuated by the Apatani community and *phut* starter cake of the Monpa community of Arunachal Pradesh (Tanti et al., 2010; Buragohain et al., 2013; Anupama et al., 2018; Sha et al., 2018). In the state of Mizoram, beverages such as *zupui, zufâng*, and *rakzu* are prepared by Mizo community from a starter

culture called *dawdim,* where the occurrence of *W. anomalus*, *C. glabrata, P. anomala*, and *S. fibuligera* were found to coexist as a consortia (Sha et al., 2018; Thanzami and Lalhlenmawia, 2020). In the state of Tripura, production of local alcoholic drinks like *gora bwtwk* and *chuwak* are customary traditions carried out by the Kalai, Jamatia, Debbarma, and Molsom communities who conserve and perpetuate a starter culture called *chowan*, where the dominance of *C. glabrata* and *W. anomalus* was reported (Anupama et al., 2018; Sha et al., 2018; Majumdar, 2020). In our previous investigations (Parasar et al., 2017), we analyzed the starter cultures conserved by the Bodo, Karbi, Rabha, Mishing, Ahom, and Kachari communities of Assam. The starter culture *emao/amao* of the Bodo community was found to be dominated by *P. burtonii, P. anomalus*, and *S. cerevisiae* while another starter culture called *thap* of the Karbi community was dominated by *S. cerevisiae* (Parasar et al., 2017). The Ahoms perpetuate a starter culture called *xajar pitha*, which is enriched with *C. tropicalis* and *C. glabrata*, an unusual observation with a great deal of research interest since no *Saccharomyces* isolates could be retrieved from the starters (Parasar et al., 2017). The Mishing community of Assam prepare and preserve *apong kusure/apop pitha* starter cake where the unusual yeast *Rhodotorula taiwanensis* was found to predominate (Parasar et al., 2017). The Rabhas, another major ethnic community, perpetuate starter cultures called *bakhor* where the persistence of *Meyerozyma carribica* was reported (Tanti et al., 2010; Narzary et al., 2016; Parasar et al., 2017; Barooah et al., 2020). The Kachari community of Assam prepare *modor pitha* starter cultures that contain a consortia of *S. cerevisiae, W. anomalus,* and *C. glabrata* (Parasar et al., 2017; Anupama et al., 2018). To our utter surprise, the entire spectrum of starter cultures studied from the region revealed the presence of *W. anomalous* except for the starter cultures collected from the Ahom community (Parasar et al., 2017; Nath et al., 2020a). Occurrence of dominant yeasts in starter cultures are summarized in **Table 1**. Additionally, the distribution of ethnic communities of the region, the starter materials used in traditional brewing, and the names of resultant beverages are summarized in **Figure 1**. Here, we have mentioned only those starter cultures whose detailed studies are available in literature and which have been investigated by researchers from the region. The repertoire of cultures is colossal and shall require far more intricate research plans to reach a final conclusion regarding the origin, distribution, and diversity of yeast types and the array of traditional fermentation procedures practiced in the region (Parasar et al., 2017; Nath et al., 2020a). Our recent findings regarding distribution of yeasts in starter cakes (Parasar et al., 2017; Nath et al., 2020a) are illustrated in **Figure 2**.

YEAST–YEAST INTERACTION AND INVOLVEMENT OF QSMs IN FERMENTED BEVERAGES

Microorganisms exist in diverse communities in both natural and man-made ecosystems. Interactions among these microbes define the complexity of food and beverages (Avbelj et al., 2016). Such intra- and interspecies interference could be observed between yeasts and other microbes that coexist in wine micro-environment (Fleet, 2003). Essential metabolic interactions and complementation physiognomies have been reviewed in food ecosystems (Ivey et al., 2013). Positive interactions such as mutualism and synergism allow the participating microbes to derive benefit from the interactions. For example, during alcoholic fermentation of starchy material, two types of yeasts have been found to play the principal role of fermentation that include amylolytic yeasts that degrade starch into simpler carbohydrates while alcohol-fermenting yeasts grow on the resulting simpler carbohydrates to yield ethanol (Tsuyoshi et al., 2005). This was further analyzed in our recent experiments and was observed that utilization of starch and subsequent ethanol production was higher when *S. cerevisiae* (non-amylolytic) was combined with *C. tropicalis* (amylolytic) in co-culture combination compared to tested combinations of *S cerevisiae* with other non-amylolytic yeasts (Nath et al., 2020a). Cooperation of yeasts with other species has also been found to be beneficial for the production of quality fermented product. For example, production of Japanese alcoholic drink *sake* employs fermentation by *S cerevisiae* var. *sake* along with *Aspergillus oryzae*, wherein *Aspergillus* break down rice starch into simpler sugars while yeasts convert resultant sugars into ethanol (Jay, 1991; Azumi and Goto-Yamamoto, 2001). Furthermore, such interactions also do enhance unique characteristics by producing secondary metabolites, higher alcohols, and ethers that produce pleasant aroma and quality texture (Hosaka et al., 1998).

Contrarily, negative interactions such as amensalism adversely affect the growth of competitive microbes. Two good examples can be cited for amensalism wherein the production of ethanol by *S. cerevisiae* eliminate all non-*Saccharomyces* yeasts present in the must during fermentation while nisin production by *Lactobacillus lactis* inhibits proliferation of other competing lactic acid bacteria (LAB) (Hutkins, 2007). Competition is another aspect of negative interaction, where the microbes compete with one another for nutrient uptake and for space to survive. Such examples can be seen during alcoholic fermentation, where *S. cerevisiae* engage in defensive mechanisms such as physical cell contact and secretion of antimicrobial peptides against other microbes that impart significant impact on wine profiles (Albergaria and Arneborg, 2016). Such microbial interactions influence the quality of the final product and impact critical parameters such as sensory quality, low toxicity, and shelf life of the fermented food or beverage (Rul and Monnet, 2015).

Application of the QSMs 2-phenylethanol, tryptophol, and tyrosol have already been reported in various biotechnological techniques, which include evaluation of wine quality (Garde-Cerdán et al., 2007; González-Marco et al., 2010) and enhancement of aroma in foods and drinks (Etschmann et al., 2003; Wang et al., 2011). In addition to enhancing food value, quorum sensing mechanisms have the potential to control food spoilage (Avbelj et al., 2016). Such observation has been reported during wine fermentation, where K2 and Klus-type yeast killer toxins were found to be responsible for

TABLE 1 | Dominant yeast species associated with starter materials from ethnic communities of northeast India.

State	Ethnic community	Starter material	Dominant yeast species	References
Sikkim	Nepali, Gorkha, Lepchas, Limboo, Bhutia,	*Marcha*	*S. fibuligera, Saccharomycopsis capsularis, P. anomala, P. burtonii, S. cerevisiae, S. bayanus, C. glabrata*	Tamang and Sarkar, 1995; Thapa, 2001; Tsuyoshi et al., 2005; Thapa and Tamang, 2020
Arunachal Pradesh	Apatani, Mongpa	*EpoPhut*	*W. anomalus*	Tanti et al., 2010; Buragohain et al., 2013; Anupama et al., 2018; Sha et al., 2018
Nagaland	Angami, Ao	*Piazu,Khekhrii*	*S. cerevisiae, C. glabrata, W. anomalus, P. anomala.*	Das et al., 2012; Buragohain et al., 2013; Sha et al., 2018.
Manipur	Meitei, Kabui	*Hamei*	*S. cerevisiae, P. anomala, Trichosporon spp., C. tropicalis, P. guilliermondi, C. parapsilosis, T. delbrueckii, P. fabianii, C. montana, P. kudriavzevi, C. glabrata*	Jeyaram et al., 2008; Sha et al., 2018; Wahengbam et al., 2020.
Meghalaya	Garo, Khasi, Jaintia	*Wanti,Thiat*	*S. cerevisiae, R. mucilaginosa, W. anomalus, S. fibuligera, P. terricola, S. fibuligera, W. anomalus, P. terricola*	Anupama et al., 2018; Mishra et al., 2018; Sha et al., 2018.
Mizoram	Mizo	*Dawdim*	*W. anomalus*, *C. glabrata, P. anomala*, *S. fibuligera*	Sha et al., 2018; Thanzami and Lalhlenmawia, 2020
Tripura	Kalai, Jamatia, Debbarma, Molsom	*Chowan*	*C. glabrata, W. anomalus*	Anupama et al., 2018; Sha et al., 2018; Majumdar, 2020
Assam	Bodo Karbi	*EmaoThap*	*P. burtonii, P. anomalus, S. cerevisiae, W. anomalu* *S. cerevisiae, W. anomalus*	Tanti et al., 2010; Narzary et al., 2016; Parasar et al., 2017; Anupama et al., 2018; Barooah et al., 2020; Nath et al., 2020a.
	Kachari	*Modor pitha*	*S. cerevisiae, W. anomalus, C. glabrata.*	
	Rabha	*Bakhor*	*M. carribica, W. anomalus*	
	Ahom	*Xajar pitha*	*C. tropicalis, C. glabrata*	
	Mishing	*Apong kusure/ Apop pitha*	*R. taiwanensis, W. anomalus*	
	Adivasi Dimasa	*DabaiHamao*	*C. glabrata* *D. hansenii, W. anomalus*	Tanti et al., 2010; Buragohain et al., 2013; Sha et al., 2018.

controlling proliferation of spoilage yeasts (Comitini et al., 2004; Rodríguez-Cousiño et al., 2011). Alternatively, quorum sensing inhibitors (QSIs) can be employed to control the proliferation of spoilage microbes thereby inhibiting the expression of undesired virulent traits (Sharma and Jangid, 2015). Recent studies reveal that different plant extracts harboring phytochemical constituents and essential oils inhibit biofilm formation of harmful yeasts/microbes, thereby rendering the product suitable for human consumption and henceforth these substances may be considered as effective QSI during wine fermentation (Avbelj et al., 2016). Likewise, a QS mechanism has been observed to control food spoilage by regulating the expression of enzymes such as cellulases, lipases, chitinases, nucleases, pectate lyase, and various proteases, thereby facilitating the management of quality control of processed foods (Skandamis and Nychas, 2012).

In traditional fermentations practiced by the communities of northeast India, different plant condiments are added during the preparation of starter cultures (Tanti et al., 2010). Metabolites and constituents from such plant additives/parts are believed to play an important role during fermentation (Nath et al., 2019). The phytohormone indole-3-acetic acid (IAA) is one of several metabolites that are known to promote rapid and long-term responses in plants (Cleland, 2010) and incidentally has also been observed to be expressed by certain yeasts inherent in traditional starters from the region (Nath et al., 2019). It is noteworthy that reports of yeasts capable of producing IAA are already available in published literature, which contemplates the importance of traditional yeasts to be exploited for producing phytohormones other than conventional metabolites (Nakamura et al., 1991; El-Tarabily, 2004; Nassar et al., 2005). Parenthetically, previous studies had reported that IAA stimulates growth and promotion of filamentous morphogenesis in *Saccharomyces* (Prusty et al., 2004), which was also supported by our recently published findings (Nath et al., 2019). From such observations, it can be presumed that IAA contributes toward the growth of yeasts while the addition of plant condiments supplements the necessary phytohormone for perpetuation and coexistence of the varied yeast species in starter cakes.

It is to be mentioned that tyrosol, the chief QSM expressed in yeasts, was reported to confer protection from oxidative stress induced by respiratory burst of neutrophils (Cremer et al., 1999) and ethanol (Nath et al., 2020b) apart from morphogenic transition and other QS-related activities. In an earlier report,

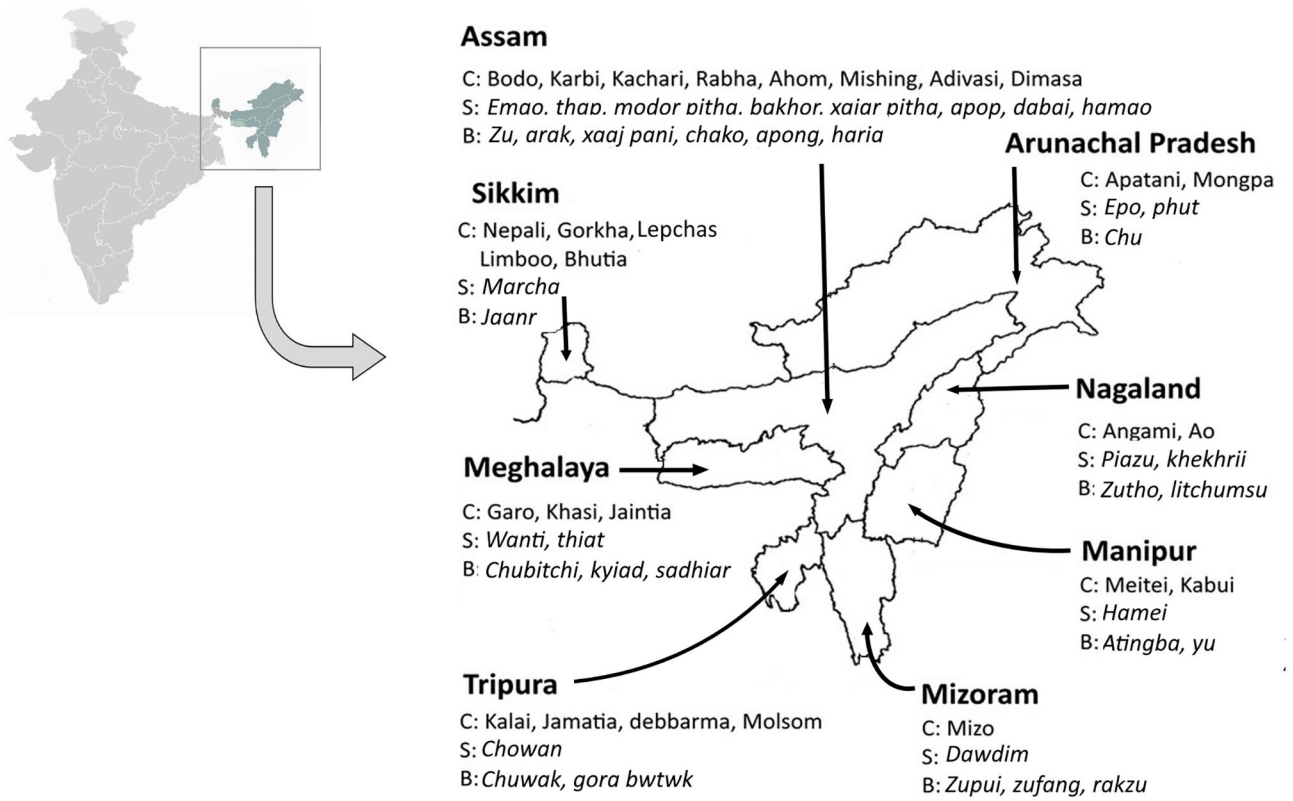

FIGURE 1 | Map of northeast India indicating ethnic communities (C), starter material/cake (S), and fermented beverages (B) associated with ethnic culture of the seven states. This illustration is based on reports by various researchers on yeast population harbored in the starter cakes and their beverages (Tamang and Sarkar, 1995; Thapa, 2001; Tsuyoshi et al., 2005; Jeyaram et al., 2008; Tanti et al., 2010; Das et al., 2012; Buragohain et al., 2013; Narzary et al., 2016; Parasar et al., 2017; Anupama et al., 2018; Mishra et al., 2018; Sha et al., 2018; Barooah et al., 2020; Majumdar, 2020; Nath et al., 2020a; Thanzami and Lalhlenmawia, 2020; Thapa and Tamang, 2020; Wahengbam et al., 2020).

tyrosol was reported to influence germ tube formation through upregulation of DNA replication and cell cycle maintenance (Chen et al., 2004). Such upregulations can presumably exert the growth-enhancing effect in yeasts concurrent with the increase in cell density. Evidently, our recently published work also demonstrated an increase in cell number under the influence of self-expressed and exogenously introduced tyrosol in both *Saccharomyces* and non-*Saccharomyces* yeasts (Nath et al., 2020b), and since ethanol production was found to be proportional to cellular density, an indirect involvement of tyrosol during fermentation could be well-justified. Nevertheless, such QS activity is believed to be limited by ethanol concentration and addition of external ethanol was found to inhibit the production of tyrosol by *S. cerevisiae*, a strong indication of an antagonistic role of ethanol on QS activity (Avbelj et al., 2015).

THE IMPORTANCE OF ASSOCIATION OF YEAST CONGLOMERATE AND QUORUM SENSING

This review was an attempt to provide an insight into yeast–yeast interrelation and the role of quorum sensing mechanism on the production of ethnic beverages during traditional fermentations. As mentioned earlier, diverse species of yeasts in fermentative cakes impart substantive effect on the shelf life and oenological properties in wines and beverages when cultured in consortium. Survival experiments of yeast isolates under co-culture conditions revealed the usefulness of non-*Saccharomyces* yeasts during fermentation and is now well-understood that apart from the enhancement of ethanol content, other metabolites also play important roles in the enhancement of quality attributes of the fermented products (Nath et al., 2020a). The unusual yeast *W. anomalus* was found to be ubiquitously present and dominant in most of the fermentative cakes representing several indigenous communities of Assam (Parasar et al., 2017), and is not a chief fermenting yeast, and yet, the species has been reported to produce volatile compounds that enhance the aroma profile of traditional wines (Clemente-Jimenez et al., 2005). Several investigations reported earlier stated that high-quality balanced wines can be produced when *W. anomalus* is mixed and co-cultured with *S. cerevisiae*, which often leads to the production of acetate esters, ethyl propanoate, phenyl ethanol, and 2-phenylethyl acetate that are known to enhance the aroma profile of wines (Rojas et al., 2003; Clemente-Jimenez et al., 2005; Swangkeaw et al., 2011, Passoth et al., 2006). Similarly,

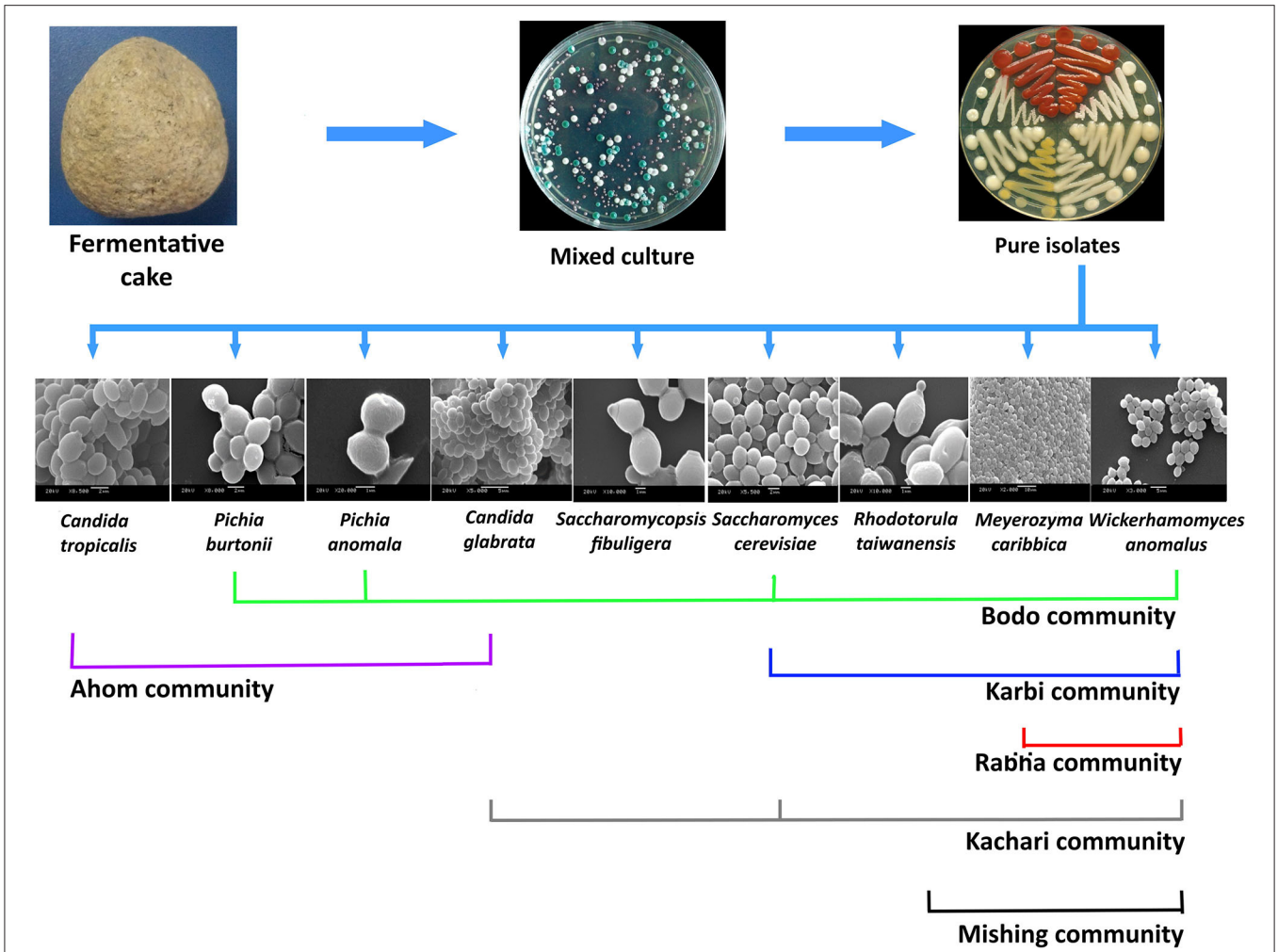

FIGURE 2 | Graphical depiction of distribution of yeasts in various starter cultures of indigenous community of Assam. The experimental data are derived from Parasar et al. (2017) and Nath et al. (2020a).

other unusual non-*Saccharomyces* yeasts like *Zygosaccharomyces bailii* (Gueguen et al., 1995), *D. hansenii* (Yanai and Sato, 1999), *P. anomala* (Manzanares et al., 2001; Swangkeaw et al., 2011), and *Hanseniaspora* (Swangkeaw et al., 2011) have been reported to express and produce several valuable metabolites, volatile compounds, and enzymes like β-glucosidase during fermentation of grape must (Rodríguez et al., 2004). It is now well-known that non-*Saccharomyces* yeasts saturate the fermentation culture during early stages of fermentation, and with an increase in ethanol concentration, the dominance of ethanol tolerant *S. cerevisiae* persists, which continues the process till the end of fermentation (Xufre et al., 2006; Lee et al., 2012a,b). Meanwhile, although the precise role of QSMs during the fermentation process has not received much attention, considering the effects of QSMs studied to date, the importance of molecules like tyrosol has proved to be beneficial, not only because of its cell growth-enhancing capability but also because it provides nutritional value to fermented products with quality traits that confers health benefits as tyrosol possesses incredible antioxidant property (Giovannini et al., 1999).

CONCLUSION

From the preceding discussion, it is evident that association of yeasts and their productive communication results in the production of beverages with distinct enological qualities inherent in fermented beverages/drinks prepared by various indigenous communities of northeast India. These unique characteristics are presumably observed due to the presence of distinct dominant species of yeasts and the addition of plant concoctions/condiments into the starter materials for perpetuation and cultivation of fermenting principle. However, several limitations such as bad odor, turbidity, inconsistency, presence of toxic metabolites, and variable texture contribute to uninvited traits rendering the fermented

products unsuitable for commercialization (Tsuyoshi et al., 2005). Although genetic or chemical engineering approaches can eliminate such limitations, natural methods of co-culturing or sequential culture procedures could prove to be more cost-effective and user-friendly. To conclude, the aforementioned yeast–yeast association experiments and studies pertaining to the role of chemical communication by QSMs like tyrosol in controlling the quality of fermented products need to be thoroughly investigated, but unfortunately, records of such investigations are scanty from northeast India, which has an abundant microflora distribution and rich in cultural heritage of a huge congregation of ethnic indigenous people. Considering the immense potential of these traditional products, more and more applications of such natural interactions in fermentation should be prioritized so that the indigenous people could benefit from these household preparations and get an opportunity for choosing sustenance through commercialization of these indigenous products that will impart sustainable livelihood options.

FUTURE DIRECTIONS AND PERSPECTIVES

The mechanism of QS in both gram-positive and gram-negative bacteria are well-established (Rutherford and Bassler, 2012). This understanding has resulted in efforts to inhibit bacterial pathogenesis and design novel antimicrobial therapeutics. Unlike bacteria, where the role of autoinducing molecules in QS like lactones and oligo-peptides and the corresponding genes that express them have been deciphered, the fungal QS system is yet to be studied in detail, particularly with reference to the effects of QS on fermentation. Processes in QS include other activities like bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion (Rutherford and Bassler, 2012) and such effects cannot be nullified in fungal quorum sensing too. Research on autoinducing molecules and transporters that release or transport these molecules in and out of cells shall complement their effects on the formation of final products as well as adduct secondary molecules that could prove to be industrially beneficial. Similarly, the effect of QSMs in starter culture perpetuation needs to be appreciated and should be studied to explicate the benefits of QS among yeasts in solid-state fermentation. At the same time, the differences of expression and activity of QSMs in starter cultures and during submerged fermentation need to be investigated. In starter materials, yeasts remain in dormant dry state that are activated upon contact with substrates. Proliferation of yeasts within starter materials without the involvement of fermentation is a question to ponder. Notwithstanding the fact that a large number of studies pertaining to the probable role of plant material concoctions in the enhancement of attributes of fermented beverages in spontaneous or traditional fermentation from starter cultures have been discussed (Nath et al., 2019), an authentic explanation to the definite role of these concoctions on QS activity concomitant to fermentation process has never been addressed. Besides these, the necessary information on the accessibility and roles of other QSMs other than tyrosol on fermentation (Nath et al., 2020b) is still missing and thus our knowledge on the array of QSMs remains limited, which needs to be addressed. More importantly, the ecology and behavior of traditional yeasts in fermentation have not been studied much and henceforth the queries on the key ecological aspects of these beneficial fungi in response to change in parameters and composition of substrates need to be studied. It has been reported that QSMs in yeasts like farnesol suppress the pathogenicity of certain pathogenic yeasts like *C. albicans* through morphogenesis (Hornby et al., 2001). Similarly, 2-phenylethanol and tryptophol regulates phenotypic changes in *S. cerevisiae* (Smukalla et al., 2008; Avbelj et al., 2016). The regulatory mechanisms of these QSMs are well-explained (Avbelj et al., 2016), but their direct involvement in fermentation is yet to be thoroughly explored. In addition, assessing the role of these QSMs during inter- and intra-species competition in a fermentation microenvironment needs validation. The role of QSMs in traditional fermentation is important to check the changes in parameters and the expression/production of both desirable and undesirable metabolites in culture. There are several future prospects of QS mechanism in the production of fermented foods. QSMs discovered to date have various health benefits (Nath et al., 2020b). As QSMs are produced in minute quantities, purification of these shall not be an economically viable option. Therefore, understanding the mechanism to develop fortified fermented food enriched with beneficial molecules could be one useful alternative. In anaerobic condition, increased production of 2-phenylethanol, tryptophol, and tyrosol was observed in *C. albicans* (Ghosh et al., 2008) and *S. cerevisiae* (Avbelj et al., 2016), implying the role of QSMs in the enhancement of fermentation during anaerobic conditions. Anaerobic condition suppresses biomass proliferation and increases more carbon conversion to end product (Huang and Tang, 2007). It is therefore envisioned that proper exploration and application of the regulating mechanisms of fermentation through QSMs can open new insights that will be beneficial in food and beverage industries. Furthermore, to the above, the interactions among yeasts, their presence, and their metabolic exchanges are key to the formation of products that will have economic value. As QS mechanism is a density-dependent phenomenon, regulation of cell population during expression of QSMs is anticipated (Nath et al., 2020b). It is evident that more experiments shall have to be conducted to reach to a conclusion as to how a fermented product is modified through QS mechanism. Co-culturing techniques could possibly help in determining the competitiveness among species, while genetic engineering approaches can facilitate in understanding the underlying molecular mechanisms of such interaction during fermentation.

AUTHOR CONTRIBUTIONS

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Sustainable Production of Pulque and Maguey in Mexico: Current Situation and Perspectives

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Pulque is a traditional Mexican fermented, non-distilled alcoholic beverage produced by fermenting the fresh sap (*aguamiel*) extracted from several *Agave* (maguey) species cultivated for pulque production (mainly *A. salmiana*). This beverage was produced and consumed since Pre-Hispanic times by Mesoamerican civilizations, mainly in the Mexican Central Plateau, and is one of the essential alcoholic beverages produced and consumed during several centuries in Mexico. By 2019, annual pulque production was reported in 171,482 billion liters. Nevertheless, traditional pulque production faces several significant limitations, including the disappearance of large agave plantations and the extent of time required (at least 5 years) to complete the plant maturation for aguamiel extraction; traditional production practices; and the lack of an efficient stabilization process of the fermented product resulting in low shelf life. In opposition, successful examples of sustainable cultivation of maguey species for aguamiel extraction and the fermentation process's industrialization resulted in high-quality pulque production, with international exportation certification. In this contribution, we present a review of the most relevant aspects of the history and commercial relevance of pulque, the causes that resulted in its production debacle during the first half of the twentieth century, the current situation of its traditional production, and the successful efforts of industrial production of the beverage. We describe recent results on the analysis of the physicochemical characteristics of aguamiel and on the microbiology of the beverage explored by metagenomic techniques that can be proposed as a baseline to redefine the quality criteria of the beverage and to define for the first time a microbiological core to optimize its production. We describe the relevance of maguey species for aguamiel production as a fundamental element of agroforestry and the relevance of its sustainable production, in four sustainable plantation models to maintain a stable plant population to ensure the continuous extraction of aguamiel and pulque production. Finally, we describe some successful examples of beverage industrialization and potential applications of several microorganisms isolated from aguamiel, pulque, aguamiel concentrates, and the maguey to produce high-value bioactive products.

Keywords: pulque, aguamiel, traditional fermented beverage, agave, maguey, sustainable production, process industrialization

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INTRODUCTION

Pulque is a traditional Mexican non-distilled, alcoholic fermented beverage, produced by fermentation of the fresh sap or *aguamiel*, extracted from several species of *Agave* or maguey *pulquero* species (plants for pulque production) (**Table 1**; Escalante et al., 2016). Pulque is probably the most important traditional fermented alcoholic beverage produced and studied in Mexico. The archeological evidence suggests the cultivation and use of maguey for various purposes (e.g., feed and raw material for fiber production) by the inhabitants from the Tehuacan Valley (Puebla State) by 6500 BC (Lorenzo Monterrubio, 2007; Moreno-Terrazas et al., 2017). The use of specialized obsidian tools for maguey scraping in the highlands of the Central and North-Central Mexico was proposed as a common practice in the early Pre-Hispanic Middle and Late Formative Periods (500 BC—100 AD) (Parsons and Darling, 2000; Lorenzo Monterrubio, 2007). The use of ceramic vessels for pulque production, consumption, and storage was dated by 200–550 AD by the detection of hopanoids present in the cytoplasmic membrane of *Zymomonas mobilis* (one of the essential bacteria during pulque fermentation) as biomarkers detected by gas chromatography-mass spectrometry in ceramic pottery collected in the neighborhood of the ancient city of Teotihuacan, the capital of a state that controlled the Basin of Mexico (Correa-Ascencio et al., 2014). The ancient Aztec culture rediscovered the maguey plants and pulque production by 1100–1300 AD (Gonçalves de Lima, 1956). Although no information is available on the extent of the cultivated maguey plantations and pulque production by the Aztec culture, they developed a total dominion of aguamiel and pulque production establishing strict rules for consumption until the down of this Empire in 1521 (Gonçalves de Lima, 1956; Ramírez Rodríguez, 2004; Instituto Nacional de Antropología, 2012).

During the Spaniard Colony in Mexico (1535–1821), the Spaniards and Creoles controlled maguey plantations, aguamiel extraction, pulque production, sale, and consumption in sites known as *pulquerías* (a type of canteen for pulque sale and consumption). By the middle and late seventeenth century, the production and sale of pulque became a relevant economic activity by the high volumes of the beverage introduced and consumed mainly in Mexico City (estimated in 2,625,000 L) (Ramírez Rancaño, 2000; Ramírez Rodríguez, 2004; Lorenzo Monterrubio, 2007). With the development of large farms for pulque production (known as Haciendas *pulqueras*) by the end of the eighteenth century, mainly in the States of Hidalgo, Puebla, Tlaxcala, and Mexico, pulque production and commerce became the fourth-highest income of the Spaniard Colony, only after the sales tax, silver production, and coining (Ramírez Rodríguez, 2004; Lorenzo Monterrubio, 2007). Pulque production and sale decreased significantly during the Mexican Independence War (1810–1821), nevertheless, its production was not interrupted (Ramírez Rodríguez, 2004). By the second half of the nineteenth century, pulque was again the preferred beverage in the main cities of the Mexican Central Plateau. From 1866 to 1880, the new *Ferrocarril Mexicano* railway connected the larger production zones in Apan (Hidalgo State) and other relevant production centers in the States of Tlaxcala and Estado de México with Mexico City, increasing the consumption of the beverage.

By 1900 the annual pulque production in Mexico was 485.56 billion liters, according to the available data in the statistical yearbook of Mexico for 1900 (*Anuario Estadístico de la República Mexicana*) reported in Ramírez Rancaño (2000). By 1907, ∼99% of the total pulque production registered in Mexico was produced only in the States of Hidalgo, Tlaxcala, Puebla, and Estado de México resulting in a second flourishing of the Haciendas pulqueras (Ramírez Rancaño, 2000). By the end of the nineteenth and the early twentieth century, from 799 to 808 haciendas reported in Mexico, 271–279 were dedicated only to pulque production. They were vast centers for maguey cultivation, aguamiel extraction, pulque fermentation, and distribution; the larger Haciendas pulqueras had a surface up to 100 ha. The central pulque production zone located in the region of Apan comprised 250,000 ha for maguey cultivation, with an estimation of more than 100 million agave plants for pulque production (Ramírez Rancaño, 2000). This economic activity resulted in the rise of a new powerful economic and political class called "the pulque aristocracy," closely related to president Porfirio Díaz (Ramírez Rancaño, 2000; Lorenzo Monterrubio, 2007). Despite the economic relevance of pulque production and consumption, by the early twentieth century, an anti-*pulque* movement came out of concern of the negative impact of pulque consumption, attributing social unrests, and relevant health problems like alcoholism, imposing strict rules on pulque distribution and sale mainly in Mexico City.

In 1906, several Haciendas *pulqueras* (mainly in Puebla State) created the first pulque company: The *Compañía Realizadora de Pulque, Sociedad Anónima*, intending to monopolize the pulque production, distribution, and sale. In 1909, was created the *Compañía Expendedora de Pulque, SCL,* by the powerful and influential pulque producers from the Apan zone in Hidalgo, Tlaxcala, and Estado de México, including 53 large haciendas. This company controlled the production of pulque, its distribution, and sale in Mexico City. In 1910, a new company, the *Compañía Expendedora de Pulques de Pachuca, Sociedad Anónima,* promoted by the first time the industrialization of aguamiel and pulque. In 1911 and 1912, the *Compañía Expendedora de Pulque, SCL* constructed a research facility in Mexico City to study the potential health properties of aguamiel and pulque. This company developed other valuable products, such as an aguamiel concentrate, a medicinal syrup (known as *Agavan syrup*), distilled 96° alcohol, vinegar, gums, and glues, some of them sold both in Mexico as in the USA (Ramírez Rancaño, 2000; Ramírez Rodríguez, 2004).

The Revolution War in Mexico (1910–1920) had a profoundly negative impact on the structure of the Haciendas pulqueras and their agroindustry. The railways had a crucial role in the civil war, and many of them were destroyed, interrupting the transport of pulque by the *Ferrocarril Mexicano* railway from the Apan zone to Mexico City. By 1914, the triumph of the constitutionalist revolution movement by Venustiano Carranza forced the owners of the Haciendas pulqueras (closer to the defeated president Porfirio Díaz) to leave Mexico. In 1916, the production, introduction, and consumption of pulque to

TABLE 1 | *Agave* species reported for aguamiel extraction (*magueyes pulqueros*).

Scientific name	Geographic distribution reported in Mexico[a]	Common names or comments
Agave atrovirens Kraw ex Salm-Dyck	Hidalgo, Mexico City, Morelos, Nuevo León, Oaxaca, Puebla, Querétaro, San Luis Potosí, Tlaxcala, Veracruz, Zacatecas	
Agave americana L.	Baja California Sur, Cólima, Coahuila, Chihuahua, Chiapas, Durango, Guanajuato, Hidalgo, Jalisco, México, Mexico City, Morelos, Nayarit, Nuevo Léon, Oaxaca, Puebla, Querétaro, San Luis Potosí, Sonora, Tamaulipas, Tlaxcala, Veracruz, Zacatecas	
Agave mapisaga Trel.	Guanajuato, Hidalgo, México, Mexico City, Morelos, Oaxaca, Puebla, Querétaro, San Luis Potosí, Tamaulipas, Tlaxcala, Zacatecas	*Carrizo*
Agave salmiana Otto ex Salm-Dyck	Aguascalientes, Chiapas, Coahuila, Colima, Mexico City, Durango, Guanajuato, Hidalgo, Jalisco, México, Morelos, Nuevo Léon, Oaxaca, Puebla, Querétaro, San Luis Potosí, Tlaxcala, Veracruz, Zacatecas	*Amarillo, ayoteco, colorado, chalqueño, chino, manso, prieto, xilometl, verde, negro, cenizo* Also reported as *Agave salmiana* subsp. *tehuacanensis* or *palmilla*
Agave hookeri	Jalisco, Michoacán, Sinaloa, Sonora	
Agave inaequidens	Durango, Hialgo, Jalisco, México, Mexico City, Michoacán, Morelos, Sinaloa, Tlaxcala	
Agave marmorata Roezl	Puebla	Wild agave used for pulque production

Based on the information reported by Alfaro Rojas et al. (2007), Mora-López et al. (2011), Escalante et al. (2016), Moreno-Terrazas et al. (2017), Peralta-García et al. (2020), Trejo et al. (2020), and Álvarez-Ríos et al. (2020).
[a] *Tropicos.org. Missouri Botanical Garden (2021).*

Mexico City were prohibited. Nevertheless, politicians allowed its production and sale because of the millions of Mexican pesos in tax revenues associated with the pulque agroindustry (Ramírez Rancaño, 2000; Juárez de Olarte and Juárez de Olarte, 2017). In the same year the *Compañía Expendedora de Pulque, SCL* was dissolved, and the large haciendas pulqueras were seized and dismantled the extensive maguey plantations for pulque production (Ramírez Rancaño, 2000; Ramírez Rodríguez, 2004). At the end of the Revolution War, several haciendas pulqueras survived, and pulque production continued. By the 1920s, pulque introduction by train to Mexico City continued; however, the dismantlement of many haciendas by the redistribution of the land after the Revolution War, the flourishment of the brewery industry, and the anti-*pulque* movement resulted in a substantial reduction in the number of maguey plantations deteriorating the pulque industry severely. In 1929, estimated pulque production was 234 million liters; by 1930, 167 million liters and maguey plantations were reduced to ∼50,000 Ha (Ramírez Rancaño, 2000; Lorenzo Monterrubio, 2007). By 1950s, official registers reported 17,000 cultivated ha of maguey pulquero, corresponding to ∼6.8% of the cultivated surface reported in the early twentieth century. The maguey plantations in pulque-producing zones were displaced by the introduction of barley cultivation, associated with unsuccessful government efforts to rescue the pulque agroindustry. Additionally, a significant increase in beer, alcoholic distilled beverages, wine, and soft drinks production and consumption in large cities and rural locations contributed to the critical decrement in pulque production and consumption (Ramírez Rodríguez, 2004; Lorenzo Monterrubio, 2007).

Nowadays, the larger maguey plantations for aguamiel extraction and pulque production are located in the States of Hidalgo, México, Tlaxcala, and Puebla (**Figure 1A**), but are distributed in a lower extent in other states such as Mexico City, Coahuila, Oaxaca, Veracruz, and Morelos (Mora-López et al., 2011; Escalante et al., 2016; Moreno-Terrazas et al., 2017; Chacón-Vargas et al., 2020; Rocha-Arriaga et al., 2020; Servicio de Información Agroalimentaria y Pesquera, 2021). From 1984 to 2019, pulque production showed two maximum production peaks by 1987 (∼550 million liters, produced in Estado de México) and 2013 (∼504 million liters, produced in Hidalgo), but in 2019 were reported the production of ∼171 million liters (a decrement of ∼66% of the maximum production peaks) (**Figure 1B**). Remarkably, Hidalgo State is the central production zone of pulque, providing an average of 73% of the pulque's national production in the last 10 years (Servicio de Información Agroalimentaria y Pesquera, 2021).

Despite the historical and economic relevance of pulque, the current traditional production of the beverage faces main significant limitations such: the considerable time required by the *Agave* species used for pulque production (at least 5 years) to complete their maturation for aguamiel extraction; the production process remains practically without changes since the golden age of production (late nineteenth and early twentieth century); the lack of an updated Mexican norm for aguamiel and pulque production quality and their microbiological, sensorial, and physicochemical properties; and the reluctance of traditional producers and consumers to change consumption habits from the fresh fermented beverage to canned or bottled options. In this review, we describe the current procedures for traditional aguamiel extraction and pulque production methods and how recent results on the analysis and stability of the physicochemical profile of aguamiel and the microbiology of the beverage led to propose a new baseline to redefine the quality criteria and to define for the first time a microbiological core to optimize its production; and the relevance of the sustainable cultivation of the maguey species used for aguamiel production as a fundamental element of an agroforestry system. In addition, we describe some successful examples of beverage industrialization resulting in high-quality pasteurized bottled or canned pulque production. Finally, we review the potential applications of several microorganisms isolated from aguamiel, pulque, aguamiel concentrate syrups, and the use of the maguey as the source of bioactive compounds and other high-value products.

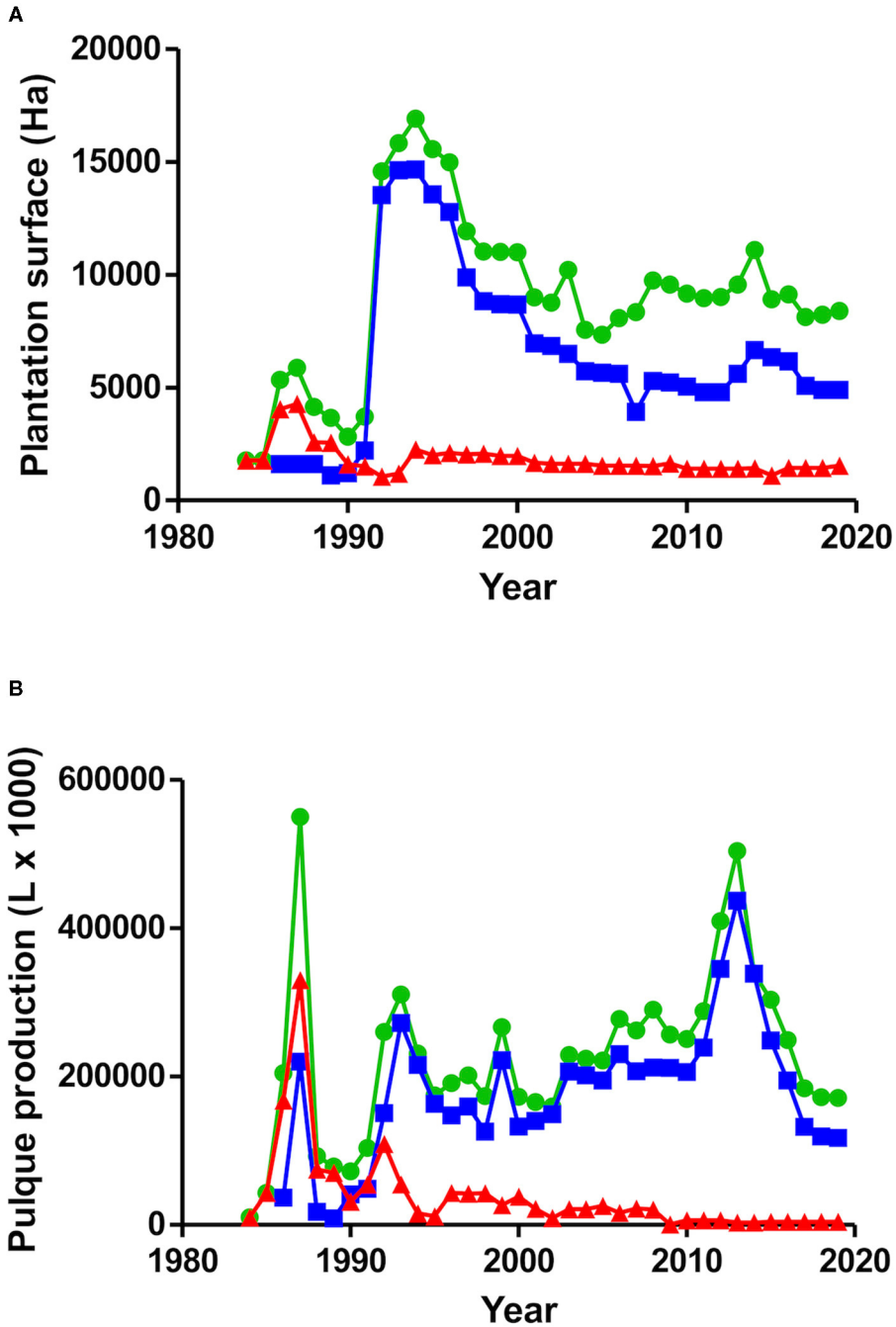

FIGURE 1 | Plantation surface of *maguey pulquero* plantation and *pulque* production. **(A)** Reported cultivated surface (ha) for *maguey pulquero*. **(B)** Reported *pulque* production associated to maguey plantations on basis of the available information at the Anuario Estadístico de la Producción Agrícola, Servicio de Información Agroalimentaria y Pesquera (2021). • National; ■ Hidalgo; ▲ Estado de México.

AGUAMIEL EXTRACTION AND PULQUE FERMENTATION PROCESSES

The pulque fermentation process starts with the selection of mature plants for aguamiel extraction with an age between 5 and 15 years (Ramírez Rodríguez, 2004; Lorenzo Monterrubio, 2007; Escalante et al., 2012, 2016; Peralta-García et al., 2020) and comprises four common steps in all the producing zones: (1) castration, (2) *cajete* scraping and aguamiel extraction, (3) seed preparation, and (4) fermentation (Ramírez

Rodríguez, 2004; Lorenzo Monterrubio, 2007; Escalante et al., 2016).

Maguey Castration

Selected mature plants are castrated by destroying the embryonic floral peduncle surrounding the floral bud (or *quiote*) (**Supplementary Figure 1**). During this operation, the plant's central leaves (heart, *meloyote*, or *cogollo*) are extracted with a pointed and sharp instrument (e.g., a pray bar), leaving a cavity known as *cajete*. The walls are then scraped with a metal scraping tool (a metal instrument like a large spoon) to shape the cavity (**Figures 2A–C**). The final cavity is covered traditionally with folded maguey leaves or with a large stone. This operation is usually performed in early spring or autumn fall. After this operation, the plant enters a maturation period ranging from 3 months to 1 year to promote the preservation of the treated plant's carbohydrate reservoir for aguamiel production (Nieto Aquino et al., 2016; Peralta-García et al., 2020). Immediately, a second scraping process is performed to enlarge the cavity, open the vessels, promoting the aguamiel outflux and its accumulation in the cavity. The castration process is performed traditionally by a person known as *tlachiquero,* who has a vast, in-depth knowledge of the maguey species for aguamiel production. The tlachiquero determines the precise moment for castration in mature plants, avoiding the floral bud's development. This a critical moment because if the floral peduncle (or *quiote*) grows, the plant is not useful for aguamiel production and dies after the inflorescence raises (Ramírez Rodríguez, 2004; Lorenzo Monterrubio, 2007; Narváez Suárez et al., 2016; Nieto Aquino et al., 2016; García Mendoza et al., 2017; Moreno-Terrazas et al., 2017; Álvarez-Duarte et al., 2018; Peralta-García et al., 2020). **Figure 2** illustrates the main activities involved in the extraction of aguamiel and pulque fermentation, and **Figure 3** shows a schematic representation of the stages for pulque production.

Cajete Scraping and Aguamiel Extraction

After the maturation period, the aguamiel extraction process is performed daily, twice (at morning and dusk), during the maguey's producing life until the plant dies. For this operation, the aguamiel accumulated in the cajete is extracted traditionally by oral suction using a large, dried gourd known as *acocote* (**Figures 2D–F**), or a device made with a hose or a plastic tube connected to a plastic soda bottle. In some rural regions, aguamiel is extracted with a plastic container, such as a cup, but some modern producers can use a pump for extraction. After aguamiel extraction, the cajete is scraped again, discarding the extracted plant material (or *metzal*) (**Figure 2G**). This action promotes the outflux of aguamiel during the day and repeats the operation at dusk. Collected aguamiel is transferred into plastic or wood containers (**Figures 2E,F**) and transported by the tlachiquero to the fermentation facilities known as *tinacal* (literally house of the tubs or vats), a facility (usually a large room or a specific zone) for aguamiel fermentation in vats, wood, or plastic containers (**Figures 2H–K**). The amount of aguamiel produced varies on the age, size (large plants can measure more than 5 m in diameter), and production lifetime (3–9 months) of the plant (Nieto Aquino et al., 2016; Álvarez-Duarte et al., 2018; Álvarez-Ríos et al., 2020; Peralta-García et al., 2020; Trejo et al., 2020). Ortiz-Basurto et al. (2008), reported an initial aguamiel production of 0.4 L/day during the first days of production. Then, the production volume increased to 6 L/day for several months, and final production of 0.4 L/day before the plant dies. Other authors reported a daily aguamiel production by several maguey species ranging from 2.5 L/day (in wild maguey plants such as *A. marmorata*) up to 10 L/day (for large plants of *A. salmiana* var. s*almiana)* in intensive maguey plantations, with a maximum total production between 1,500 and 1,960 L during the productive life of the plant (Tovar et al., 2008; Álvarez-Ríos et al., 2020).

Seed Preparation

Traditional tinacales contained large vats for aguamiel fermentation made initially of cow leather, where the shaved hairy side faces the inner side of the vat; these vats were replaced by wood and more recently by masonry, fiberglass, or plastic vats, some of them with a larger volume up to 1,000 L (Lorenzo Monterrubio, 2007; Moreno-Terrazas et al., 2017; Álvarez-Ríos et al., 2020). The operator and administrator of the tinacal, known as *mayordomo* (butler), had a higher status among workers in the former Haciendas pulqueras and was responsible for the seed preparation and pulque fermentation (Ramírez Rodríguez, 2004; Lorenzo Monterrubio, 2007). The seed is the starting material or inoculum for the fermentation of freshly collected aguamiel. The seed preparation process varies between pulque producing zones, but generally, for its preparation, 10–50 L of the best quality aguamiel is fermented in a small vat used only for seed preparation by pouring a volume of previously fermented aguamiel. The seed container is covered, and the fermentation develops at room temperature during 1–4 weeks until an upper layer called *zurrón* appears (Moreno-Terrazas et al., 2017). The seed is used to start a small volume fermentation for pulque production or prepare a second inoculum or seed known as *pie de cuba*. The pie de cuba is prepared by pouring into a new vat, one-fourth of the first seed, and three-fourths of high-quality fresh collected aguamiel (Crist, 1939; Moreno-Terrazas et al., 2017). The name used to refer to the seed varies among producing zones and practices: *Pie de pulque, pulque fuerte, asiento,* or *nancle* (Álvarez-Ríos et al., 2020).

Pulque Fermentation

The proper fermentation process for pulque production starts in a second fermentation vat where a volume of the pie de cuba or second seed is poured; then, freshly collected aguamiel is poured twice daily (in the morning and dusk) until it fills the vat. Then, the content of this vat is divided into two halves, and each half is poured into two new containers, respectively, starting the process again, now in two vats; then freshly collected aguamiel is poured twice daily until the vats fill. The mayordomos determine the fermentation time based on the development of characteristic sensorial properties, such as the alcohol content, acid notes, foam, the development of a characteristic viscosity, and tasting the fermented beverage (Escalante et al., 2016). A more extensive fermentation time, as 36 h, is reported as common practice for pulque production,

FIGURE 2 | Aguamiel collection and relevant traditional pulque production operations. **(A)** Metallic scraping tools or *raspador.* **(B)** Scraping process of the cajete's wall. **(C)** A close view of *the cajete* with accumulated aguamiel. **(D–F)** Aguamiel extraction by the tlachiqero using the traditional acocote and the sap's transference into a plastic container for transport to the tinacal. **(G)** Residual wall material or *metzal* in a plastic bucket after the scraping of the cajete. **(H–K)** Diversity of tinacales, fermentation vessels and vats. Images **(A,B,F,G–K)** were kindly provided by Mrs. E. Velázquez Gutiérrez.

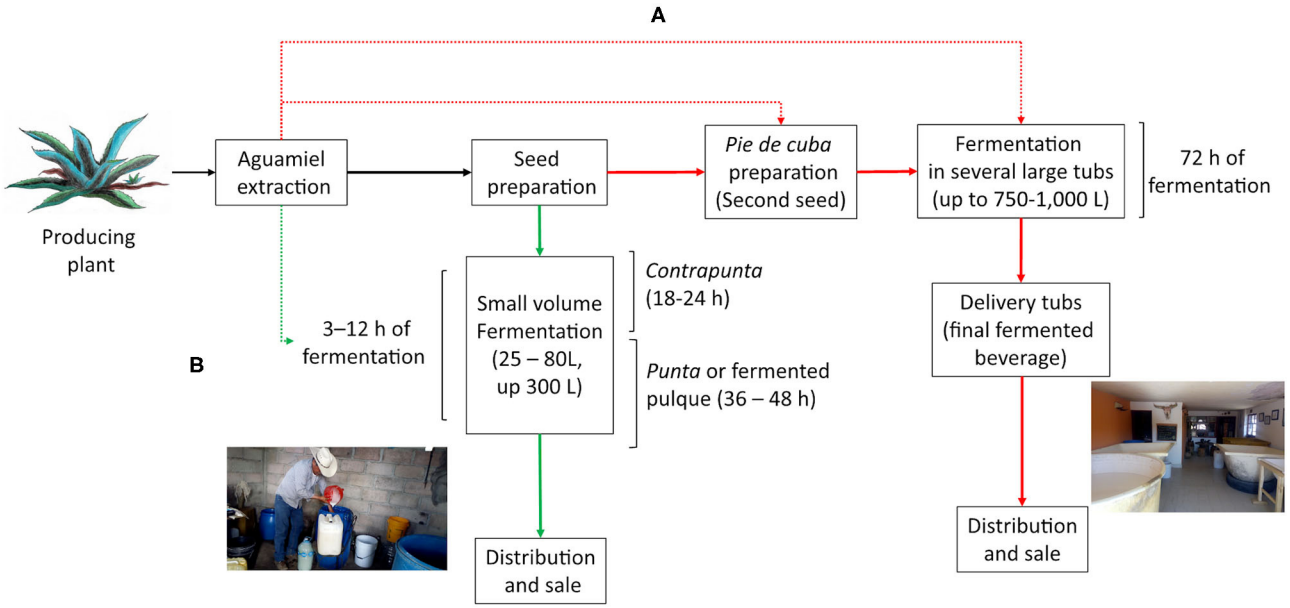

FIGURE 3 | Traditional pulque production flowchart. **(A)** Medium-large volume production process as described in section Types of Pulque and Properties. Dotted lines indicate pouring of freshly collected aguamiel to feed the pie de cuba and the fermentation tanks. **(B)** Small volume production process as described in section Types of Pulque and Properties. Dotted lines indicate freshly collected aguamiel to feed the fermentation tank. *Contrapunta* and *punta* are colloquial terms used by pulque producers to refer to an intermediate fermentation stage (contrapunta) and the final fermented pulque fermented (*punta*). This flowchart was elaborated from information reported in Crist (1939), Ramírez Rancaño (2000), Ramírez Rodríguez (2004), Escalante et al. (2016), Moreno-Terrazas et al. (2017), Álvarez-Ríos et al. (2020), and Rocha-Arriaga et al. (2020).

including seed preparation and further fermentation in vats as reported from three locations of Hidalgo State (Zempoala, Tepeapulco, and Epazoyucan) (Rocha-Arriaga et al., 2020). For a large-scale production process, the fermentation takes up to 72 h and is performed in several vats (up to 10) of 750–100 L each one. Larger producers deliver and sale the fermented pulque to pulquerías or restaurants offering this beverage (Álvarez-Ríos et al., 2020). In the traditional fermentation process, the final fermented pulque is not subjected to a stabilization process (e.g., pasteurization) to stop the microbial activity responsible for the fermentation. Small traditional pulque producers perform all the above-described stages and sold the fermented pulque directly from the tinacal during the day or in local markets (Ramírez Rancaño, 2000; Ramírez Rodríguez, 2004; Escalante et al., 2012, 2016; Moreno-Terrazas et al., 2017).

AGUAMIEL AND PULQUE SENSORIAL AND PHYSICOCHEMICAL PROPERTIES

Aguamiel Properties

Freshly collected aguamiel is a lightly amber, thick, sweeter, fresh plant flavored, and slightly acid sap (Escalante et al., 2016; Peralta-García et al., 2020). The Mexican norm (Norma Mexicana) defining the sensorial properties and quality criteria for aguamiel was published in 1972: NMX-V-022-1972 (*hidromiel, aguamiel*) (Secretaría de Economía, 1972a), and is still in force today. This norm defines the aguamiel as a light amber liquid with a characteristic smell and taste and is used as the fermentable substrate for pulque production. It also defines aguamiel as the "juice" obtained by scraping the cajete or the central cavity from maguey pulquero plants of the genus *Agave* and cultivated mainly in the States of México, Hidalgo, and Tlaxcala. The norm defines two types of aguamiel, Type I (high-quality aguamiel with high sugar content, used for seed preparation) and Type II (lower quality aguamiel, lower sugar content used for pulque production).

This norm establishes that acceptation or rejection of an aguamiel batch for pulque production requires the minimal determination of the pH (6.6–7.5 for Type I and 4.5 for Type II) and total reducing sugars (8–12 g/100 mL for Type I and 6.0 g/100 mL for Type II), determined in a sample of 100 mL for each aguamiel container. These analyses should be determined in the tinacal previously to deliver the freshly collected aguamiel for fermentation. The norm also establishes that high-quality aguamiel or Type I should be collected 15 days after the first cajete's scraping in the producing plant and during the next 30–60 days. Both Type I and Type II aguamiel should not show advanced fermentation.

Types of Pulque and Properties

As shown in **Figure 3**, two primary processes for pulque production are performed by traditional producers: A medium-large volume production process (**Figure 3A**) including the steps of aguamiel extraction, preparation of seed with high-quality aguamiel, the preparation of a second seed or pie de cuba, and the final fermentation process in large vats or tinacales. In this

process, the fermentation in the vat is a feed-batch process, where collected aguamiel is continuously poured into the fermentation vat twice daily (morning and dusk) for several days until the producer (mayordomo) determines the "end" of the process. A fermentation time of 72 h was reported for process in vats of 750–1,000 L. Then, the complete batch is usually transferred into wood barrels and delivered for sale. The second process is performed at smaller volume containers (**Figure 3B**), comprising aguamiel extraction, preparation of a seed, transference into a plastic container or wood barrels to start the fermentation, where twice daily collected aguamiel (morning and dusk) is poured and fermented. After several hours of fermentation, the beverage is withdrawn for *in situ* sales or distribution, usually in small plastic containers. Fermentation times vary from 3 to 12 for traditional producers, but the fermentation can extend up to 48 h. When the producer considers that the fermentation has finished, the beverage is delivered for sale. The remaining fermented product in the container is feed with new aguamiel, and the process is maintained for several days until the producer decides to discard the remaining beverage, then prepares a new seed and start a new fermentation process (Lorenzo Monterrubio, 2007; Escalante et al., 2016; Narváez Suárez et al., 2016; Moreno-Terrazas et al., 2017; Rocha-Arriaga et al., 2020).

The physicochemical and sensorial properties of fermented pulque are described in the Mexican norm (Norma Mexicana) NMX-V-037-1972 (Pulque—handled in bulk) (Secretaría de Economía, 1972b). This norm is still in force and describes the bulk handled pulque as the fermented beverage, with lower alcohol content, white color, not clarified, and viscous beverage produced from the fermentation of aguamiel extracted from maguey pulquero. It also defines the seed and pie de punta (described above) and the pulque's industrial production.

The norm defines two types of pulque, Type I (seed and pie de cuba) and Type II or commercial pulque. Relevant physicochemical properties for Type I are a final pH >3.5–4.0; sugar content of 0.10–0.8 g/100 mL of reducing sugars, expressed as equivalents of glucose; and 6–9% of alcohol content (v/v). For Type II, the norm defines a final pH of 3.0–4.0; sugar content of 0.20–0.50 g/100 mL of reducing sugars; and alcohol content of 4.0–0.6% (v/v). The norm defines the *biochemical properties of pulque* as a beverage resulting "from the equilibrated fermentative activity of the associated microbiota without evident alterations in the sensorial properties affecting its final quality." Sensorial properties described are a white color beverage for both types of pulque and "*sui generis*" taste and smell. The norm defines the quality criteria and sampling procedure for acceptance or rejection of a batch of pulque. These analyses were performed in the former customs facilities for pulque control in Mexico City, such as the customs facilities in the railway station of Pantaco (North of Mexico City), where pulque transported by train from the producing zones of Hidalgo and Tlaxcala was analyzed before final delivery to the sale sites. The norm establishes as mandatory that if a sampled 250 L cask of pulque was rejected, the entire batch should be disposed into the drainage. The former railway line for pulque transport to Mexico City and the custom of Pantaco closed in 1993 by high operating costs (Ramírez Rodríguez, 2004). Small traditional pulque producers have their quality criteria for the final fermented beverage and vary according to the producer; these criteria are mainly based on their own traditional tasting experience (Escalante et al., 2016).

STUDIES ON AGUAMIEL AND PULQUE'S MICROBIOLOGY: DEFINITION OF THE MICROBIAL CORE FOR THE FERMENTATION AND PROPOSING THE DEVELOPMENT OF INOCULUM FOR THE BEVERAGE PRODUCTION

Earlier studies on the microbiology of aguamiel and pulque described the isolation of *Zymomonas mobilis*; the lactic acid bacteria (LAB) *Leuconostoc mesenteroides, L. dextranicum, Lactobacillus* sp.; and the yeast *Saccharomyces cerevisiae* in aguamiel and pulque samples (Sánchez-Marroquín and Hope, 1953; Sánchez-Marroquín and Echegaray, 1954). These microorganisms were used as a mixed inoculum to start a controlled fermentation with sterilized aguamiel as substrate (inoculum 5% v/v, pH 5.0, 28°C). The sequential inoculation with the LAB followed by *S. cerevisiae* resulted in a high-quality fermented beverage (final alcohol content = 5.43°GL; final pH = 4.6, and total acidity (lactic acid) = 0.348) (Sánchez-Marroquín and Hope, 1953). Further attempts included *Z. mobilis* in the mixed inoculum, proposing the use of these microorganisms as a starter to ferment sterilized aguamiel to produce a good quality and a hygienic beverage (Sánchez-Marroquín and Hope, 1953; Sánchez-Marroquín et al., 1957, 1967).

Recent studies reported a high diversity of bacteria and yeasts both in aguamiel and pulque. A complete compilation of the microorganisms isolated and identified in aguamiel and pulque was reported previously, indicating the presence of a complex bacterial and yeast diversity (Lappe-Oliveras et al., 2008; Escalante et al., 2016). Nevertheless, recent non-culturable and metagenomic analysis of aguamiel and pulque from different geographical origins (Villarreal Morales et al., 2019; Escobar-Zepeda et al., 2020; Peralta-García et al., 2020; Rocha-Arriaga et al., 2020), as during a 6-h laboratory pulque fermentation (Chacón-Vargas et al., 2020), revealed the presence of non-previously reported bacterial and yeasts diversity and provided relevant biochemical and metabolic traits associated to aguamiel and the process of fermentation. Remarkably, only two reports correlate the microbial diversity dynamics with the biochemical y physicochemical properties of aguamiel (Peralta-García et al., 2020) and during the fermentation process (Chacón-Vargas et al., 2020).

Biochemistry and Microbiology of Aguamiel

Aguamiel quality and sugar composition during the plant producing lifetime has been analyzed to propose a baseline of quality for standardization purposes (Ortiz-Basurto et al.,

2008; Peralta-García et al., 2020). The study of the production lifetime (3 months) of three plants of *A. salmiana* from the location of Tecamachalco (18° 53′ N, 97° 44′ W; altitude 2,020 m), in Puebla State, showed that aguamiel composition has no relevant differences among samples collected at different time points during the producing life of the studied plants. Samples had a relatively constant pH (∼4.5) and a dry matter (11.5 wt %) content composed mainly by 74 wt % sugars, including 8.8% sucrose; 26.5% glucose; 32.4% fructose; and 10.2% fructooligosaccharides (FOS) with a high degree of polymerization (DP), mainly inulin-type fructans and agavins with few β-fructofuranosyl units linked by β1-2 and β2-6 linkages. These authors concluded that aguamiel is a stable raw material for pulque production suitable for the standardization of pulque industrial production (Ortiz-Basurto et al., 2008).

The analysis of sugar composition in aguamiel collected from three plants from the pulque producing locality of Huitzilac, Morelos (19°01′42″ N 99°16′02″ O; altitude 2,561 m), showed variation during the producing lifetime (4–9 months) among three analyzed plants: *A. mapisaga* (two plants: 1 and 2) and *A. salmiana* (one plant: 3) (Peralta-García et al., 2020). These authors reported for the first time the characterization of the sap accumulated in the cajete immediately after the scraping of the cavity (15–20 mL collected immediately after scraping, named as *fresh aguamiel*) and the accumulated sap 7 h after scraping, which is collected for pulque production. Remarkably, in the first 9–13 weeks of the producing lifetime, the fresh sap's pH was neutral to slightly alkaline (7.0–10), but the accumulated aguamiel was slightly acid (5.0–3.5) among the three plants. The fresh sap contains just sucrose and FOS but not fructose or glucose (resulting from the hydrolysis of sucrose). During the production lifetime, sucrose content remains relatively constant in the fresh aguamiel but decreases between 30 and 50% after the accumulation time, associated with the presence of glucose and fructose. Nevertheless, fructans showed the highest concentration in fresh aguamiel between the first third and the midpoint of the production lifetime with a full FOS content between weeks 9 and 15 (Peralta-García et al., 2020).

FOS analysis in fresh and accumulated aguamiel of the three analyzed plants showed three types of fructan profiles: Type I found in the three studied plants during the early sap accumulation period after scraping. These FOS showed a close related profile to inulin or isomalto-oligosaccharides, different to the FOS of the plant, possibly synthesized by the glycosyltransferase activities of microorganisms present both in the plant as in the sap. These FOS were proposed to be partially or hydrolyzed or consumed by the sap's microbiota. Type II was found in aguamiel collected from plants at intermediate and late production times, identified as a complex mixture of oligosaccharides such as 1-kestose and 6-kestose signals, proposed as the early intermediates of the synthesis of inulin or levan, respectively. These FOS's presence was proposed due to the hydrolysis of agavins or by enzymatic synthesis mediated by microbial fructosyl transferase enzymes. Type III, corresponding to FOS found in the plant's intermediate and late producing lifetime, with a similar profile to that of the FOS detected in fresh sap after scraping and diluted as they accumulated in the cavity. FOS identified in aguamiel Types I and II have a β2-1 or β2-6 structure, respectively, a different structure from that α1-6 type corresponding to dextran, the primary polymer present in fermented sap. These authors also estimated an aguamiel production of 676.6 L for plant 1, 662.3 L for plant 2, and 334 L for plant 3; total sucrose present in aguamiel was estimated at 70.6 Kg for plant 1, 61.5 Kg for plant 2, and 36.8 Kg for plant 3; and fructans were estimated at 3.0 Kg for plant 1, 1.7 Kg for plant 2, and 2.1 Kg for plant 3 (Peralta-García et al., 2020). These authors proposed that the described FOS profile could be considered a marker for high-quality aguamiel as the substrate for pulque production and suggest this profile as the baseline for quality control purposes. Remarkably, the DP of the FOS found in producing plants from Huitzilac, Morelos (two plants of *A. mapisaga* and one of *A. salmiana*) agrees with the FOS profile reported for three plants of *A. salmiana* previously studied from the location of Tecamachalco, Puebla (Ortiz-Basurto et al., 2008). Finally, the analysis of the bacterial diversity present in fresh sap by a metagenomic approach showed a significant abundance at the genus level for *Leuconostoc* (46.08%), *Zymomonas* (35.98%), *Acetobacter* (5.0%), *Lactococcus* (4.67%), and *Acinetobacter* (3.22%). Among these bacteria, *Leuconostoc* and *Zymomonas* are proposed to synthesize additional FOS detected in aguamiel by their fucosyltransferase enzymes (Peralta-García et al., 2020).

Biochemistry and Microbiology of Pulque Fermentation

Chacón-Vargas et al. (2020), analyzed by a metagenomic shotgun sequencing approach the diversity present in overnight accumulated aguamiel (collected at daybreak) and in overnight fermented pulque from the locality of Huitzilac, Morelos, and during a laboratory fermentation of 6 h started by mixing a sample of the daybreak collected aguamiel and a sample of the overnight fermented pulque. Their results showed a great bacterial diversity determined by the Simpson's diversity (D) and Shannon's (H) diversity indexes in aguamiel, compared to that in fermented pulque and during three fermentation times analyzed (0, 3, and 6 h). Aguamiel showed a D = 9.03 and H = 2.86. At the genus level, the highest diversity was composed of *Acinetobacter* (21.95%), *Leuconostoc* (13.92%), *Lactococcus* (13.72%), *Zymomonas* (4.77%), and *Lactobacillus* (0.97%). At the species level, the highest diversity was composed by *Lactococcus plantarum* (8.50%), *Z. mobilis* (4.78%), *A. nectaris* (2.68%), *L. gelidum* (1.67%), *L. citreum* (1.68%), *L. piscium* (1.65), *A. buissieri* (1.59%), and *L. lactis* (0.97%). Remarkably, the yeast *Saccharomyces* was present in a lower percentage (0.03%). Diversity at T0 (start of the laboratory fermentation by mixing 2:3 L of aguamiel:pulque, and maintained at 23°C) showed a D = 5.88 and H = 2.43. The highest diversity at the genus level was composed of *Lactococcus* (19.52%), *Leuconostoc* (17.34%), *Zymomonas* (12.57%), *Saccharomyces* (10.79%), *Acinetobacter* (5.73%), and *Lactobacillus* (4.94%). At species level: *Z. mobilis* (12.57%), *L. plantarum* (12.11%), *L. citreum* (2.44%), *S. cerevisiae* (2.42%), *Lactobacillus sanfranciscensis* (2.22%), *L. gelidum* (1.77%), *L. citreum* (1.68%), and *L. lactis* (0.79%). At T3,

D = 4.19 and H = 2.21. At the genus level, diversity was composed of *Zymomonas* (19.91%), *Saccharomyces* (19.08%), *Leuconostoc* (12.38%), *Acinetobacter* (5.45%), and *Lactobacillus* (3.61%). At the genus level, *Z. mobilis* (19.91%), *L. plantarum* (7.08%), *S. cerevisiae* (4.05), *L. sanfraciscensis* (1.41%), *L. piscium* (1.31%), *L. geliudum* (1.29%), and the yeast *S. eubayanus* (0.99%). At T6, D = 3.71 and H = 2.14. Diversity at genus level was *Zymomonas* (22.27%), *Lactococcus* (11.73%), *Acinetobacter* (7.17%), *Lactobacillus* (7.08%), and *Saccharomyces* (5.65%). At the species level: *Z. mobilis* (22.27%), *L. plantarum* (7.33%), *L. sanfranciscensis* (2.2%), *L. gelidum* (1.72%), *L. piscium* (1.39%), S. *cerevisiae* (1.25%), and *L. citreum* (1.2%). Finally, fermented pulque had a D = 4.10 and H = 2.13. At the genus level *Z. mobilis* (21.48%), *Leuconostoc* (14.30%), *Saccharomyces* (13.51%), *Lactococcus* (13.03%), *Lactobacillus* (7.53%), and *Acinetobacter* (2.85%). At the species level, the highest diversity was composed by *Z. mobilis* (21.48%), *L. plantarum* (8.11%), *L. sanfranciscensis* (3.71%), *S. cerevisiae* (2.86%), *L. piscium* (1.57%), *L. gelidum* (1.47%), and *L. citreum* (1.04%).

Rocha-Arriaga et al. (2020), explored the microbial diversity during a traditional fermentation process, including the analysis in the collected aguamiel (0 h), in the middle stage of the fermentation (18 h) (known by producers as *contrapunta*), and in the final fermented pulque (36 h) (also known as *punta*) from three locations of Hidalgo State (Epazoyucan 20°01′ 03″ N; 98° 38′ 11″ W, an altitude of 2,456 m; Tepeapulco 19° 47′ 06″ N; 99° 33′ 11″ W, altitude 2,508 m; and Zempoala 19° 48′ and 20° 03′ N; 98° 50′ W and altitude of 2,400–2,900 m) by sequencing of the 16S rDNA V3-V4 regions for bacterial diversity and ITS sequencing for yeasts diversity. Contrary to the results by Chacón-Vargas et al. (2020), these authors found a lower diversity of OTUs in aguamiel (H = 1.47, D = 0.39), an important increment at the middle stage (contrapunta, 12 h of fermentation, H = 2.5, D = 0.61), and a further decrement in diversity in fermented pulque (36 h of fermentation H = 1.58, D = 0.45). These authors identified in all the studied stages as the most abundant OTUs *Sphingomonas, Acetobacter, Lactobacillus, Acinetobacter, Enterobacter, Gluconobacter, Halomicronema, Lactococcus, Leuconostoc, Marivitia, Serratia*, and *Weissella*. Among these, the most abundant OTUS found in analyzed sap were *Sphingomonas, Lactobacillus*, and *Acinetobacte*r; in contrapunta, the most abundant OTUs were *Acinetobacter, Sphingomonas*, and *Lactobacillus;* whereas the most abundant OTUs in pulque were *Sphingomonas, Lactobacillus*, and *Acetobacter*. Remarkably these authors did not find *Zymomonas* in the analyzed samples. Regarding yeasts diversity, the most abundant yeasts identified in all production stages were *Candida zemplinina* (25% in sap, 1.7% in contrapunta, 17.98% in pulque); *Clavidospora lusitaniae* (23% in sap, 16% in contrapunta, 9% in pulque), and *Candida stellate* (0.6% in sap, 32.96% in contrapunta, 6.19% in pulque), but *Saccharomyces* was found as the 9th most abundant yeast in all samples (0.74% in sap, 13.31% in contrapunta, 8.49% in pulque) (Rocha-Arriaga et al., 2020). The absence of *Zymomonas* in the analyzed samples by these authors is a relevant result as this bacteria has been reported in pulque samples since the earlier studies of Linder on the microbiology of aguamiel and pulque between 1923 and 1924 (described as *Thermobacterium mobile*) (Swings and De Ley, 1977) and by Sánchez-Marroquín and Hope (1953). *Zymomonas* sp. was also reported in studied pulque samples from Oaxaca State, outside of the main producing zone of Hidalgo State (Valadez-Blanco et al., 2012).

Remarkably, *Sphingomonas,* the most abundant genus reported in aguamiel and pulque fermentation identified by Rocha-Arriaga et al. (2020), was reported in the analysis of a mixed pulque sample of 24 and 48 h of fermentation from Huitzilac, Morelos by a whole metagenomic sequencing strategy, showing a relative abundance of 2.47% (Escobar-Zepeda et al., 2020), and also reported by Chacón-Vargas et al. (2020), but detected in a lower abundance (above 0.1% in T6 and fermented pulque samples).

The studies on the microbiology of aguamiel and during the fermentation process showed high microbial diversity with common microbial groups among studied samples independently of the plant's geographical origin, species, and local variations involved in the fermentation process. Nevertheless, a systematic and standardized methodology is required to provide information on the relevance of the significant and minor microbial groups present in aguamiel and pulque fermentation from diverse geographical origins. This information should be correlated with the sap and fermented pulque's main physicochemical characteristics to propose a microbial core responsible for the beverage's main sensorial properties to define a mixed starter culture to standardize the fermentation process.

TOWARD THE DEFINITION OF A MICROBIAL STARTER CULTURE FOR PULQUE PRODUCTION

A starter culture is defined as a selected microbial preparation developed to increase the efficiency of a fermentation process, providing a quick start of the process, reducing the risk of fermentation failure, and improving safety, stability, and the final sensorial value of the fermented product. The production of fermented products with a superior quality depends on the presence, growth, and metabolic activity of specific microorganisms (Vinicius De Melo Pereira et al., 2020). It is necessary to establish a microbiological core and a new physicochemical and sensorial definition of aguamiel and pulque to improve and standardize the fermentation process. Escalante et al. (2016) proposed that fermented pulque's final sensorial properties are defined by the simultaneous development of four fermentation types: (i) An alcoholic fermentation performed by *Z. mobilis* and *S. cerevisiae* to ferment available sugars in aguamiel (sucrose, fructose, and glucose), resulting in ethanol production. (ii) A lactic acid fermentation developed mainly by LAB including several species of *Leuconostoc, Lactobacillu*s, and *Lactococcus,* which result in the production of lactic acid, acetic acid, and possibly other minor metabolic products such acetoin, diacetyl, and butanediol. (iii) An acetic acid fermentation by acetic acid bacteria such as *Acetobacter* and *Gluconobacter*. (iv) The production of exopolysaccharides like dextran and levan

by *L. mesenteroides* and *Z. mobilis,* respectively, is responsible for developing the fermented beverage's characteristic viscosity. Nevertheless, recent metagenomic reports on the diversity of pulque from Huitzilac (Morelos) and three Hidalgo State locations showed that *S. cerevisiae* was detected as a minor microorganism in aguamiel as in the fermentation process. Likewise, the acetic acid bacteria *Acetobacter* and *Gluconobacter*, previously reported (Escalante et al., 2004, 2008), were not detected or were detected as a minor microorganism by the two metagenomic approaches (Chacón-Vargas et al., 2020; Rocha-Arriaga et al., 2020).

A remarkable effort to propose a microbial core with aguamiel and pulque fermentation's physicochemical properties was also reported (Chacón-Vargas et al., 2020). These authors correlated the temporal patterns of the major genera and species present at $\geq$1% in the aguamiel and at least one fermentation stage and their association with the significant fermentative products of pulque such as lactic and acetic acids and ethanol from available sugars (sucrose, fructose, and glucose). Results showed that *Acinetobacter, Lactococcus*, and *Leuconostoc* were highly abundant in the sap (>13%). Both *Lactococcus* and *Leuconostoc* showed a slight fluctuation during the 6-h fermentation but were present at the same abundance in fermented pulque. Nevertheless, *Acinetobacte*r decreased during the fermentation process (6 h) but increased to 2.85% in overnight fermented pulque. The abundance of this bacteria was proposed as positively associated with glucose content and negatively with fructose (resulting from the hydrolysis of sucrose or FOS) and ethanol. The abundance of *Zymomonas* correlated positively with the increased production of ethanol, lactate, and an increment in fructose concentration, and negatively respect to sucrose content. Regarding *Lactobacillus* abundance, it was positively correlated with lactate and ethanol production. Remarkably, *Saccharomyces* showed lower abundance in aguamiel (0.033%), fluctuated during the 6-h fermentation, and increased to 13.51% in fermented pulque. As stated above, a relevant conclusion from this work was that the abundance of *Saccharomyces* did not correlate significantly with sugar or ethanol abundance, focusing on the relevance of *Z. mobilis* in ethanol production during the fermentation of pulque (Chacón-Vargas et al., 2020). It is relevant to highlight that *Acinetobacter* was detected by both metagenomic approaches as one of the most abundant genera in aguamiel, during the fermentation process, as in the final fermented product (Chacón-Vargas et al., 2020; Rocha-Arriaga et al., 2020). The genus *Acinetobacter* has been reported in traditional fermented foods such as the *chicha* from Brazil, the *koko* from Ghana, and the *chikwangue* from Zaire (Tamang et al., 2016). This bacterium was previously reported in aguamiel and pulque samples from Huitzilac, Morelos, but in a lower proportion (Escalante et al., 2008; Escobar-Zepeda et al., 2020). *A. nectaris* (reported by Chacón-Vargas et al., 2020) is a Gram-negative bacterium reported as the most abundant bacteria found in the floral nectar of some cultivated plant species from Northern Israel and wild plants in the Southern Spanish Mediterranean Sea. This bacterium can grow on glucose and oxidizes D-fructose and sucrose. Remarkably, produces levan from sucrose by a periplasmic levansucrase (Álvarez-Pérez et al., 2013; González-Garcinuño et al., 2017). High sugar content in aguamiel supposes a suitable environment for this bacterium.

The metabolic activities shown in **Figures 4**, **5**, resulting in the production of ethanol, lactic acid, acetic acid, CO_2, dextran, and levan, could be considered as a basal line for the development of a well-defined inoculum for pulque fermentation, composed by *Z. mobilis, Leuconostoc, Lactobacillus, Lactococcus, Acinetobacter,* and possibly *S. cerevisiae*. The use of these well-adapted microorganisms to the physicochemical conditions in aguamiel and pulque represent an opportunity to reduce some stages during the fermentation process, the fermentation time, and increasing reproducibility of the process. It is necessary to determine which species of *Leuconostoc* and *Lactobacillus* could be considered essential for a precise mixed inoculum development for controlled pulque production. According to Chacón-Vargas et al. (2020), *L. plantarum* was the second most abundant bacterium after *Z. mobilis* in the sap, during a 6-h fermentation, as in 12-h fermented pulque. In contrast, *Lactobacillus sanfranciscensis* was the most abundant lactobacilli detected by these authors, but other *Leuconostoc* species such as *L. citreum, L. kimchi*, and *L. mesenteroides* have been detected in aguamiel and pulque from Huitzilac as main microorganisms (Escalante et al., 2004, 2008; Escobar-Zepeda et al., 2020).

Recent metagenomic, physicochemical, and metabolic characterization of aguamiel and pulque fermentation process has provided valuable information to propose for the first time the basis for a new definition of the physicochemical and biochemical quality of the aguamiel used as a substrate for pulque production and the development of a defined mixed inoculum suitable to improve the quality, stability, and end products of the pulque fermentation process. It is necessary to use this valuable information to propose a new Mexican norm (or to update the existing ones) to define the quality and sensorial properties for aguamiel and pulque, based on precise analytical criteria and the standardization of the fermentation process under controlled production conditions.

RELEVANCE AND CURRENT SITUATION OF THE CULTIVATION OF MAGUEY FOR PULQUE PRODUCTION AND DERIVATIVE TRADITIONAL AND VALUABLE PRODUCTS

Cycle of Life of Maguey Pulquero

The agaves are plants with unique biological and ecological characteristics. These plants, known as maguey or *mezcales*, are endemic to the American Continent and are members of the Family Asparagaceae, Subfamily Agavoideae, genus *Agave,* comprising ∼210 species (Colunga-GarcíaMarín et al., 2007; The Angiosperm Phylogenetic Group, 2009). In Mexico, the genus is widely distributed, covering 76% of its territory with 61% of endemic species. Showed a tremendous significant diversification as they grow from the sea level up to 3,000 m altitude (García Mendoza, 2011; García Mendoza et al., 2017, 2019).

The agaves for pulque production are plants of vital aspect, perennial, formed by rosettes of lanceolate leaves arranged in

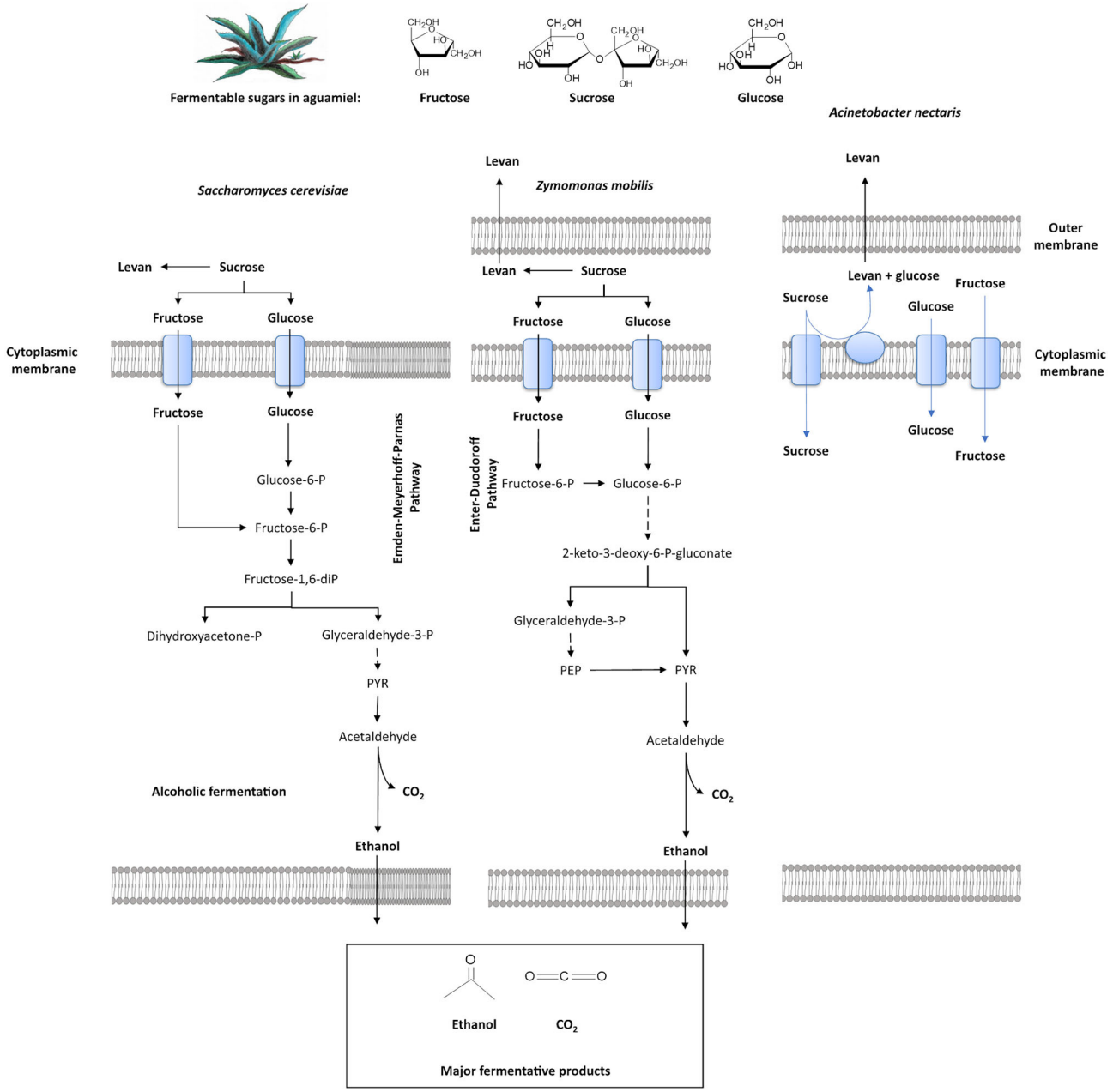

FIGURE 4 | Schematic representation of key metabolic pathways from the possible microbial essential core for pulque fermentation: The role of *Saccharomyces cerevisiae, Zymomonas mobilis,* and *Acinetobacter nectaris.* Sugar transporter systems are shown as blue structures in the cytoplasmic membrane. Continue arrows represent single metabolic enzyme-catalyzed reactions. Dotted lines represent several enzymatic reactions. Adapted from Swings and De Ley (1977), Sahm et al. (2006), Álvarez-Pérez et al. (2013), Walker and Stewart (2016), Weir (2016), Yang et al. (2016), Jansen et al. (2017), Chacón-Vargas et al. (2020), Rocha-Arriaga et al. (2020), and Carvalheira et al. (2021).

a spiral, succulent, fibrous and thick, thorny in the margins of the apex. It has a panicle inflorescence of 5–12 m, with greenish-yellowish flowers (or *gualumbos*) (García-Mendoza, 2002). These plants have a sexual reproduction by seeds resulting from pollination of the inflorescence and vegetative propagation by transplanting of stoloniferous shoots (named *hijuelos* or *matecuates*) of the mother plant; from bulbils, which are seedlings born from leaves, buds, rhizomes, and by micropropagation (**Supplementary Figure 2**; García Mendoza, 2007; García Mendoza et al., 2017; Torres-García et al., 2019). The sexual reproduction of this plant is semelparous or monocarpic, which indicates that the plant dies after a unique event of sexual reproduction (García Mendoza, 2007). The maguey cultivation cycle comprises the following stages

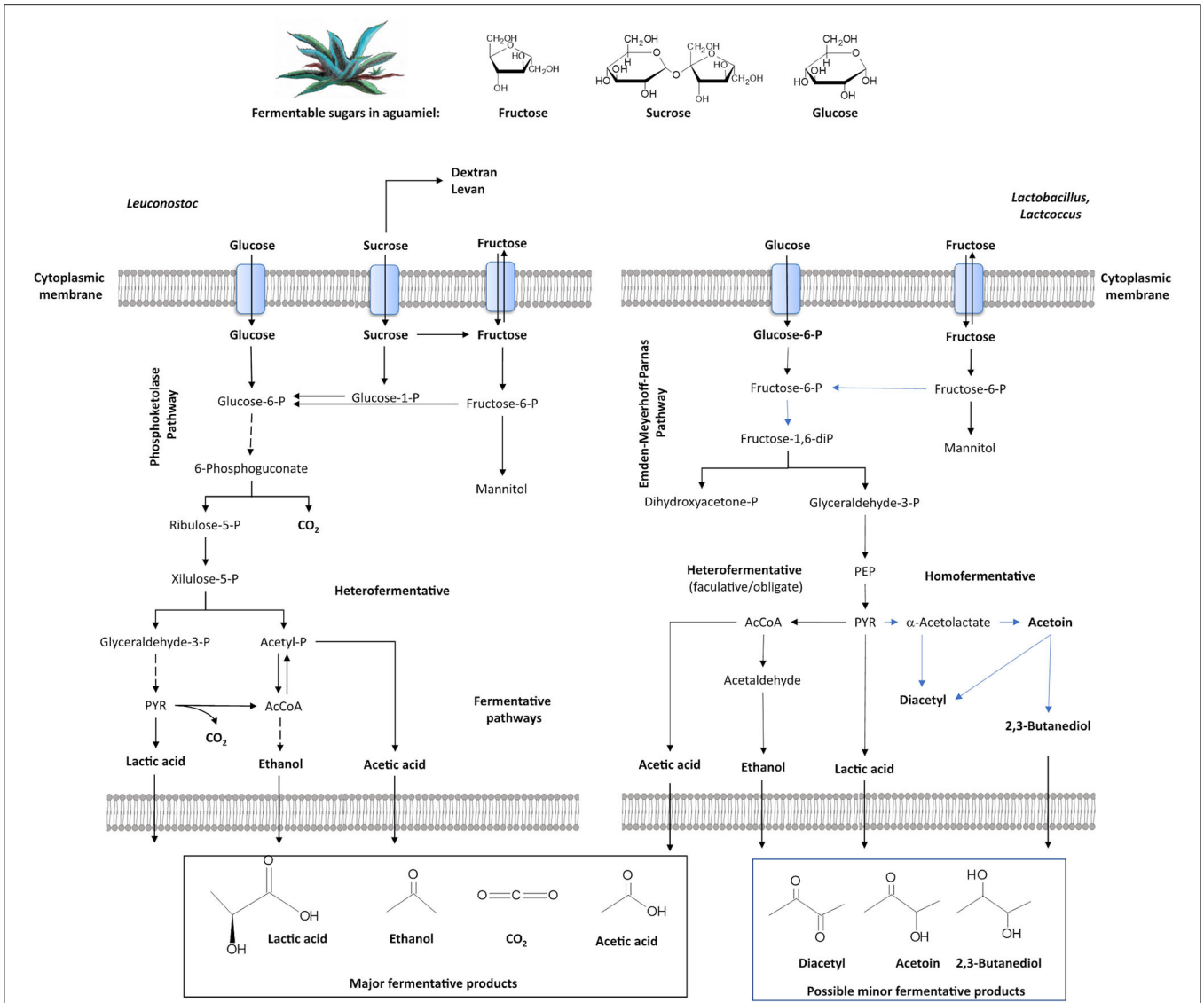

FIGURE 5 | Schematic representation of key metabolic pathways from the possible microbial essential core for pulque fermentation: The role of *Leuconostoc, Lactobacillus,* and *Lactococcus*. PEP, phosphoenolpyruvate; PYR, pyruvate. Sugar transporter systems are shown as blue structures in the cytoplasmic membrane. Continue arrows represent single metabolic enzyme-catalyzed reactions. Dotted lines represent several enzymatic reactions. Adapted from Dols et al. (1997), Melchiorsen et al. (2001), Zotta et al. (2017), Chacón-Vargas et al. (2020), Rocha-Arriaga et al. (2020), and Carvalheira et al. (2021).

(**Supplementary Figure 2A**; Ramírez Rodríguez, 2004; Nieto Aquino et al., 2016): (i) Reproduction. Young plants from germinated seeds, consolidated seedlings (2–6 months), and the stoloniferous shoots of 1-year-old and a height of 50-60 cm are cultivated in a greenhouse. Young plants are transplanted to the field when they reach 1 m in height or are 1–2 years old (**Supplementary Figure 2B**). (ii) Transplanting of stoloniferous shoots. When the plants reach adulthood (up to 8 years) produce several *hijuelos*. Vegetative propagation by transplanting the stoloniferous shoots led to the exchange of different species or varieties of plants from a diverse geographic origin, resulting in maguey plantations with mixed populations (Torres-García et al., 2019; Álvarez-Ríos et al., 2020). (iii) Physiological maturity: The adult plants are ready for flowering, their growing activity decreases, and channel resources for the rise of the floral bud. It is in this phase where the preparation for the extraction of aguamiel (castration) takes place. This process lasts from at least 5 years depending on the species (García Mendoza, 2007; Nieto Aquino et al., 2016; Álvarez-Ríos et al., 2020).

The use and management of these plants for thousands of years have resulted in an artificial selection process, with gigantism being the most evident result. In the case of *A. mapisaga*, these plants can reach up to 4 m in height, representing higher mead yields. Other effects of artificial

selection are reducing morphological mechanisms (e.g., smaller size and lesser number of teeth or lateral spines) and chemical defense mechanisms, making these plants easier to manipulate. The propagation by offsprings over sexual reproduction resulted in the development of a stable genotype maintained for several generations, but with a decreased genetic diversity, larger population structure, and less gene flow, in addition to increasing its vulnerability to diseases (Álvarez-Duarte et al., 2018; Torres-García et al., 2019).

The Maguey Pulquero in the Agroforestry Systems and Large Plantations for Aguamiel and Pulque Production

The agroforestry systems are spaces where wild and agricultural diversity coexist (Torres-García et al., 2019). In Mexico, agaves for aguamiel production have been used since Pre-Hispanic times with this purpose in an agricultural model known as *metepantle* (*metl* = agave and *pantli* = wall). The metepantle is a cultivation terrace characterized by having a soil containment board where magueys are planted. The space between the board or rows of cultivated magueys retains the soil and is used to grow other crops in zones with orography of steep slopes and high erosion, mainly in the States of México, Tlaxcala, Hidalgo, and Oaxaca (Álvarez-Duarte et al., 2018; Torres-García et al., 2019; Álvarez-Ríos et al., 2020; Viniegra-González, 2020). Agave plantations in the model of the metepantle are essential components of the agroforestry systems, as these plants retain soil and humidity, promoting the infiltration of water into the soil. If the plants bloom, they attract various pollinators such as bees and various species of nectar feeders, promoting pollination. These systems are also habitats for different species of animals (Torres-García et al., 2019).

Current maguey plantation for pulque production comprises four models classified based on the type and intensity of management to which they are subjected, the diversity and number of plants cultivated and managed for production, and the amount of aguamiel and pulque produced (Álvarez-Ríos et al., 2020):

a. *Intensive maguey plantation.* This model includes private or communal properties where magueys are grown at a high density. An example of this plantation model is located in Nanacamilpa, Tlaxcala State. This high-density cultivation area comprising 44 ha with 2,500 plants per ha, of which 500 plants are used for aguamiel production, yielding 2,500–3,000 L/day. The magueys used belongs to the varieties *ayoteco* and *chalqueño* with a maturity age of 12–14 years; and *manso* and *púa larga*, with a maturity age of 8–10 years. The aguamiel production life depends on the maguey's size: For larger plants, it is 5–7 months, and for smaller ones, 4–6 months. Pulque production is carried out in 10 fiberglass vats with a capacity of 750–1,000 L each, and the fermentation process takes 72 h. The producer uses sensorial criteria to define the fermentation's end, and the naturally fermented pulque is mixed with fruits and pasteurized to kill associated microbiota and stop the fermentation. The pasteurized product is canned and exported. This production model produces up to 20,000 L of pulque monthly.

b. *Remaining maguey plantations*. This small system (<1 ha) corresponds to the remaining maguey plantations enclosed in urban zones, represented by a maguey plantation in the west of Mexico City (municipality of Cuajimalpa). This plantation is an example of a familiar plantation where the magueys are planted every four meters in rows. The plants have a maturity age of 10 years and correspond to the varieties *chalqueño, manso*, and *mano larga*. In this plantation, 15 plants are used for production, yielding 68–80 L/day for 3 months. Pulque fermentation is performed in 25 L buckets, and the fermentation time is 3–6 h. The pulque is produced for self-consumption or local sale.

c. *Metepantle.* An example of this system is located in the Rancho la Coyotera, municipality of Zacualtipán de Angeles, Hidalgo State. The magueys are planted in staggered rows with native vegetation on a surface of 20 ha. The planted magueys include eight varieties, of which the most abundant are the *manso de zoqui* and a wild variety or *corriente*. In minor proportion the varieties *penca ancha, púa larga, penca larga, espina china, corriente cenizo*, and *corriente colorado*. Thirty-five plants with an age of 8 years are used for production, yielding 30–100 L/day. The fermentation is performed in an 80 L plastic vessel located in a tinacal, and the fermentation process takes 2 h. The product is sold locally, by order, in neighboring localities, or producers' fairs in large cities.

d. *Bordering plants.* This plantation is a standard system in the rural landscape where the magueys border a crop or grazing surfaces (2–4 ha) functioning as living fences to delimit and protect plots of land, forming small agave communities, or bordering roads. One example of this model is located in the municipality Santiago Undameo, Michoacán State. The maguey species planted are the varieties *verde, negro, cenizo,* and *tarimbaro*. Twenty plants are used for aguamiel production at the age of 10 years old, with a producing life of 4 months, yielding 30–60 of L aguamiel daily. The fermentation is performed in 80 L plastic vessels located in the producers' house. In this location, the collected aguamiel is boiled for several minutes to be drunk or fermented. The fermentation occurs in up to 80 L containers for 3 h and is sold in local markets or nearby towns.

e. *Wild maguey*. This system remembers the ancient maguey utilization for pulque production as it uses wild agave species. The example of this system is located in San Juan Raya and Zapotitlán Salinas, municipality of Zapotitlan, Puebla State, where the wild agave species of *A. marmurata* Rozel is used for aguamiel production in the wild, by transplanting offsprings or by the transplantation of the entire young plants closer to the producer's home. The plants used for aguamiel extraction are 12–15 years old and produce 1.5 L/day for 2 months. In this system, 35 plants are used for production, and aguamiel is collected three times daily. The fermentation is performed for 3 h in 25 L buckets located inside the house's producer, and the fermented beverage is sold.

Each one of the systems described above maintains self-sustaining plant cultivation practices, trying to maintain a stable plant population by transplanting offspring to ensure new plants' availability. Sexual reproduction of plants is not allowed in

any system (Álvarez-Ríos et al., 2020). Nevertheless, maguey cultivation for pulque production faces different situations that have resulted in a significant decrease of plants in some regions, where pulque production has practically disappeared. A fundamental problem for pulque producers is the lack of regulation for clandestine activities such as *desmixiotado*, an illegal activity that consists of removing the cuticle (*mixiote*) of the leaves or *pencas* or the mutilation of the entire leaves of the plant for cuisine purposes, which significantly affects its development (José-Jacinto and García Moya, 2000; Álvarez-Duarte et al., 2018). The extraction of two species of lepidopteran caterpillars or maguey worms (see below), which have a high gastronomic value, results in the plant's destruction. The disappearance of the maguey due to its irrational or clandestine use, the scarce and inadequate propagation strategies, and the lack of interest in pulque production by new generations have seriously affected the pulque agroindustry in some regions, becoming its production as a secondary economic activity (Álvarez-Duarte et al., 2018).

Production of Traditional and New Products From Maguey, Aguamiel, and Pulque

The cultivation and propagation of maguey pulquero are activities performed mainly to extract aguamiel for pulque production. However, the plant's leaves are used to extract fibers (*ixtle*) to produce bags, ropes, brush bristles, and broom fibers. The dry leaves and the dry plant's pine are used as fuel, and the flowers of the inflorescence (*gualumbos*) are the main ingredient of several dishes in traditional Mexican cuisine. The caterpillars from the lepidoptera *Comadia redtenbacheri* (red worm or *chunicuil*) and *Aegiale hesperiaris* (white worm or *escamol*) infecting the leaves, roots, and the pine of the plant are highly appreciated in the traditional and gourmet Mexican cuisine (García Mendoza, 2007; Narváez Suárez et al., 2016; Torres-García et al., 2019). Additionally, the scraped bagasse or metzal from the cajete during aguamiel extraction is a rich-sugar material (containing sucrose, glucose, fructose, and FOS) used as fodder (**Supplementary Figure 3**). The total mass of extracted metzal during the aguamiel producing life of a plant varies from 21.5 to 37.2 Kg (Peralta-García et al., 2020).

The use of maguey plants as raw material for the production of several of the byproducts mentioned above or as a food ingredient is only carried out on a local scale with a low commercial impact profile, leading to the extraction of aguamiel for pulque production as the main product obtained from the species of maguey pulquero. Narváez Suárez et al. (2016), studied the producing maguey plantations and pulque production zones of Zempoala, Singuilucan, and Epazoyucan (Hidalgo, State); Españita and Nanacamilpa (Tlaxcala State), Texcoco and Tepetlaoxtoc (Estado de México); Tepeyahuaco (Puebla State); and Perote (Veracruz State), to determine whether its cultivation still represents a viable economic activity. These authors conclude that the sale of aguamiel, pulque, red, and white worms, and the sale of the entire cut leaves for barbecue preparation are the activities with higher economic value for these rural agricultural locations. Finally, they consider the production of concentrated aguamiel syrups, candies, pulque distillates, and rural *pulque tourism* as relevant products/activities with higher economic value for these communities (Narváez Suárez et al., 2016).

Nowadays, the industrial production of canned and bottled pulque is an important economic activity in the main pulque-producing zones. As the USA and Europe as primary markets, several brands are successful examples of the pulque industrialization process incorporating tank-fermentation homogenization, pasteurization of the final product, quality control, and bottled or caning for final sale. Relevant examples are the *Corporativo Maguey San Isidro* established in 2003 in Tlaxcala State, with Pulque del Razo Group, which produces about 300,000 units/month of canned pulque brand *Pulque Hacienda 1881*, a product with certification from the Foods and Drugs Administration (FDA), USA. Pulque del Razo also provides high-quality pulque to 70% of the pulquerías in Mexico City and produces byproducts such as aguamiel concentrated syrups, inulin (inulin-type fructans), pulque distillates, flavorings, and sugars. Additionally, the *Corporativo Maguey San Isidro* offers pulque tourism to the *Rancho San Isidro*. This company possesses an own sustainable production of maguey varieties for pulque production by propagating and plantation of the varieties *manso, púa larga, ayoteco*, and *chalqueño* (corpmasir.com). *La Flor Pura* company established in 2009 in the Estado de México, produces bottled aguamiel and pulque with *La Flor Pura*'s brand (pulquelaflorpura.com). The most recently established company was *Embotelladora Pulquemanía* (established in 2020 in the city of Texcoco, Estado de México), producing bottled pulque with the brand *Pulque Penca Larga* with a monthly production of 260,000 bottles from aguamiel collected from the locality of Nanacamilpa, Tlaxcala State. This brand is also certified by the FDA for exportation to the USA (pencalarga.com).

Potential New Products From Maguey, Pulque, and Aguamiel

Several studies have focused on the potential probiotic benefits of specific bacteria isolated from aguamiel and pulque: The *Leuconostoc mesenteroides* strain P45 showed higher antimicrobial activity against pathogenic bacteria such as EPEC *Escherichia coli, Listeria monocytogenes, Salmonella* Typhi, and *S.* Typhimurium, both *in vitro* and *in vivo* mice models, compared to the antimicrobial activity shown by a commercial probiotic bacteria (Giles-Gómez et al., 2016); the *Lactobacillus sanfranciscensis* strain LBH1068 improved mice health by a significant reduction of weight loss, decreasing the gut permeability, and cytokine modulation (Torres-Maravilla et al., 2016); the isolated LAB identified as *Lactobacillus* sp. and *Pediococcus* sp. showed antimicrobial activity *in vitro* against *Staphylococcus aureus* and *Helicobacter pylori* (Cervantes-Elizarrarás et al., 2019). Other relevant biological activities have been identified in other bacteria isolated from aguamiel concentrated syrups (agave sap concentrate), including the

potential *in vitro* anticancer enhancement effect of the isolated bacteria *Gordonia* sp. and *Arthrobacter globiformis;* and the ability to survive and grow in the colonic environment and the production of short-chain fatty acids by microorganisms such as *Lactobacillus* and *Leuconostoc* suggest their potential use as probiotics (Figueroa et al., 2017, 2021).

Several reports also showed the potential biological activity of byproducts from the maguey. The *in vitro* activity of fructans with high molecular weight and branched fructans extracted from a 6-year-old plant of *A. angustifolia* as a growth-promoting agent of several *Bifidobacterium* and *Lactobacillus* species suggest a potential prebiotic activity (Velázquez-Martínez et al., 2014). Recent studies have focused on the potential properties of agave bagasse from *A. salmiana* as a source of bioactive compounds such as steroidal saponins with potential biological activities such as antifungal, anticarcinogenic, or anti-inflammatory (Santos-Zea et al., 2020), and the role of polysaccharides present in the bagasse of this maguey species as a natural emulsion stabilizer and enhancer of topical anti-inflammatory activity of indomethacin (Jiménez-Rodríguez et al., 2021). These reports open an area of opportunity to use different bacterial strains isolated from aguamiel, pulque, or byproducts such as aguamiel concentrates as probiotic agents that can be incorporated into the formulation of functional foods and beverages, using just aguamiel or a diluted preparation of concentrated aguamiel syrups as a substrate for their growth or formulation.

Finally, the maguey plantation model of meteplantle was proposed as a potential bioresource for the production of several products with higher economic added value, including the use of aguamiel for the production of a maguey beer, the use of the fiber ixtle as raw material for the production of biodegradable food bags, or the use of aguamiel as a substrate for the production of lactic acid as food acidulant or as raw material for the production of the valuable polymer polylactate (PLA), as possible alternative commercial scenarios. Particularly for lactic acid production from aguamiel as substrate by industrial fermentations, Viniegra-González (2020) projects a scenario where lactic acid production for PLA synthesis could consume 3 billion liters of aguamiel to be produced in 100,000 ha. Nevertheless, this scenario does not contemplate a sustainable system of replacing mature magueys for aguamiel production. In any case, it is a potential scenario that, with adequate investment, infrastructure conditions, and sustainable maguey replacement, would require at least 20 years for its development (Viniegra-González, 2020).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Pulque production and maguey cultivation are essential elements of identity, millenary tradition, and contextualized in a close-related environmental, alimentary, social, religious, cultural, and productive relationship as they form part of the same production system, mainly in rural communities. Today there is a general perception that pulque is a popular beverage that has practically disappeared from urban markets and just sold in pulquerías, some restaurants, and rural locations. Although it is a preferred beverage in rural areas, its traditional production is in a critical situation and at risk of disappearing for different reasons, such as the irrational use of maguey by producers, inadequate or inexistent propagation strategies, displacement of its cultivation by other agricultural products, its use for other activities such as desmixiotado, mutilation of leaves, or the collection of maguey worms, or because of a lack of interest in continuing with this activity. All these situations have significantly affected the economy and food sufficiency of several rural communities.

However, another scenario in which different producers have successfully industrialized the production of pulque and marketed canned or bottled, reaching a production of more than 1,300,000 bottles per year, equivalent to more than 1,138,000 L of pulque (just for one producing company), have shown the successful development of this agroindustry. Remarkably, some of these companies have their own maguey propagation and cultivation practices, but others buy an average of 5,000 L of aguamiel per day, generating a permanent market and an economic benefit to aguamiel producers. The beverage's industrial production has made it possible to standardize the traditional fermentation process (extraction of aguamiel, preparation of the seed, and fermentation), incorporating quality standards and the final product's pasteurization process. These operations solve an ancestral problem with the production of pulque and stop the fermentation process, increasing the shelf life of the final product, which in some cases has achieved international quality standards. Similarly, some companies sustainably cultivate the maguey varieties used to produce aguamiel by propagating and planting to avoid a shortage of plants and ensure continuous beverage production. However, it is paradoxical that regular pulque consumers do not like industrialized pulque despite some international certifications.

This situation has a relevant impact on the pulque agroindustry, where two cultural visions of pulque production are opposed. The cultivation of maguey pulquero for its traditional production represents the continuity and conservation of culture and traditional knowledge. It also provides environmental benefits in the agroforestry system, such as reducing soil erosion, conserving native flora and fauna, capturing carbon, and recharging groundwater. Nevertheless, this activity represents a minor economic activity for many traditional producers due to the lower volume production and market. Although the industrial production of pulque represents an economic opportunity for traditional aguamiel producers as providers of the sap, they must comply with the quality standards required for the producer, resulting in sometimes opposite to the traditional production customs. It is necessary to promote in the Mexican market the canned or bottled pulque. Current pulque production in Mexico is ∼0.1% of the current beer market reported for 2020 (Instituto Nacional de Estadística Geografía e Informática, 2021). The possibility of increasing the Mexican market to canned or bottled pulque at least at the same level of the exported production would generate, for example, a greater demand for aguamiel. It

would result in this agroindustry's reactivation in many rural communities where it is currently considered a secondary economic activity.

The species of agaves for aguamiel production, the collected sap, and traditional pulque fermentation represent an invaluable resource from the genomic, metabolic, and microbiological point of view, resulting as a strategic activity the elucidation of the natural microbiota responsible for the fermentation among different producing zones and the long-term maintenance of stocks of these microbial cultures. The microbiological and functional studies of the associated microbiota have provided valuable information to propose a defined culture starter to optimize the fermentation. It has also provided the scientific basis of some of the traditional health benefits associated with the beverage's consumption, proving the source for developing new potential probiotic and functional foods or beverages and developing future bio-industries.

AUTHOR CONTRIBUTIONS

All the authors contributed equally in the preparation and review of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.678168/full#supplementary-material

Supplementary Figure 1 | Pulque castration process. The *tlachiquero* selects the proper mature plants on the basis of the thinness of the central leaves (heart or *meloyote*). **(A)** The *tlachiquero* uses a sharpened knife to cut off some leaves surrounding the heart. **(B,C)** Cutting of the heart. **(D–F)** Destruction and extraction of the remaining floral bud with a sharpened pray bar. **(G)** Scraping of the wall of the resulting cavity with a scraping tool or *raspador* to shape the final size of the *cajete*. **(H)** Once the cavity was completed, it is covered with leaves to start the maturation process. Images kindly provided by Mrs. E. Velázquez Gutiérrez.

Supplementary Figure 2 | The cycle of life of maguey for pulque production. **(A)** Seedling, (b) adult plant, (c) flowering, (d) flower, (e) seeds, (f) bulblet, and (g) stolon. Sexual reproduction by seeds resulting from pollination of the inflorescence (c–e, a). Vegetative propagation from bulblets (f) or by transplanting stoloniferous shoots or *hijuelos* of the mother plant (g). **(B)** Vegetative propagation by transplanting stoloniferous shoots of 1-year-old and a height of 50–60 cm (a) are transplanted to a greenhouse. Young plants are transplanted to the field when they reach 1 m in height or are 1–2 years old (b) to complete its growth for aguamiel production (c) (García Mendoza, 2007; Nieto Aquino et al., 2016; Álvarez-Ríos et al., 2020).

Supplementary Figure 3 | Main products obtained from the maguey, aguamiel, and pulque. *Gualumbos, mixiote,* maguey worms, and leaves (*pencas*) are used in traditional and gourmet Mexican cuisine. *Ixtle* fibers are used for the production of bags, ropes, brush bristles, and broom fibers.

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Preparation of Alcoholic Beverages by Tribal Communities in the Indian Himalayan Region: A Review on Traditional and Ethnic Consideration

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The Indian Himalayan Region (IHR) is the center of the diverse food culture comprising fermented and non-fermented ethnic foods and alcoholic beverages. Diverse tribal communities in IHR (Uttarakhand, Himachal Pradesh, Jammu and Kashmir, Laddakh, Sikkim, Assam, Arunachal Pradesh, Manipur, Meghalaya, and Tripura) have been long known for their rich culture and food habits. Having strong ritual importance among the ethnic people of the IHR, alcoholic beverages are being consumed in various cultural, social, and religious events for ages. Consumption of in-house prepared alcoholic beverage is the socio-cultural tradition in India as well as across the globe. The processes and ingredients involved in alcoholic beverage preparations vary with raw material availability in different regions. The majority of the fermented drinks are cereal-based with a significant proportion of various plants and fruits as the main raw material, making a beverage more unique in taste. Some plant ingredients used for traditional alcoholic beverages have potential nutraceutical as well as therapeutic properties that are well documented. These properties could constitute an additional economic value for traditional alcoholic beverages commercialization, which, in turn, could promote the local rural economy. Until now, such beverages have only received marginal attention by ethnobotanists and few studies concern traditional fermented beverages in the IHR. In this view, the current review focused on preparation, diversity, cultural, and economic significance and health benefits of ethnic beverages used by tribal communities in the IHR.

Keywords: alcoholic beverages, socio-cultural tradition, fermented drinks, indigenous people, Indian Himalayan Region

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INTRODUCTION

The Indian Himalayan Region (IHR) is host to the world's highest ecosystems, which includes Jammu and Kashmir, Ladakh, Himachal Pradesh, Uttarakhand, Sikkim, Darjeeling hills, Arunachal Pradesh, and some hilly regions of northern Assam (Tamang, 2001; Nehal, 2013). The ethnic tribes of IHR, living in high-altitude areas, are known for their traditional knowledge and complex life system all over the world. They are unique, due to their geographical condition, food preference, and lifestyle (Tamang, 2010a). Being a distinctive component, uses and preparations of alcoholic

beverages among the tribal communities have been a part of traditional knowledge for thousands of years (McGovern, 2009; Dutfield, 2010; Egea et al., 2015).

Fermentation of beverages is a 5000-year-old tradition in India. Soma is the most talked about and most mysterious of the Indian beverages. The entire 9th Mandala in Rig-Veda (1700 BC) is dedicated to Soma and it is elevated to the position of moon Goddess (https://www.arishtam.com/indian-traditional-beverages/home-brew-tutorials/accessed on October 24, 2020). The preparation of the ethnic beverages using the fermentation process by the tribal people of India is well known and documented by several workers (Kumar and Rao, 2007; Rivera et al., 2012a,b). It is reported that more than 350 types of major traditional beverages are prepared using indigenous knowledge. These homemade beverages, prepared informally at the local or family level, are region-specific and prepared manually by mixing old starter culture with the raw material (Tamang, 2020). The World Health Organization (WHO, 2014) has also reported the importance of traditional beverages in cultural and social events. It is important to mention that these traditional alcoholic beverages also contain extract of plant parts and a good source of minerals and bioactive compounds beneficial for health (Darby, 1979; Campbell-Platt, 1994; Steinkraus, 1996; Tamang and Fleet, 2009).

Although traditional alcoholic beverages are an important part of the cultural and social life of tribal communities, these beverages received minor attention from researchers and ethnobotanists so far especially in India. Several ethnobotanical studies on traditional alcoholic beverages used by the tribal communities have been published, but the details of ingredients, preparation methods, cultural significances, etc. have not been explored properly (Kishor et al., 2013; Nath et al., 2019). It is also observed that the investigation based on ethnobotanical field surveys, social and cultural engagements, and possible nutraceutical values are substantially lacking in IHR. Therefore, this review has been focused on the preparation and cultural significance of traditional alcoholic beverages in the IHR.

MATERIALS AND METHODS

In the present comprehensive review, an extensive database on various aspects of traditional alcoholic beverages in the IHR was searched using the most relevant search engines. For the compilation of the review, online original research articles, review articles, book chapters, published books, conference proceedings, and reports available on authentic and reputable scientific search engines like ScienceDirect (www.sciencedirect.com), PubMed (https://pubmed.ncbi.nlm.nih.gov/), Scopus (https://www.scopus.com), National Library of Medicine (https://www.nlm.nih.gov/), Google Scholar (https://scholar.google.com/), and Web of Science (https://webofknowledge.com) were searched. The literature was searched for alcoholic beverages in the IHR using keywords like indigenous beverages, Himalayan beverages, traditional beverages, ethnic beverages, starter culture, substrates etc. A detailed bibliographic search was made using 83 references from 1974 until 2020 in the present review.

DIVERSITY AND PREPARATION OF TRADITIONAL ALCOHOLIC BEVERAGES

There are a variety of traditional alcoholic beverages, commonly or lesser known (Chhang, Judima, Jann, Chakti, Angoori, Daru, etc.), that are produced by tribes of IHR using fruits, cereals, grains, etc. as raw material (Tamang, 2001). Based on the used raw materials and preparation methods, these beverages can be classified into various types such as rice and cereal wine, palm wine, distilled spirit, and distilled alcoholic beverages (Bluhm, 1995; Thakur et al., 2004; Franz et al., 2011). Traditional alcohol brewing is a home-based industry run by tribal women using indigenous knowledge of the fermentation process. Tribes of the high-altitude Himalayan region have developed their own cultures for the preparation of fermentation-based alcoholic beverages using the available natural resources in the region (Roy et al., 2004). The preparation of traditional beverages is not only a means of livelihood in the hill regions but also an important household-cum-societal drink associated with religious ceremonies (Sharma and Mazumdar, 1980). As listed in **Table 1**, quite a few indigenous beverages are known by different local names for their unique taste, aroma, and process of preparation. However, all tribes across the Himalayan region mostly share a similar method, which is cooking of raw material, drying, and incorporation of starter culture, fermentation, and the extraction of the final product.

PREPARATION OF STARTER CULTURE

The majority of the alcoholic beverages are prepared from cereals, mostly rice (46%) followed by millets (17%), barley (12%), wheat (10%), maize (10%), and sorghum (5%), as mentioned in **Supplementary Figure 1**. The raw materials for different beverages are used as a substrate for the growth of microorganisms such as yeasts, molds, and bacteria (Dung et al., 2006; Nath et al., 2019). The microbial starter culture is prepared through grinding the rice and local herbs and plant parts followed by mixing and preparing starter cake in some places whereas some communities prefer to use it in powdered form (Tsuyoshi et al., 2005). The details of medicinal plants used for the preparation of starter culture are presented in **Table 2**. There are a variety of starters used by the tribes of IHR to prepare alcoholic beverages. Keem is a starter cake used to prepare an alcoholic beverage popularly known as Soor in Garhwal Himalaya of Uttarakhand state (India). It is prepared using *Cannabis sativa* and *Sapindus mukorossi*, including 40 other herbs (Rana et al., 2004; Sekar and Mariappan, 2007). Balam is a wheat-based starter used to prepare Jaan and Daru. It is made by the Bhotiya community of high-altitude regions of Uttarakhand Himalaya. During the preparation of Balam, roasted wheat flour as a substrate is mixed with various herbs and spices such as *Cinnamomum zeylanicum*, *Amomum subulatum*, *Piper longum*, *Ficus religiosa*, and wild chillies (Das and Pandey,

TABLE 1 | Description of ethnic alcoholic beverages and their use in the Himalayan Region.

State	Tribes/Community/Region	Ethnic Beverages	Substrate	Starter/Microbes	Health Benefits	References
Laddakh	Bhoto community in Leh, Nubra valley	*Chhang/Lugri*	Barley	*Phab (Yeast)*	Treatment of arthritis, joint pain	Targais et al., 2012; Angmo and Bhalla, 2014
Himachal Pradesh	Lahaul and Spiti, Kinnaur region	*Chhang*	Wheat	*Phab (Yeast)*	Protection against cold	Kanwar et al., 2011
	Kinnaur region	*Lugari*	Rice	*Phab (Yeast)*	Protection against cold	Savitri and Bhalla, 2019
	Kangra region	*Lugdi*	Rice	*Phab*	Rich in protein and phenolic compounds	Kumar et al., 2019
	Lahaul and Spiti, Kinnaur region	*Aara*	Barley	*Phab (Yeast)*	Protection against cold	Savitri and Bhalla, 2007
	Kullu, Kangra, Mandi region	*Sura, Sur*	Finger millet	*Dhaeli or Dhehli*	Rich in Vitamin B	Thakur et al., 2004; Joshi et al., 2015
	Kinnaur region	*Angoori*	Grapes	*Phab*	Protection against cold	Thakur et al., 2004; Savitri and Bhalla, 2019
	Kinnaur region	*Chulli*	Wild apricot	*Phab*		
	Kinnaur region	*Behmi*	Apple	*Phab*		
	Kinnaur region	*Ara/Arak*	Apple, barley, pear	*Phab*		
	Shimla and Kulllu region	*Chakti/Jhol*	Jaggery	*Phab*		
Uttarakhand	Bhotiya tribes in Johar valley	*Jaan*	Rice	*Balam (Yeast)*	Treatment of fever, dysentery, cough and cold, stomach ailments	Sekar and Mariappan, 2007; Kishor et al., 2013
	Bhotiya tribes in Johar valley	*Kacchi*	Barley	*Balam (Yeast)*	Cholera, treating weakness of cattle	Kishor et al., 2013
	Jaunsari community in Tons valley	*Soor*	Barley	*Keem*	Protection against cold	Sekar and Mariappan, 2007
	Bhotiya tribes in Johar valley	*Daru*	Rice, jaggery	*Balam (Yeast)*	Protection against cold	Roy et al., 2004; Sekar and Mariappan, 2007
Sikkim and Darjeeling	All tribes	*Jnards*	Finger millet	*Murcha (Bacteria and Yeast)*	Protection against cold	Tamang et al., 1988; Sekar and Mariappan, 2007
	Gorkha, Bhutia, Lepcha, Monpa tribes	*Kodo ka jaanr*	Finger millet	*Chyang*		Chetia and Borgohain, 2020
	Gorkha tribes	*Poko*	Rice	*Manapu*		
	All tribes	*Chhayang*	Finger millet/barley	*Yeast, Lacto acid bacillus*		Tamang, 2010b
	Gorkha tribes	*Baati jhar*	Finger millet	*Marcha*		Chetia and Borgohain, 2020
	Nepalese, Tibetan community	*Chhaang*	Finger millet	*Marcha*		Nath et al., 2019
	Lepcha, Bhutiya and Nepali community	*Rokshi*	Maize	*Mold and Yeast*		Sekar and Mariappan, 2007

(Continued)

TABLE 1 | Continued

State	Tribes/Community/Region	Ethnic Beverages	Substrate	Starter/Microbes	Health Benefits	References
	All tribes	*Jhara*	Various plants	*Ranu Dabai*		
Arunachal Pradesh	Monpa, Apatani, Nishi tribes	*Apong*	Rice	*Ipo*	Protection against cold	Tiwari and Mahanta, 2007; Chetia and Borgohain, 2020
	Monpa, Apatani, Nishi tribes	*Ennog*	Black rice	*Ipoh*		
	All tribes	*Madua*	Finger millet	-		Shrivastava et al., 2012
	All tribes	*Apong*	Rice	*Khamtip (fermented mixture)*		
	Monpa tribes	*Themsing*	Finger millet	-		
	Karbi tribes	*Bankhe-kham*	Tapioca (*Mannihot esculenta*) a tuber	*Khamtip (fermented mixture)*		
	Karbi tribes	*Shhang or Ccharo-kham*	Barley	*Khamtip (fermented mixture)*		
	Adi, Nyshing, and Mishmi tribes	*Opo*	Rice	*Pee*		Shrivastava et al., 2012; Nath et al., 2019
	Monpa tribes	*Mingri, Lohpani, Bhang chang*	Finger millet, rice, maize, or barley	*Pham*		
	Hill miri tribes	*Mingri*	*Rice*	*Bokha*		Nath et al., 2019
	Apatani tribes	*Opo*	*Rice*	*Chu*		
	Monpa, Miji, Mishmi tribes	*Rakshi*	Finger millets, rice, barley grains	*Ipoh*		Shrivastava et al., 2012; Nath et al., 2019
	Deuri and Khampuri tribes	*Poka*	Cereals	*Si-ye*		Nath et al., 2019
	Singpho tribes	*Rice bear**	Cereals	*Chho*		
	Nyshing tribes	*Rice bear**	Cereals	*Paa*		
	Tagin tribe tribes	*Rice bear**	Cereals	*Phab*		
	Lisu or Yobin tribes	*Rice bear**	Cereals	*Aje*		
	Sulung tribes	*Rice bear**	Cereals	*Epop*		Nath et al., 2019
	Nocte tribes	*Rice bear**	Cereals	*Pee*		
	Thangsa tribes	*Apong*	Cereals	*Ipoh*		
	Adi Galos tribes	*Kala-apang, Ennog*	Rice	*Kshai*		
	Monpa, Apatani, Nishi tribes	*Pona*	Rice	*Ipoh*		Chetia and Borgohain, 2020
	Adi Galos tribes	*Opo*	Rice	*Siiyeh*		

(Continued)

TABLE 1 | Continued

State	Tribes/Community/Region	Ethnic Beverages	Substrate	Starter/Microbes	Health Benefits	Reference
Assam	Mishings tribes	*Apong*	Rice	*Ipoh, Apop-pith*	Protection against cold	Tiwari and Mahanta, 2007; Kardong et al., 2012; Bhuyan and Baishya, 2013; Handique and Deka, 2016; Chetia and Borgohain, 2020
	Ahom tribes	*Haj pani or Koloh pani or Xajpani*	Bora rice	*Vekur pitha*		Chakrabarty et al., 2009; Das and Deka, 2012; Handique and Deka, 2016, Bhuyan and Baishya, 2013; Chetia and Borgohain, 2020
	Zemenaga tribes	*Dekuijao*	Sproutrd rice grain	*Saccharomyces cerevisiae*		Chakrabarty et al., 2009; Das and Deka, 2012
	Dimasa tribes	*Judina*	Rice	*Humao*		
	Rabha tribes	*Jonga Mod, Chako/phab*	Rice	*Bakhor, Surachi or Phap*		Deka and Sharma, 2010; Bhuyan and Baishya, 2013; Chetia and Borgohain, 2020
	Sonowal tribes	*Rohi*	Rice	*Saoul pitha*		Bhuyan and Baishya, 2013
	Deori tribes	*Sujen*	Rice	*Mod-pitha, Perokkushi*		Deori et al., 2007, Nath et al., 2019, Chetia and Borgohain, 2020
	Bodo tribes	*Jou bishi/Jumai*	Rice	*Angkur, Amao*		Bhuyan and Baishya, 2013; Chetia and Borgohain, 2020
	Karbi tribes	*Hor-Alank, Horlang*	Rice	*Thap*		Teron, 2006, Bhuyan and Baishya, 2013; Chetia and Borgohain, 2020
Manipur	Tankhul tribes	*Yu angouba*	Sticky rice	*Hamei*	Beneficial like milk	Devi and Kumar, 2012
	Meitei tribes	*Atingba/Yu*	Rice	*Hamei*		Chetia and Borgohain, 2020
	Tankhul tribes	*Atingba*	Rice	*Ham*	Protection against cold	Jeyaram et al., 2009; Devi and Kumar, 2012
	Sherdukpen tribes	*Rice bear**	Cereals	*Paa*		Nath et al., 2019
	Naga tribes	*Banana wine*	Ripe banana	-		Devi and Kumar, 2012
Meghalaya	Pnar/Jaintias, Khasis tribes	*Kiad*	Red rice	*Thiat (Yeast)*		Samati and Begum, 2007; Chetia and Borgohain, 2020

(Continued)

TABLE 1 | Continued

State	Tribes/Community/Region	Ethnic Beverages	Substrate	Starter/Microbes	Health Benefits	References
	Garo tribes	*Chu*	Rice	*Wansi*		Chetia and Borgohain, 2020
Nagaland	Angami tribes	*Litchumasu/Peyazu, Zutho*	Sprouted, glutinous rice	*Piazu/Yei, Piazu*		Das and Deka, 2012; Chetia and Borgohain, 2020
	Dimasa Kacharis	*Zudima*	Rice	*Humao*		Chetia and Borgohain, 2020
	Naga tribes	*Zutho/Ijadijang, Duizou*	Rice	*Grist, Khekhrii*		Teramoto et al., 2002; Deka and Sharma, 2010; Chetia and Borgohain, 2020
Tripura	Kalai tribes	*Chuwak*	Rice	*Chuwan*	Protection against cold	Ghosh et al., 2016; Nath et al., 2019
	Jamatia tribes	*Chuwak*	Rice	*Chuwan*		
	Debbarma tribes	*Chuwak*	Rice	*Chuwan*		
	Molsom tribes	*Rakju*	Rice	*Chuwan*		
	Tripuris tribes	*Langi/Chuwak*	Rice	*Chuwan*		Chetia and Borgohain, 2020

**The local name of the particular traditional alcoholic beverage is not available; thus, the common name is provided.*

2007). Mana is a granular-type starter culture prepared from wheat flakes (Tamang, 2010a). Another ethnic starter is Ragi, which is prepared by rice or millet mixed with herbs and spices (Tamang, 2012). The mixture is mixed with water and 2–4% powder of old Ragi and mixed thoroughly. The prepared mixture is shaped into balls for fermentation (72 h at 23–25°C) in a humid environment. After sun drying, these starter balls are used to prepare alcoholic beverages (Saono et al., 1974). Koji is another starter made with steamed rice. It is a mold starter that is prepared until mycelium growth in the fermentation process (Lotong, 1985; Tamang, 2010b). Dhehli is a herbal mixed starter used to prepare Sur or Sura beverage in Himachal Pradesh, India. It is prepared from 36 fresh herbs such as *Pistacia integerrima*, *Solanum canthocamptid*, *Clitoria ternatea*, *Aegle marmelos*, *Viola cinerea*, and *C. sativa* collected from the forest by elderly people in an annual community effort (Thakur et al., 2004; Tamang, 2010a; Sharma, 2013; Joshi et al., 2015). The extract with plant biomass is added to roasted barley flour to prepare Dhehli (Thakur et al., 2004; Sharma, 2013). Phab is another traditional starter used in Ladakh and the hilly areas of Himachal Pradesh (Thakur et al., 2004; Tamang, 2010b). It is used to prepare Chhang. Phab is prepared using roasted barley with black pepper, dried ginger, crushed paddy, wild herbs, and earlier made Phab starter. Marcha is another known starter used in Darjeeling hills and Sikkim in India (Tamang et al., 1988, 2010; Dung et al., 2006; Bhuyan and Baishya, 2013). It is prepared by crushing soaked glutinous rice. Plant parts, e.g., *Plumbago zeylanica*, *Buddleja asiatica*, and *Vernonia cinerea*, along with old starter powder, were also added to it (Tamang et al., 1988; Thapa, 2002; Tsuyoshi et al., 2005). Hamei is a rice-based starter of northeast hilly areas of India. It is prepared by mixing *Albizia myriophylla* in soaked rice and a pinch of old Hamei (Jeyaram et al., 2009; Tamang, 2010b; Tamang et al., 2010).

PREPARATION OF SOME POPULAR ALCOHOLIC BEVERAGES

The preparation of alcoholic beverages is very common in tribal communities of IHR. They use different types of starters (described above) to prepare a variety of alcoholic beverages. Jann is a traditional beverage made from rice, wheat, jau, etc. (Roy et al., 2004). It is prepared and consumed by the Bhotiya community of Uttarakhand state in India. High-quality Jaan is made from local millet koni (*Setaria italica*). The quality of Jann is judged by its taste (sweetness), smell, and strength. Generally, it is known for low alcohol concentration. The preparation of Jann is very common and prepared by the community efforts (Roy et al., 2004). Tribes of Uttarakhand and Himachal Pradesh (both are hill state of the IHR) also prepare a local alcoholic beverage Sur (30–40% alcohol) using cereals and fruits (Rana et al., 2004; Sharma, 2013). Tribes of these regions consume Sur to tolerate adverse climatic conditions. They also consume Sur at family functions and festivals. Chhang is a popular indigenous alcoholic beverage that is also called Jhol and Chakti. Tribes of Lahaul and Spiti, Kullu, and Kangra prepare this ethnic beverage by a solid-state fermentation process using the sherokh (huskless) variety of

TABLE 2 | List of plant species used to prepare some popular starter cultures in the Indian Himalayan Region.

State (Tribe/Region)	Tribes/Community/Region	Starter/Microbes	Medicinal Herbs	References
Ladakh	Bhoto community	*Phab* (Yeast)	*Artemisia* sp.	Angmo and Bhalla, 2014
Himachal Pradesh	Lahul and Spiti, Kannaur, Kangra region	*Phab* (Yeast)	*Artemisia* sp.	
	Mandi region	*Dhaeli or Dhehli*	*Varbascum thapsus, Bistorta amplexicaule, Viburnum grandifolia, Impatience, recemosa/sulcata, Arisaema* sp., *Arisaema tortuosum, Ajuga brevifolia, Ajuga bractiosa, Viola canescens, Morus seratta, Cuscuta europiana, Cannabis sativa, Solanum pseudocapsium*	Joshi et al., 2015
	Kullu region	*Dhaeli or Dhehli*	*Varbascum thapsus, Bupleurum lanceolatum/ Valeriana jatamansi, Cannabis sativa*	
	Kangra region	*Dhaeli or Dhehli*	*Swertia chirayata, Selinum tenuifolium, Silene grifithii, Polygonum allatum, Polygonum* sp., *Centella asiatica, Picrorhiza kurrooa, Varbascum thapsu*	
	Lug valley of Kullu region	*Dhaeli or Dhehli*	*Pistacia integerima, Solanum xanthocarpum, Clitoria ternatea, Aegel marmelos, Viola cinerea, Cannabis sativa, Trachyspermum copticum, Micromeria biflora, Spiranthes aurstralis, Saussurea* sp., *Bupleurum lanceolatum, Drosera lunata, Salvia* sp., *Arisaema helleborifolium, Fragaria* sp.	Thakur et al., 2004
Uttarakhand	Bhotiya tribes	*Balam* (Yeast)	*Cinnamomum zeylanicum, Ammomum subulatum, Piper longum, Ficus religiosa*	Das and Pandey, 2007; Sekar and Mariappan, 2007
	Janusari community	*Keem*	*Cannabis sativa, Sapindus mukorossi, Melia azedarach, Zanthoxylum armatum, Leucas lanata, Dicliptera roxburghiana*	Sekar and Mariappan, 2007
Sikkim and Darjeeling	Nepalese, Tibetan, Gorkha community	*Marcha*	*Plumbago zeylanica, Buddleja asiatica, Vernonia cinerea, Zingiber officinale*	Tamang et al., 2012; Nath et al., 2019
	All tribes	*Ranu Dabai*	*Coccinia grandis, Vernonia cinerea, Clerodendrum viscosum, Plumbago zeylanica, Stephania japonica, Stephania glabra, Oroxylum indicum, Mussaenda roxburghii, Scoparia dulcis, Rauvolfia serpentina, Artocarpus heterophyllus, Wattakaka volubilis*	Sekar and Mariappan, 2007
Arunachal Pradesh	All tribes	*Ipoh*	*Cinnamomum glanduliferum, Cissampelos pareira, Cynadon dactylon, Leucas aspera, Lygodium salcifolium, Piper betle, Scoparia dulcis, Veronica cinera*	Greeshma et al., 2006
	Adi, Nyshing, and Mishmi tribes	*Pee*	*Clerodendum indicum, Cissampelos*	Nath et al., 2019
	Monpa tribes	*Pham*	*Solanum khasianum*	
	Hill miri tribes	*Bokha*	*Cinnamomum glanduliferum, Solanum nigrum*	
	Apatani tribes	*Chu*	*Solanum khasianum*	
	Thangsa tribes	*Ipoh*	*Scoparia dulcis, Leucas lanata*	
	Deuri and Khampuri tribes	*Si-ye*	*Leucas aspera, Piper betle*	
	Singpho tribes	*Chho*	*Piper longum, Scoparia dulcis*	
	Nyshing tribes	*Paa*	*Cissampelos pareira, Clerodendron viscosum*	
	Tagin tribes	*Phab*	*Cinnamonum glanduliferum*	
	Lisu or Yobin tribes	*Aje*	*Albizia myriophylla*	
	Sulung tribes	*Epop*	*Veronia cinerea, Amomum aromaticum*	
	Nocte tribes	*Pee*	*Piper betle*	
	Miji tribes	*Ipoh*	*Artocorpus lakoocha, Mangifera indica*	
Assam	Mishing tribes	*Ipoh*	*Scoparia dulcis, Amblovenatum opulentum, Justicia adhatoda, Zanthoxylum nitidum, Phlogacanthus thyrsiflorus, Centella asiatica, Andrographis paniculata, Cheilocostus speciosus, Piper nigrum, Selaginella* sp., *Piper longum, Naravelia zeylanica, Solena amplexicaulis*	Kardong et al., 2017

(Continued)

TABLE 2 | Continued

State (Tribe/Region)	Tribes/Community/Region	Starter/Microbes	Medicinal Herbs	References
	Karbi tribes	*Thap*	*Croton joufra, Amomum corynostachyum, Acacia pennata, Artocarpus heterophyllu, Oryza sativa, Phlogacanthus thyrsiflorus, Solanum torvum*	Teron, 2006; Bhuyan and Baishya, 2013
	Ahom tribes	*Vekur paitha*	*Centella asiatica, Cinnamonum bejolghota, Cissampelos pareira, Cledendrum viscosum, Croton caudatus, Hydrocotyle sibthorpioides, Lygodium flexuosum, Naravelia zeylanica, Oryza sativa, Pteridium aquilinum, Piper nigrum, Sida rhombifolia, Smilax perfoliata*	Bhuyan and Baishya, 2013
	Mishing tribes	*Apop-pitha*	*Ananas comosus, Artocarpus heterophyllus, Adhatoda vasica, Actinodaphne obovata, Cinnamomum tamala, Costus speciosus, Centella asiatica, Drymeria cordata, Hydrocotyl rotundifolia, Hydrocotyle sibthorpioides, Lygodium flexuosum, Lygodium japonicum, Melothrea heterophylla, Naravelia zeylavica, Oldenlandia corymbosa, Oryza sativa, Piper longum, Piper nigrum, Phogocanthus thyrsiflorus, Pteridium aquilinum, Scoparia dulcis, Selaginella* sp., *Swertia chirata, Saccharum officinarum, Vitex negundo, Zanthoxylum hemiltonian*	Kardong et al., 2012
	Rabha tribes	*Bakhor, Surachi or Phap*	*Ananas comosus, Artocarpus heterophyllus, Calotropis gigantean, Capcicum fructescens, Cleodendrum viscosum, Dennstaedtia scabra, Ochthochloa coracana, Plumbago indica, Saccharum officinarum, Scoparia dulcis, Sida rhombifolia*	Deka and Sharma, 2010; Bhuyan and Baishya, 2013
	Bodo tribes	*Angkur*	*Clerodendrum viscosum, Oryza sativa, Scoparia dulcis, Xanthium strumarium*	Bhuyan and Baishya, 2013
	Sonowal tribes	*Saoul pitha*	*Centella asiatica, Clerodendrum viscosum, Corchorus olitorius, Naravelia zeylanica, Oryza sativa, Pteridium aquilinum, Sida rhombifolia*	Bhuyan and Baishya, 2013
	Deori tribes	*Mod Pitha*	*Allium sativum, Artocarpus hererophyllus, Ananus comosus, Alpinia malaccensis, Alternanthera sessilis, Capsicum annum, Cinnamomum bejolghota, Centella asiatica, Coffea bengalensis, Costus speciosus, Cyprus* sp., *Desmodium* sp., *Desmodium pulchellum, Equisetum* sp., *Lygodium flexuosum, Melastoma malabathricum, Mussaenda roxburghii, Myxopyrum smilacifolium, Naravelia zeylanica, Oryza sativa, Psidium guajava, Pothos scandens, Pteridium aquilinum, Pycnarrhena pleniflora, Rubus* sp., *Saccharam officinarum, Selaginella semicordata, Scoparia dulcis, Solanum torvum, Thunbergia grandiflora, Zanthoxylum oxyphyllum, Zingiber officinale*	Deori et al., 2007
Manipur	Tankhul tribes	*Hamei*	*Albizia myriophylla*	Jeyaram et al., 2009
	Sherdukpen tribes	Paa	*Buddleia macrostachya, Plumbago zeylanica*	Nath et al., 2019
Meghalaya	Pnar tribes	*Thiat*	*Amomum aromaticum, Musa paradisiaca*	Samati and Begum, 2007
Tripura	Kalai tribes	*Chuwan*	*Dysoxylum blume, Litsea monopetala, Moringa oleifera, Saccharum officinarum*	Ghosh et al., 2016
	Jamatia tribes	*Chuwan*	*Ananas comosus, Casearia aculeate, Dysoxylum, Markhamia stipulate*	
	Debbarma tribes	*Chuwan*	*Allophyllus serrarus, Ananas comosus, Aporusa diocia, Combretum indicum, Cirtus sinensis, Markhamia stipulate*	
	Molsom tribes	*Chuwan*	*Artocarpus heterophyllus, Litsea monopetala, Markhamia stipulate, Nyctanthes arbor-tristis*	

barley locally called grim (Thakur et al., 2004; Targais et al., 2012). It is presented to visitors, priests, and even deities during every social gathering like childbirth, marriage, or other celebrations. Chhang is known to provide energy and refreshment (Targais et al., 2012). All these alcoholic beverages are prepared by women tribes at the family level. After preparation, beverages are carefully tested and evaluated by older ladies of the family or community.

FERMENTATION

Fermentation is a microbiological process. The knowledge of microbial activities or fermentation is hardly recognized by indigenous people. Fermentation takes place when raw materials are mixed with starter culture and kept in a closed container. Starter cultures are nothing but inoculums containing microorganisms that are required to initiate the fermentation process. A schematic diagram has been presented (**Supplementary Figure 2**) to show the fermentation steps involved in ethanol production from cereal grains and microorganisms associated with it. These microorganisms could be present in the environment, raw materials, or the utensils used to prepare the drink. The selection of the microorganism depends on adaptation to the substrate and the fermentation conditions (Tamang, 1998). Many research groups indicated that the fermentation process varies from 5 to 25 days based on the flavor, taste, temperature, and alcohol content (Kanwar et al., 2011; Tamang et al., 2015; Chetia and Borgohain, 2020). The fermentation processes are used to prepare alcoholic beverages in the Himalayan region, which experiences low temperatures; therefore, resilient microorganisms capable of surviving and performing fermentation at low temperature are used for the preparation of foods and/or beverages.

The Bhotiya community in the Himalayan regions of Uttarakhand uses the wheat-based starter culture Balam, which is known to have as many as 32 microbial isolates. They are dominated by *Bacillus* (two species) and yeasts (*Saccharomycopsis fibuligera*, *Kluyveromyces marxianus*, *Saccharomyces* sp.; Das and Pandey, 2007; Kumari et al., 2016). Previous studies by a group of researchers reported many yeasts, molds, bacteria, and fungi, viz., *Saccharomyces cerevisiae*, *S. fibuligera*, *Wickerhamomyces anomalus*, *Candida glabrata*, *K. marxianus*, *Meyerozyma* sp., and *Pichia* sp., among yeasts, and molds like *Aspergillus penicillioides* and *Rhizopus oryzae*. These studies were performed using culture-dependent approaches and have drawbacks if estimating total microbial communities. Therefore, with the advancement in sequencing technologies, especially Next-Generation Sequencing, it has become possible to look into the complete microbial community composition. Recent high-throughput sequencing analysis of different fermented foods revealed the presence of bacterial community, which depict phyla Proteobacteria, Firmicutes, and Actinobacteria and genera *Leuconostoc*, *Lactobacillus*, *Acetobacter*, *Gluconacetobacter*, etc. Several studies also revealed the presence of fungal phyla Ascomycota and Zygomycota along with the genera *Saccharomyces*, *Zygosaccharomyces*, *Aspergillus*, *Aureobasidium*, *Mucor*, *Candida*, etc., in traditional starter cultures (Thiat, Marcha, Phut, Humao, Chowan, etc.) used by indigenous people of the Indian Himalayas (Thakur et al., 2004; Sha et al., 2017).

During the preparation of the starter, microorganisms could be added in the form of old starter powder or may be present already in the raw material as indigenous microbiota. These microorganisms have been found beneficial for health (Tamang et al., 2015). The health benefits of *Saccharomyces* sp., *Lactobacillus*, and *Bacillus* are recently well documented and considered as potential probiotic candidates. Studies on the genes responsible for the probiotic properties have been performed on the starter cultures and fermented foods to document the role of microbes in probiotic properties in ethnic foods (Das and Pandey, 2007; Kumari et al., 2016; Syed et al., 2020; Elkhalifa et al., 2021). Microorganisms present in these starter cultures are also known to be responsible for suppressing pathogenic population, carbohydrate metabolism, protein metabolism, etc. (Jani and Sharma, 2021). Additionally, microorganisms associated with these foods are also known for the production of several enzymes, flavoring substances, vitamins, etc., which are used in the fermentation industry for commercial purposes (Tamang et al., 2015).

CULTURAL SIGNIFICANCE OF ETHNIC BEVERAGES

Fermented foods and beverages have a strong connection with the socio-cultural lives of the various ethnic groups of the country. Traditional alcoholic beverages are not only a refreshing drink but also an integral part of the social and cultural occasions of the tribal communities in IHR in various ways (Tamang et al., 2010). These beverages are served in various functions such as wedding ceremonies, crop harvesting celebrations, offerings to traditional Gods, worshipping rituals, and death commemorations of loved ones to express togetherness, unity, joy, and sorrow. These beverages also related to the origin, habitat, religion, and overall life of tribes; therefore, they regard these ethnic beverages as their cultural heritage (Jeyaram et al., 2009; Ghosh et al., 2016). However, there is no such large-scale production unit or industry that can be seen based on a local alcoholic beverage in this region. It is only confined to each ethnic group or community of the respective state, especially women who are associated with preparing these beverages. New Year celebration and farm activities are one of the major events of tribes of Himachal Pradesh and Ladakh. They prepare Chhang, which is a traditional alcoholic beverage of Buddhists of Laddak (also described in the *Diversity and preparation of traditional alcoholic beverages* section). Judima is another traditional alcoholic beverage that is very intimately related to the ritual of Dimasa tribes. Freshly prepared Judima is offered to family gods and goddesses during religious occasions, marriage ceremonies, and festivals (Chakrabarty et al., 2009). Tribes also believe that a drop of Judima to a newborn baby will be helpful for good health and also protect the baby from any evil force (Chakrabarty et al., 2009).

Traditional alcoholic beverages consumed by the local tribes in the Himalayan region not only are related to rituals and occasions but also are known to provide increased nutrition such as proteins, vitamins, added minerals, phytochemicals, phytosterols, and dietary fibers to the consumer (Vijayendra and Halami, 2015). Tribal people used to drink these alcoholic beverages mostly in the morning before having breakfast, for health benefits. Alcoholic beverages have also discussed "Ayurveda" for their medicinal importance. Several workers have reported the health benefits of traditional alcoholic beverages such as rice beer, which has been found to be effective in diarrhea and urinary problems, headache, body ache, inflammation, worms treatment, etc. (Samati and Begum, 2007; Deka and Sharma, 2010).

In the tribal community, most of the people are very much hooked to rice beer and sometimes do not realize the harmful effects of these beverages. Tribes of IHR usually believe that traditional alcoholic beverages help reduce diseases and generate energy in the body to tolerate the very low temperature of hilly areas. Due to this belief, tribes drink alcoholic beverages very frequently (Seale et al., 2002). Tribes of IHR start alcohol consumption at an early age, mostly in the teenage years, and become addicted to alcohol. In most regions, beverage consumption is very common among all family members except infants. It is regularly consumed by all male members and elder women. Young male members (16–30 years) consume ∼4–5 glasses per day. However, young women used to consume it at weekly intervals or during occasions (Shrivastava et al., 2012). Gradually, such young generation of tribes have become highly addicted to alcohol, and sometimes, this habit ruins their life and livelihood. It is well known and studied that consumption of traditional alcoholic beverages in small amounts is good for the health but frequent intake of alcohol badly affects the body and leads to health problems (Luu et al., 2014). According to Luu et al. (2014), the level of risk associated with traditional beverage varies from low-risk (family and neighbors) to high-risk (by an agent) distribution. Furthermore, dilution, adulteration, and waste release increase the chances of health risk, which is related to direct consumption as well as associated local air pollution, water pollution, and bad public behavior. Therefore, awareness programs for tribal people in India is a necessity of the current scenario.

DISCUSSION AND PROSPECTS

The preparation and consumption of indigenous alcoholic drinks have been known for centuries. All tribal communities in the IHR prepare their specific beverage for livelihood. The local brews or traditional alcoholic beverages also play a very significant role in the cultural and traditional aspects of tribal people residing in a particular part of the country. Besides having tremendous nutritional properties, viz., proteins, carbohydrates, ash, crude fiber, and macro- and micronutrients, ethnic beverages also have many beneficial microflorae, which may exert health benefits such as probiotics, especially those that are consumed as an undistilled drink like rice beer (Tamang et al., 2012, 2015).

It is well known that tribes use many plant species that have medicinal properties to prepare traditional rice beer, which helps to reduce the toxic effect of traditional drinks (Samati and Begum, 2007; Deka and Sharma, 2010). Tribes used these traditional beverages, e.g., rice beer, for treating fever, colds and cough, body ache, etc. Tribal women used these alcoholic beverages to treat menstruation problems (Darby, 1979; Campbell-Platt, 1994; Steinkraus, 1996; Samati and Begum, 2007; Tamang and Fleet, 2009; Deka and Sharma, 2010). Tribes collect medicinal plants directly from their natural habitat for the preparation of ethnic drinks. Due to uncontrolled collection from the natural habitat and rapid urbanization, these plant species (which are used in the preparation of traditional beverages) will be depleted. Therefore, the quality, taste, and medicinal properties of traditional drinks are compromised seriously. However, medicinal properties of traditional alcoholic beverages have been documented by several workers (Darby, 1979; Campbell-Platt, 1994; Steinkraus, 1996; Tamang and Fleet, 2009); until now, very limited studies have been carried out to establish the medicinal properties of traditional alcoholic beverages. Therefore, it is required to urgently carry out the work that elaborates the nutritional and medicinal aspects of traditional alcoholic beverages.

Documentation of the fermentation process and plant species used for the production of traditional alcoholic beverages is not adequately addressed by the scientific community. Only limited and fragmented pieces of literature are available regarding the production of traditional beverages, especially in the Himalayan region. The plant parts used by tribal communities and sanitary conditions during preparation regulate the quality of the starter culture, alcohol content, and overall quality of the drink (Basumatary et al., 2014; Nath et al., 2019). Sometimes, contamination or toxicities found in prepared drinks become lethal to the community, and due to the lack of knowledge about the facts, tribal people start blaming the person who was involved in the preparation of the beverage. It has also been reported that the indigenous microorganisms (yeast strains) involved in fermentation processes during the alcoholic beverage preparation lose their effectiveness due to the use of inorganically grown substrate, i.e., rice, over the polluted areas (Kumari et al., 2016). Therefore, scientific studies are required to urgently establish the knowledge about the microorganisms (potential isolates) used in the fermentation process of ethnic alcoholic beverages so that harvesting can be done effectively. Also, it should be noted that reduction in the manufacturing of ethnic alcoholic beverages for daily use has been observed due to economic and legal bindings. Moreover, due to lack of interest and ignorance of traditional values of the young generation, there is a constant decrease in traditional knowledge. This has created a gap leading to the lack of knowledgeable and experienced people among the tribal communities.

Scientific studies may be helpful to reveal some new scope for value addition in traditionally prepared alcoholic beverages, and of course, application of modern science could be incorporated for improvement, wherever it is required (Syed et al., 2021). It is necessary to also conserve traditional alcoholic beverages and culture. As already discussed, these alcoholic beverages are enriched with some nutritional and medicinal properties, and there is a scope of improvement and drug development using biotechnological, medicinal, and food and nutrition-based research that will

be helpful for the commercialization of traditional alcoholic beverages (Sekar and Mariappan, 2007).

CONCLUSION

The socio-cultural life in the IHR is associated with the people of various ethnic origins, languages, faith, and traditional practices. These ethnic origins and traditional practices give rise to some unique food habits such as fermented food and beverages. The tribal communities prepare these ethnic beverages by a fermentation process and consumed them in almost every family or social gatherings. Preparations of these alcoholic drinks are well mingled with the cultural and indigenous healthcare systems of these tribes. These alcoholic beverages are enriched with many nutritional components like vitamins and proteins. Phytochemical and ethnobotanical studies have also revealed that traditional alcoholic beverages have a medicinal property to cure various diseases and have healing capacity. Based on available literature, it can be concluded that traditional alcoholic beverages play a very important role in preserving the long-standing traditions of tribes. Therefore, documentation of traditional culture is a prime necessity, which will be a valid way to conserve the ancient heritage of traditional alcoholic beverages for the future. It will be helpful to transfer this knowledge or technique from one generation to another.

Furthermore, there is an urgent need to work on the value addition (nutritional value) of these ethnic beverages by research on method improvement, microorganisms selection, raw material improvement genetic improvement, etc., which may suggest the due market value of traditional alcoholic beverages and lead to their industrialization. Such initiatives might be advantageous for mankind and the economic sustainability of the tribal communities.

AUTHOR CONTRIBUTIONS

BR and JR: manuscript design and final manuscript writing and revision. SP, PD, and JR: data collection. BR, SP, and PD: data analysis. BR, JR, and PD: data interpretation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.672411/full#supplementary-material

Supplementary Figure 1 | Percentage of various cereals used by the tribal communities for the preparation of alcoholic beverages.

Supplementary Figure 2 | Schematic diagram depicting different stages of fermented beverage production.

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Use of the Coyol Palm (*Acrocomia aculeata*) for the Production of "Taberna," a Traditional Fermented Beverage in México

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Acrocomia aculeata is a palm, which is used for various purposes in different rural communities in southeastern Mexico; among which is the obtaining of a traditional fermented beverage called "taberna." The objective of this study was to know the management that taberna producers give to the coyol palm. The work was carried out between march and april 2016 in two communities in Chiapas. The information on palm management was obtained by applying semi-structured interviews to the families that make the beverage in each community, who narrated the process of using the palm; from its care in the field, to its court for the elaboration of a taberna and the uses and customs that revolve around this activity were described. With this work, it was possible to know the details of the elaboration of the beverage from a social and cultural perspective, which is surrounded by traditional knowledge, with great biocultural importance that gives identity not only to the communities that possess the resource, but also to the families that they take advantage of it. With this, it is expected to contribute to a management and conservation plan for the species, to guarantee the resource to the communities and preserve the biological and cultural diversity associated with this activity, given that the use of natural resources is ultimately a biocultural heritage that implies a relationship between natural resources, people, regions and local culture.

Keywords: taberna, palm, beverage, Acrocomia, traditional

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INTRODUCTION

Acrocomia aculeata, native palm; also called coyol palm, it is distributed from Mexico to Costa Rica (NOTIMEX, 2011). Particularly in Mexico, this palm is found along the coastal plains of the Gulf of Mexico and the Pacific; in Chiapas it is located especially in the region of La Frailesca, Centro and Soconusco (Zuart-Macías et al., 2013). It is a palm of rapid reproduction, the same that is observed in pastures and savannas (NOTIMEX, 2011; Mondragón, 2015); adapted to habitat with disturbances, it reaches 15–20 m in height. The importance of this palm (*A. aculeata*), lies in the diversity of uses by the regional population; among which its medicinal properties stand out, as well as artisanal (Zuart-Macías et al., 2013), the use of the fruits, which are consumed in the form of a preserve known as "dulce de coyol" (Díaz Montesinos et al., 2011; Ramírez Hernández et al., 2013; Farrera et al., 2020) and the flowers; which are also edible. The taberna is a drink product of the fermentation of the sap obtained from the trunk of the palm and is produced in different countries,

from Mexico to Central America (Payas, Honduras, where it is known by the name of sap wine; Guanacaste) (Lentz, 1990). In Mexico, the extraction and consumption of this drink is of utmost importance among the populations that produce, becoming even in some ejidos of the Isthmus of Tehuantepec, one of the main traditions that are carried out in different festivities (Mondragón, 2015). In Chiapas the taberna is consumed in a large part of the communities of the "Sierra Madre." Traditionally, in some regions of Chiapas known as La Concordia, Cintalapa, Villaflores and Villa Corzo, the inhabitants are accustomed to consume a large amount of taberna during Holy Week, as well as preferably in the months of february to may (NOTIMEX, 2011). Despite the importance of this resource, *A. aculeata* is a species little studied from the perspective of its management and conservation; in Chiapas, Toledo Espinoza (2014); with the purpose of generating basic information for its conservation, it carried out a micropropagation study through embryos and organogenesis from seedlings, as well as an analysis of landscape genetics, finding that 100% of the somatic embryos extracted from the Municipality of Villaflores germinated successfully; while only 22 and 24% of those extracted from El Ocote and La Sepultura, respectively, did so. Likewise, encouraging results were obtained regarding the use of growth regulators for organogenesis and the results of the population genetics analysis showed that there is a significant differentiation between the physiographic regions of the state. The objective of this work was to know the management that the taberna producers give to the coyol palm, in the studied communities,

FIGURE 1 | *Acrocomia aculeata* in field, ready for cutting for taberna production.

to contribute in the near future; with a management and conservation plan for the species, which guarantees the resource to the communities and helps preserve the biological and cultural diversity associated with this activity, highlighting relationship between natural resources, people, regions and local culture.

FIGURE 2 | Cutting of *Acrocomia aculeata* in the field. Appearance of trunk cut **(A)**, removal of foliage in preparation for taberna production **(B)**.

FIGURE 3 | Cutting of the palm trunk, for the formation of the "canoe," from which the taberna will be obtained.

MATERIALS AND METHODS

The present work consisted of a case study carried out in two localities of the state of Chiapas [ejido Tierra y Libertad (TyL), municipality of Jiquipilas and Benito Juárez (BJ), municipality of Villaflores]. During the months of march and april 2016.

Firstly, authorization for access to the community was requested with the Ejidal Commissariat, who was asked to request the approval of the ejidatarios in general in order to conduct the research.

Once approval was obtained, we proceeded to identify the families in charge of the production of the taberna, who, through semi-structured interviews, were asked to describe and demonstrate in a practical way, the production process of the beverage, from the selection of the palm, the cutting and preparation of the plant, to the production of the Taberna.

RESULTS

Once permission was obtained from the authority of each locality, two taberna producing families were identified, one in each community, who reported that the taberna's production process has been transmitted orally from generation to generation. According to the information obtained, in both communities, traditionally the taberna was consumed and produced by different inhabitants, however, at present this activity is only carried out by some people, who we can consider as specialists; also called "Maestros Taberneros." In the case of Benito Juárez, there are two maestros taberneros, over 60 years old and in Tierra y Libertad, only one. The interviewees reported that the taberna is consumed during the Easter season, which coincides with the season of the year when the temperature is high (hot weather), in this way, the producers have observed that the sap is prevented from having a sticky consistency. The age of the palm should range between 5 and 15 years of age; it is important to mention that the longer the palm is, the taberna production will take place for a longer time (about 2 months). The specialists reported that the palms are obtained directly from the field, that sometimes they are developed on their farms and they use them freely to make the drink, however, when they do not have them available in this way, they can buy them from other inhabitants who do have them on their lands, and acquire them at a cost of up to $ 300.00 pesos. The taberna production process is detailed as described by the producers as follows:

Cutting and cleaning process: It consists of choosing the palms of considerable age for the production of the tavern, the trees that are more than 5 m high are used to obtain the taberna (**Figure 1**). Once chosen, the palm is cut from the base of the trunk (**Figure 2A**), on the ground it is cleaned trying to remove from it as many thorns and leaves (**Figure 2B**), leaving the trunk clean. In the town of Benito Juárez, the producer transports the palms to his backyard, which is where the taberna-making process continues, while in the town of Tierra y Libertad, the producer leaves the palms in the place where they were cut, which is located on his property and goes to the place every day to collect the sap.

FIGURE 4 | Coyol palms once the "canoe" has been excavated and covered with a piece of wood, fastened with a stone.

Opening of the canoe: 24 h after cutting, the farmer makes a hole called a "canoe" (**Figure 3**), which is measured from 30 cm from the apex of the trunk toward the stem and the first cut is made parallel to the base of the trunk, the following cuts are perpendicular to the first cut of the width of the machete used, and a final cut to obtain a square of the stem, which is extracted to verify that the cuts were made at the height of the so-called "palmito" (medullar part of the plant); The stem is then left to rest for another 24 h and the process of extracting the sap and cleaning the palm continues. The "canoe" is covered with a piece of the same palm or with a piece of wood, to avoid the entry of dust or garbage on the sap, in this study; the canoe was covered with a piece of wood, held with a stone (**Figure 4**).

Extraction of the sap: After 24 h, the producer is ready to extract the sap that emanates from the trunk, with the help of a plastic hose; he sucks the liquid and deposits it in a jug or plastic bottle, collecting around 2 to 3 L of taberna every 12 h; of each palm (**Figure 5**). The drink is transferred to a plastic container, where it is stored for sale and/or consumption by the family. At the beginning of the process, the drink has a sweet taste and with the passage of days it acquires an alcoholic flavor, so the producer

FIGURE 5 | Extraction of the sap accumulated in the palm "canoe," using a plastic hose.

must leave the container between open to avoid excess gases and its explosion.

Scraping and cleaning of the canoe: After extracting the sap, the canoe is scraped and cleaned, which consists of scraping the walls of the canoe in its four cardinal points with the help of a machete previously cleaned with water; in this way, the scraping consists of cutting between one centimeter each side of the canoe, trying not to leave residues of the trunk or the heart of palm inside the hole, which is then washed with water and cleaned with a previously washed spoon (**Figure 6**). The extraction of the sap, as well as the scraping and cleaning of the canoe, is done every 12 h (between six in the morning and six in the afternoon; during 15–30 days) during the season of taberna production.

Marketing, beliefs and traditions: The two producers who collaborated in this study refer that each year, they can produce taberna of around 20 to 30 palms, so that on average, the annual production oscillates around 2,700 L per year, considering the first 15 days of production, since from that time on, the flow of sap is less and the taberna production gradually decreases. The sugar content is also lower, after this time; which impacts the taste of the drink, making it less attractive to consumers, although producers report that in some years, production can extend up to a month. The taberna is sold at a price of $25.00 per liter; marketing is done directly by the producer to consumers or distributed informally to people who transport it to other locations where it is also customary to consume the drink in the Easter season. In this case study, the interviewees described some bodily effects of consuming it, mainly the weakening of the legs. The interviewees stated that the consumption of the taberna in addition to being part of recreational and ceremonial activities, they also do it for health reasons, mentioning that the drink can be used to alleviate gastrointestinal diseases, they also pointed out the custom of taking the drink directly from the palm using a reed to inhale it, another custom among the consumers of the taberna; is to take it in the form of "bolis" (the frozen drink inside a small plastic bag), or served in glasses adding ice, water and table sugar and drinking it as a soft drink, this practice is done after the sap has been kept preserved bulk in the plastic containers, that is, not from the freshly extracted palm sap. Likewise, they indicated that they have the custom of decorating the stems that are being

FIGURE 6 | Scraping and cleaning process of the "canoe" after as much sap as possible was extracted.

used for taberna production with red ribbons, to avoid the "mal de ojo," caused by people with "strong eyes" and that decompose the drink.

DISCUSSION

The information provided by the families of the producers in the communities under study coincides with data on the use of the palm in other localities, as mentioned by different authors (Díaz Montesinos et al., 2011; Ramírez Hernández et al., 2013; Zuart-Macías et al., 2013; Farrera et al., 2020), who describe *A. aculeata* as a multipurpose species used by the inhabitants of the Frailesca region, in these reports they mention that they use the fruits and flowers as food and for their medicinal properties, as well as for the preparation of beverages. Other authors also report the use of flowers in a ceremonial way in some localities of Chiapas (Bermúdez, 2011). The season of production and consumption of taberna during Holy Week is the same as in some ejidos of the Isthmus of Tehuantepec, in one of the main traditions carried out in different festivities (Mondragón, 2015). Prior to this study, the elaboration of taberna has been reported, using *A. aculeata* as raw material in the Maya Frailesca region (Alcántara-Hernández et al., 2010; Santiago-Urbina et al., 2013; Coutiño et al., 2020), where the municipalities under study belong. The sap is fermented naturally, without the producers adding any inoculum, carrying out acidic, alcoholic and sometimes acetic fermentations, which gives the drink its characteristic flavor. The sweet taste of the sap produced in the 1st days of collection is due to the presence of sucrose and other sugars, as indicated by Luján-Hidalgo et al. (2019), who reported a concentration of 110 g/L of sucrose, as well as 48 and 51 g/L of glucose and fructose; respectively, and its decrease with the passing of the days explains the change of flavor to one of alcoholic taste, due to the fact that these sugars are used by the developed native microbiota; transforming them into a mixture of compounds, among which are mainly ethanol, whose concentration can reach 4.7–10.31% w/v, depending on the environmental conditions and the time of collection of the drink (Santiago-Urbina et al., 2013; Coutiño et al., 2015, 2020). Regarding the use of the taberna as

a remedy for some gastrointestinal diseases; Romero-Luna et al. (2017) pointed out that the taberna, among other traditional drinks, contain beneficial microorganisms for health, since they improve the balance of the intestinal flora, thereby reducing the risk of gastrointestinal diseases. In studies carried out in taberna, an autochthonous microbiota composed of lactic and acetic bacteria and yeasts has been found (Alcántara- Hernández et al., 2010; Santiago-Urbina et al., 2013, 2015; Luján-Hidalgo et al., 2019), which possibly confer probiotic potential to the beverage, as pointed out by Coutiño et al. (2020), who also point out that the taberna contains bioactive compounds, such as phenolic compounds, vitamins, short-chain fatty acids, among others; that can be generated by the microbiota developed during the fermentation of the sap, or come directly from the plant. Regarding the custom of attaching a red ribbon to the stems, Zuart-Macías et al. (2013) also describe this practice, to avoid excessive and sudden acidification of the taberna, caused by the admiration of people who are not familiar with it, even commenting that some producers prevent the passage of strangers to the place where the stems of the palms in production are located.

CONCLUSIONS

The production of taberna is a predominantly family economic activity among the inhabitants of the communities of the Frailesca Region of the State of Chiapas, which has been transmitted from generation to generation, and is surrounded by myths and beliefs among them. Until a few years ago, the plant was used directly from the field, but in recent years, it has begun to be cultivated to use it, but those who use it for taberna production are people with around 60 years of age. The use of the palm to make taberna reflects a cultural aspect of the central depression of Chiapas. The growing interest in disseminating the uses of traditional fermented beverages can contribute to the generation of an adequate management plan for *A. aculeata*, to ensure that this activity lasts and the tradition is preserved, also taking advantage of the biotechnological potential of the taberna as a probiotic. The article contributes to continue documenting the elaboration of this drink and begins its use in a context of resource management, because its cultivation for these purposes is already beginning to be a valued drink, locally and regionally, it is important to preserve local knowledge for its elaboration. The use of natural resources, finally, is a biocultural heritage that implies a relationship between natural resources, people, regions and local culture.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ejidal Commissariat, who requested the approval of the ejidatarios to conduct the research. The participants provided their informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JA-R conducted fieldwork and contributed to the writing of the manuscript. AV-V, CO-G, and MS-C contributed to conception and design of the study and contributed to manuscript revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Rational Selection of Mixed Yeasts Starters for *Agave* Must Fermentation

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Tequila and mezcal are both traditional Mexican liquors that are produced from cooked *Agave* spp. must fermentation and usually rely on spontaneous or pure *Saccharomyces cerevisiae* strain inoculation. In order to contribute to the rational selection of yeast starters for tequila and mezcal productions, we tested a collection of 25 yeasts originally isolated from mezcal musts, spanning 10 different yeast species. These strains were first characterized in a semi synthetic medium (labeled as M2, having 90 g/L fructose and 10 g/L glucose of initial hexoses) at 48 h of culture, observing a differential pattern in the consumption of sugars and productivity. Selected *Saccharomyces* strains left around 10 g/L of fructose and showed higher fermentation performance. However, some non-*Saccharomyces* strains, specifically from *Torulospora* (Td), *Kluyveromyces* (Km), and *Zygosaccharomyces* (Zb) genera, consumed almost all the sugar (i.e., Km1Y9 with < 5 g/L) and had a high productivity of ethanol. In general, all *Saccharomyces* strains presented a high production of ethyl-butyrate, ethyl-decanoate, and ethyl-hexanoate with peaks of 10, 38, and 3 μg/L, respectively. In addition, some *Kluyveromyces* and *Torulospora* strains showed a high production of phenyl ethyl acetate (i.e., Km1D5 with up to 1400 μg/L); isoamyl acetate (i.e., Km1D5 and Td1AN2 with more than 300 μg/L), and hexyl acetate (i.e., Td1AN2 with 0.3 μg/L). Representative strains of the most productive genera (*Saccharomyces*, *Torulospora*, and *Kluyveromyces*) were selected to evaluate their fermentative performance and survival in a mixed culture on a medium based on *Agave tequilana* must, and their population kinetics was characterized using specific fluorescent *in situ* hybridization (FISH) probes in a qualitative and semi-quantitative analysis during fermentation. We observed that the mixture ratios of 0.1:1:1 or 1:1:1 (*Saccharomyces:Kluyveromyces:Torulospora*), maintained good fermentation productivities, with alcohol yields above 0.45 g/g, and allowed a high survival rate of the non-*Saccharomyces* strains during the fermentation process. Finally, mixed inoculum fermentations on *A. tequilana* must medium, including different *Saccharomyces* strains and the finally selected *Torulospora* and *Kluyveromyces* strains, showed the best production parameters in terms of ethanol, carbon dioxide, glycerol, and acetic acid

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values, as well as improved volatile metabolite profiles as compared to the pure cultures. All these data were used to propose a methodology of selection of strains to be used as a pure or mixed starter for tequila and mezcal fermentations, with high primary metabolite productivity and desired aromatic profile.

Keywords: *Saccharomyces*, *Kluyveromyces*, Torulaspora, mezcal, tequila, mixed starter, aroma profile, *Agave*

INTRODUCTION

The aroma and flavor of alcoholic beverages obtained from *Agave* spp. plants (commonly known as *Agave* or maguey) are probably the most complex in the liquor world, due to the fact that they are the result of many volatile compounds that may be contained in the raw material and that vary among *Agave* species (Vera Guzmán et al., 2009). They are further increased and transformed during the cooking of the *Agave* fructan-rich tissues, previous to carrying out the fermentation. A comprehensive review of all the aromatic compounds found in different liquors, as well as all the different yeast species detected in these fermented products, including tequila and mezcal, can be found in De la Torre-González et al. (2017). This complex mixture defines the sensory attributes and consumer acceptance of these *Agave* alcoholic beverages. It has been reported in general that the end of the *Agave* must alcoholic fermentation process is usually carried out almost exclusively by *S. cerevisiae* (Cedeño, 1995). However, there is an initial presence of other yeasts (non-*Saccharomyces*), which are usually naturally co-cultured with *S. cerevisiae*, which give unique organoleptic characteristics to the products produced during fermentation (Lachance, 1995; De la Torre-González et al., 2017). These non-*Saccharomyces* yeasts have become increasingly important, since they contribute to the production of compounds that provide the aromatic characteristic to *Agave* beverages (Amaya-Delgado et al., 2013; González-Robles et al., 2015) and wine (Tufariello et al., 2021). The co-existence of different species or strains in the mezcal fermentative process can have positive effects such as the increase of the hexose consumption and alcohol yields (Nolasco-Cancino et al., 2018). The non-*Saccharomyces* yeasts have shown potential as starters for the *Agave* alcoholic beverage industry (Arrizon et al., 2006; Amaya-Delgado et al., 2013; Segura-García et al., 2015; De la Torre-González et al., 2017; Nolasco-Cancino et al., 2018). However, most of the reported kinetics of these consortia of *Saccharomyces* and non-*Saccharomyces* yeasts have been analyzed for wine (grape juice), where both yeast populations maintain similar growth rates during the first phases (2–3 days) of fermentation, and then present a reduction in the non-*Saccharomyces* yeast number as the fermentation time progresses (Pina et al., 2005). Fortunately, an increasing number of reports are slowly unraveling the microbial consortia complexities of *Agave* fermented beverages (Lachance, 1995; Narváez-Zapata et al., 2010; Verdugo Valdez et al., 2011; Páez-Lerma et al., 2013, Kirchmayr et al., 2017; Nolasco-Cancino et al., 2018). A very specific group of yeasts were obtained from Tamaulipas (Mexico) mezcal fermenting musts (De la Torre-González et al., 2017), which have been characterized in terms of their stress tolerance, particularly, when fermenting on high fructose contents (Oliva Hernández et al., 2013; De la Torre-González et al., 2016; Vergara-Álvarez et al., 2019). This is important due to the fact that cooked musts of *Agave* spp. plants contain, as fermentable sugar, a high fructose content (90%), fructose oligosaccharides (FOS), and also toxic Maillard compounds, furfural, methanol and saponins, among others (Díaz-Montaño et al., 2008; Arroyo-López et al., 2009; Amaya-Delgado et al., 2013; Nava-Cruz et al., 2014), explaining in part why some of our yeasts have been recently characterized as genomically belonging to a separate, specific domestication event, as compared to other *S. cerevisiae* strains worldwide (Peter et al., 2018). Also, pure and dual mixes of these mezcal strains have been evaluated during a wine-type fermentation, observing that *S. cerevisiae*/*T. delbrueckii* inoculum presented a fruitier aroma profile, showing the feasibility of using these mezcal yeasts as inoculum for wine fermentation (De la Torre-González et al., 2017, 2020). However, the specific behavior of the yeast populations and their viability during fermentation of an actual *Agave* must, and the application of these mezcal yeasts as pure or mixed inoculum on *Agave* spp. must fermentations, remained to be conducted. Therefore, in this study we evaluated the fermentative performance of 25 *Saccharomyces* and non-*Saccharomyces* mezcal strains on a semi synthetic (M2) medium, to assess and screen the strains, and then on an *Agave tequilana* must-based medium for the selected yeasts, by using pure and mixed cultures to gain information to propose a minimum characterization methodology to select a starter for *Agave* must (mainly, tequila and mezcal) fermentative applications.

MATERIALS AND METHODS

Yeast Strains and Inoculum Growth Conditions

The 25 yeast strains used belong to the mezcal LBI-CBG yeast collection and are conserved in 60% glycerol at −70°C, and the commercial wine strain *Saccharomyces cerevisiae* Fermichamp (DSM Food Specialties B.V., The Netherlands) was used as a control for its fructophilic character, which is used to reactivate stuck wine fermentations. The strains used are representative of the yeast diversity found in the fermentation of mezcal from Tamaulipas (Mexico), which comprises almost 100 different isolated yeasts, and the studied strains were identified by their 26S rDNA sequences, amplified by using the primers NL1 5′ GCATATCAATAAGCGGGAGGAAAAG 3′ and NL4 5′ GGTCCGTGTTTCAAGACGG3′, reported as adequate for most of the ascomycetous yeasts, which can be reliably identified with their D1/D2 LSU regions (Kurtzman and Robnett, 1998). The D1/D2 LSU gene marker was chosen

as it exhibits a lower heterogeneity than the widely used ITS1-ITS2 region, which renders a better species identification (De Filippis et al., 2017) as also suggested by Libkind et al. (2020) in the specific case of yeasts, when comparing the use of these two ribosomal markers with whole-genome analyses. The conditions used for amplification and sequencing of the D1/D2 were reported by Campos-Rivero et al. (2019), as follows: PCR was conducted on a thermocycler (2720 Thermal Cycler; Applied Biosystems, Carlsbad, CA, USA), with a final volume of 25 μl, which consisted of 1.5 mM of $MgCl_2$, 1× of PCR Buffer, 0.8 mM of dNTP, 0.4 μM of each primer, 1.5 U of Taq DNA polymerase, and 10 ng of DNA template. The D1/D2 LSU region (26S rDNA) was amplified with an initial denaturation at 95°C for 10 min, 35 cycles of repeated reactions (denaturation at 94°C for 30 s, annealing at 61°C for 30 s and elongation at 72°C for 1 min) and final elongation of 10 min at 72°C. Amplification was visualized on 1% (w/v) agarose gels stained with SYBR Green (Invitrogen; Thermo Fisher Scientific, Carlsbad, CA, USA). PCR products were purified using Wizard® SV gel and PCR clean-up Kit (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. D1/D2 LSU amplification products were directly sequenced by using both original primers. Sequencing was performed using an ABI Prism 3130 sequencer (Applied Biosystems). Sequences are deposited on the Genbank and belong to the species *S. cerevisiae* LCBG- Sc3Y2 (JQ824877), -Sc3Y3 (JQ824872), -Sc3Y4 (JQ824875),- Sc3Y5 (JQ824869), -Sc3Y8 (JQ824874), -ScMosca3 (KT945088), -Sc4Y3 (KT945087), -Sc3D2 (JQ824871), -Sc3D4 (KT945086), -Sc3D5 (KT945085) and -Sc3D6 (JQ824876), *Kluyveromyces marxianus* strains -Km1D5 (KT945093), -Km4D3 (KT945094) and -Km1Y9 (KT945092), *Torulaspora delbrueckii* strains -Td1AN1(KT945091), -Td1AN2 (KT945089) and -Td1AN9 (KT945090), *Pichia kluyveri* strain -Pk4D6 (KT945083), *Meyerozyma guilliermondii* strain -Pg1Y12 (KT945082), *Yamadazyma mexicana* Pm1AN3 (KT945081), *Candida parapsilosis* strains -Cp1Y7 (KT945079), *Clavispora lusitaniae* strain -Cl4Y4 (KT945080), *Rhodotorula mucilaginosa* strain -RmP12 (KT945095), and *Zygosaccharomyces bailii* strain -Zb3Y1 (KT945084) (De la Torre-González et al., 2020). An initial preculture of the tested yeast was grown on YPD agar plates containing 1% yeast extract, 2% peptone, 2% w/w D-glucose, plus 2% bacteriological agar (Difco Laboratories, Franklin Lakes, NJ, USA), all on a w/w basis and incubated at 30°C for 48 h. A loop of this preculture was used as inoculum for liquid YPD medium (3 ml) incubated 24 h at 30°C with shaking at 200 rpm. The optical density of the cultures was determined at 600 nm and the initial inoculum concentration was adjusted using sterile Ringer solution if needed. This active culture was used as the inoculum for a second round of growth on YPD incubated for 48 h at 200 rpm and 30°C before using it as inoculum in the fermentation experiments carried out as described below.

Fermentation Media

All fermentation experiments were carried out in minibioreactor tubes of 50 ml with four-hole vent caps (Corning Science de México, Reynosa, Mexico) containing 20 ml of semi-synthetic medium labeled M2 (Oliva Hernández et al., 2013), to simulate basic composition of sugars of diluted *Agave* must, and containing per liter: 1 g of yeast extract, 2 g of $(NH_4)_2SO_4$, 0.4 g of $MgSO_4{\cdot}7H_2O$, 5 g of KH_2PO_4, 10 g of glucose, and 90 g of fructose. Sugars were autoclaved separately from the rest of the components, and pH was adjusted on the mineral solution to 5 using either concentrated solutions of NaOH or HCl prior to autoclaving. For *Agave* must-based medium, a freshly collected 8-year-old *A. tequilana* Weber stem (*piña*) was ground and mixed with an equal amount of distilled water, and then autoclaved for 1 h at 121°C. The resulting caramelized liquid was filtered, hexose concentration quantified by HPLC as described below, and then diluted with sterile water to an initial hexose concentration of 107 g/L, then supplemented with 2 g/L of $(NH_4)_2SO_4$ and autoclaved again for 15 min at 121°C. Glucose and fructose concentration in this *Agave* must medium (and denominated from here on as *Agave* medium) was 11.6 and 94 g/L, respectively, an approximate glucose/fructose ratio of 1:9. The pure *Saccharomyces* inoculum used was 3×10^6 cells/ml. Inoculum for fermentations used for FISH analysis consisted of one *Saccharomyces* and two non-*Saccharomyces* strains in two proportions: 0.1:1:1 and 1:1:1, respectively. Inoculum in mixed cultures for microbiological enumeration was 3×10^4 cells/ml in a 0.1:1 dilution of *Saccharomyces* and non-*Saccharomyces* strains, to allow the sampling and processing directly without dilution for the microscopical FISH analysis. Incubation was performed at 30°C using an agitated incubator at 200 rpm. Each experiment was run in triplicate for each experimental point, and the measurements for each minibioreactor were performed at least two times.

Biomass and CO_2 Production Quantification

Biomass was quantified as dry weight by centrifuging 2 ml of each sample in pre-weighed 2 ml Eppendorf tubes; the supernatant was recovered and filtered through a 0.45 μm membrane for further HPLC analysis, and the tubes containing the biomass pellet were stove-dried at 60°C overnight, placed in a silica-gel glass dessicator for at least 4 h, and then weighed. Biomass production was calculated as the difference in the weight of the tube divided by the volume of the centrifuged sample. Duplicate samples were taken from each of the three minibioreactor tubes that comprised one experimental sampling point. The production of carbon dioxide per liter was followed by weighing each minibioreactor every 24 h and comparing with the initial weight after considering the loss of weight due to evaporation, which was measured to be 0.0034 g/h ($R^2 = 0.9998$) per hole in the vent cap under the conditions used in this work: 20 ml of M2 or *Agave* medium, 30°C and 200 rpm.

Sugar Consumption and Metabolite Quantification by HPLC

The residual concentration (consumption) of hexoses (D-glucose and D-fructose) and the production of primary metabolites (ethanol, glycerol, and acetic acid) in the centrifuged and filtered sample supernatants were measured by HPLC as reported by Narváez-Zapata et al. (2010) by triplicate using an Agilent 1100

series HPLC equipment, coupled to an autosampler and using a BioRad^{TM} Aminex HPX (30 × 7.8 ml) column. The mobile phase was H_2SO_4 0.005 M. The volume of the injection loop was 25 μl with each run lasting around 30 min with a flow rate of 0.5 ml/min at 35°C. The peaks were detected by IRD or UVD at 190 nm. Calibration curves were constructed using ethanol, glycerol, acetic acid, fructose, and glucose standards ranging from 0.125 to 20 g/L, using linear regression with a Pearson coefficient of not less than 0.95 were chosen.

Mixed Yeasts Populations Quantification by Fish

The specific detection of main yeast species was carried out microscopically by using fluorescence *in situ* hybridization microscopy technique (FISH). This method allows the quantification of complex mixed populations (two or more strains) by using a combination of species-specific probes and universal probes based on D1/D2 region of the 26S rDNA (Xufre et al., 2006). For this study, the hybridization of the species-specific probes was optimized for Sc (5′-TGACTTACGTCGCAGTCC-3′), Td (5′-GCAGTATTTCTACAGGAT-3′), and Km (5′-AGCTACAAAGTCGCCTTC-3′) for *S. cerevisiae*, *T. delbrueckii* and *K. marxianus*, respectively. Universal EUK probe (5′-ACCAGACTTGCCCTCC-3′), designed in general for eucaria, was also used (Xufre et al., 2006). The isolates were aerobically activated in YM medium (20 g/L of glucose, 5 g/L of yeast extract, and 3 g/L of peptone), under stirring conditions of 250 rpm at 25°C. After the fermentation was carried out on M2 or on *Agave* medium, 1 μl of sample was taken and deposited on the slide, previously covered with gelatin (0.1%) and 0.01% of KCr $(SO_4)_2$, and it was left to dry at room temperature for 40 min. Subsequently, dehydration was carried out with EtOH at three different concentrations (50, 70, and 96%) for a period of 3 min in each one. Then, samples were dried on environmental temperature during 20 min. During this time the hybridization solution was prepared, which consisted of 70% of (NaCl 0.9 M, Na_2EDTA 5 mM, Tris/HCl 20 mM pH 7, SDS 0.01%) and 70% of formamide; 9 μl was taken from this solution for every 1 μl of probe, then 10 μl was placed on the already dehydrated and dried sample to be placed in a 50-ml falcon tube to be hybridized in the oven at 42°C from 1 to 4 h. Once the hybridization was finished, a careful washing was given with distilled water, and it was subjected to a washing buffer (0.9M NaCl, 5 mM Na2EDTA, 20mM Tris/HCl pH 7, 0.01% SDS) for 15 min. Once the buffer wash was finished, another wash was given with distilled water, and it was left to dry at room temperature. Once the slide was dry, a few drops of Citifluor® oil (Cargille Laboratories, Cedar Grove, NJ, USA) were placed on the slide to avoid oxidation of the sample when in contact with UV light. Finally, the visualization of the fluorescent detection under the 100× objective was carried out using an Olympus Microscope (Olympus BX51; Olympus, Tokyo, Japan) with a BX-URA2 reflector illuminator, the BX-RFA fluorescence illuminator with filter U-RSL6EM (U-RSL6), U-MWIG2 filters (maximum excitation 552 nm and maximum emission 565 nm) for probes with Cy3 labeling, and U-MNUA2 filter (maximum excitation 372 nm and maximum emission 456 nm) for DAPI (4′, 6-diamidino-2-phenyl indole dilactate). The microscope had an RGB infinity camera attached to the trinocular connected to a personal computer with the Image-Pro Plus version 6.3 analysis and image processing program (Media Cybernetics, Rockville, MD, USA). The summatory of the area of the cells present in 30 randomly selected fields of view were measured by duplicate for each slide with the different probes, and expressed as squared micrometers of cells, throughout the duration of the fermentations. It is worth commenting that after 48 h, the samples presented a diminished fluorescence (as also observed by Andorra et al., 2011); nevertheless, it did not interfere with the area measurement.

Mixed Yeasts Populations Quantification by Differential Plate Counts

A second population analysis, considering standard microbiological enumerations on these mixed cultures, was also conducted, by enumeration of the culturable yeasts in mixed cultures by using WL Differential and Nutrient Agars (Sigma-Aldrich, St. Louis, MO, USA). The count plating technique allows the differentiation of *Saccharomyces* and non-*Saccharomyces* colony populations and their quantification, which in turn allows the whole culturable yeast population determination, as established in De la Torre-González et al. (2016), based on the work of Hedges (2002). Limitations of the differential WL media include that it was only reliably useful to determine unequivocally dual mixed cultures of *Saccharomyces* and non-*Saccharomyces* strains (De la Torre-González et al., 2020) due to the indistinguishable appearance of *Torulaspora delbrueckii* and *Kluyveromyces marxianus* colonies used in this work. In parallel, total and viable cell counts were also determined microscopically by counting on a Neubauer chamber, using methylene blue staining as an indicator of viability. All the samples were analyzed in triplicate.

Aromatic Profile Quantification by GC-MS

Analysis of volatile compounds was performed according to the methodology of Márquez-Lemus et al. (2019) with some modifications. Fermentation samples were centrifuged at 7000 rpm (5697×*g*) for 15 min at 10°C in a Sigma 6K15 centrifuge. One gram of the centrifuged sample was deposited in a 20-ml vial with a magnetic cap and a teflon-silicon septum, and volatiles were extracted by a 2-cm PDMS/DVB/CAR solid-phase micro extraction fiber (Supelco; Sigma-Aldrich) while being heated at 45°C and agitated at 200 rpm for 1 h. The fiber was desorbed at 250°C, splitless mode, to the injection port of a 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a capilar column DB-5MS (60 m × 250 μm diameter, 0.25 μm film thickness, Agilent Technologies Inc.) and coupled to a 5975C single quadrupole mass spectrometer (Agilent Technologies, Inc.). The carrier gas was helium (99.99 % purity) at a flow rate of 1.0 ml/min, and the following temperature program was used: 3 min at 40°C, first ramp of 5°C/min up to 180°C, second ramp of 20°C/min up to 260°C, and held for 5 min. The transfer line was set at 280°C. A solution

TABLE 1 | Primary metabolite production and residual hexoses for all yeasts strains tested at 48 h of fermentation in semi-synthetic medium M2.

Strain	Glucose	Fructose	Ethanol	CO_2	Glycerol	Acetic ac.	Dry weight
				g/L			
Sc3D4	**0.0** ± 0.0 ^{a}	**11.9** ± 4.50 ^{bcd}	**29.4** ± 5.10 ^{bcd}	**57.7** ± 4.19 ^{abc}	**1.80** ± 0.26 ^{c}	**0.4** ± 0.18^{a}	**3.8** ± 0.20
Sc3D5	**0.0** ± 0.0 ^{a}	**16.9** ± 1.26 ^{efg}	**25.1** ± 0.60 ^{fghij}	**52.2** ± 2.15 ^{cdefg}	**1.52** ± 0.17 ^{def}	**0.2** ± 0.17 ^{defg}	**3.8** ± 0.13
Sc3Y2	**1.4** ± 0.0 ^{b}	**23.7** ± 1.41 ^{i}	**23.4** ± 1.49 ^{ij}	**52.2** ± 7.91 ^{defgh}	**0.99** ± 0.03 ^{ij}	**0.2** ± 0.10 ^{defghi}	**3.2** ± 0.34
Sc3D6	**0.0** ± 0.0 ^{a}	**15.4** ± 2.36 ^{def}	**25.6** ± 1.52 ^{fghij}	**50.7** ± 2.12 ^{cdefgh}	**1.57** ± 0.15 ^{cde}	**0.3** ± 0.10 ^{defghij}	**4.4** ± 0.13
ScMosca3	**0.0** ± 0.0 ^{a}	**9.90** ± 4.80 ^{bc}	**29.5** ± 3.62 ^{bcd}	**56.7** ± 6.79 ^{bcd}	**1.58** ± 0.24 ^{c}	**0.1** ± 0.11 ^{defghij}	**4.4** ± 0.24
Sc4Y3	**0.0** ± 0.0 ^{a}	**12.8** ± 0.92 ^{cde}	**25.2** ± 1.21 ^{efghij}	**55.7** ± 1.10 ^{abc}	**1.43** ± 0.05 ^{def}	**0.2** ± 0.10 ^{efghij}	**4.2** ± 0.25
Sc3D2	**0.5** ± 0.4 ^{b}	**12.8** ± 6.48 ^{ef}	**26.8** ± 6.18 ^{ghij}	**50.4** ± 7.80 ^{fghi}	**1.46** ± 0.31 ^{efg}	**0.3** ± 0.12 ^{bcde}	**3.2** ± 0.64
Sc3Y3	**0.0** ± 0.0 ^{a}	**14.8** ± 0.78 ^{def}	**27.7** ± 1.15 ^{cdefg}	**49.9** ± 0.50 ^{cdefgh}	**1.67** ± 0.03 ^{c}	**0.4** ± 0.08 ^{abc}	**3.3** ± 0.04
Sc3Y5	**0.6** ± 0.0 ^{a}	**15.9** ± 0.09 ^{ef}	**27.4** ± 0.35 ^{defghi}	**48.6** ± 1.03 ^{defgh}	**1.63** ± 0.04 ^{cd}	**0.3** ± 0.01 ^{abcd}	**2.8** ± 0.10
Sc3Y4	**0.0** ± 0.0 ^{a}	**12.4** ± 0.86 ^{hi}	**26.3** ± 2.20 ^{defghi}	**53.7** ± 0.50 ^{bcdef}	**1.46** ± 0.02 ^{def}	**0.2** ± 0.01 ^{fghijk}	**3.2** ± 0.17
Sc3Y8	**0.0** ± 0.0 ^{a}	**10.8** ± 0.40 ^{ghi}	**29.7** ± 1.70 ^{cdef}	**60.2** ± 2.21 ^{a}	**1.48** ± 0.13 ^{def}	**0.3** ± 0.12 ^{bcde}	**3.0** ± 0.25
Fermichamp	**0.2** ± 0.5 ^{cd}	**10.8** ± 8.01 ^{fghi}	**29.8** ± 6.01 ^{abc}	**59.3** ± 7.76 ^{abc}	**1.34** ± 0.25 ^{def}	**0.2** ± 0.09 ^{ghijkl}	**4.8** ± 0.52
Km4D3	**0.2** ± 0.4 ^{a}	**19.6** ± 2.50 ^{fgh}	**23.7** ± 1.60 ^{hij}	**47.9** ± 0.23 ^{efghi}	**1.99** ± 0.20 ^{b}	**0.4** ± 0.02 ^{ab}	**2.8** ± 0.04
Km1D5	**0.0** ± 0.0 ^{a}	**7.13** ± 2.83 ^{ab}	**28.9** ± 0.96 ^{ced}	**59.1** ± 1.15 ^{ab}	**1.96** ± 0.10 ^{b}	**0.3** ± 0.09 ^{abcd}	**2.9** ± 0.23
Km1Y9	**0.0** ± 0.0 ^{a}	**3.43** ± 1.83 ^{a}	**33.2** ± 2.19 ^{ab}	**57.5** ± 3.19 ^{abc}	**2.22** ± 0.10 ^{a}	**0.3** ± 0.05 ^{defg}	**3.1** ± 0.22
Td1AN9	**0.0** ± 0.0 ^{a}	**8.27** ± 5.47 ^{bcd}	**29.4** ± 3.66 ^{cdefgh}	**53.6** ± 5.38 ^{cdefg}	**1.94** ± 0.20 ^{b}	**0.2** ± 0.02 ^{defgh}	**3.0** ± 0.31
Td1AN2	**2.0** ± 0.3 ^{fg}	**28.6** ± 1.94 ^{k}	**15.8** ± 1.45 ^{k}	**40.5** ± 0.99 ^{jk}	**1.21** ± 0.09 ^{gh}	**0.1** ± 0.02 ^{ijkl}	**3.0** ± 0.13
Td1AN1	**4.6** ± 0.1 ^{h}	**44.7** ± 0.45 ^{l}	**5.09** ± 0.19 ^{l}	**30.4** ± 3.00 ^{l}	**0.65** ± 0.04 ^{kl}	**0.1** ± 0.01 ^{ijkl}	**2.2** ± 0.13
Pk4D6	**1.6** ± 0.0 ^{fg}	**30.9** ± 0.83 ^{k}	**14.4** ± 1.47 ^{k}	**35.9** ± 2.35 ^{kl}	**1.13** ± 0.15 ^{hi}	**0.2** ± 0.07 ^{fghijk}	**2.2** ± 0.06
Pm1AN3	**1.9** ± 0.2 ^{g}	**27.9** ± 1.54 ^{k}	**17.7** ± 1.60 ^{k}	**49.5** ± 10.7 ^{hij}	**0.59** ± 0.03 ^{l}	**0.1** ± 0.01 ^{jkl}	**2.7** ± 0.36
Pg1Y12	**5.5** ± 0.2 ^{h}	**46.4** ± 1.63 ^{l}	**1.79** ± 0.29 ^{l}	**23.8** ± 0.13 ^{m}	**0.22** ± 0.02 ^{m}	**0.1** ± 0.02 ^{l}	**2.3** ± 0.19
Zb3Y1	**5.0** ± 0.4 ^{h}	**7.11** ± 2.51 ^{fghi}	**29.5** ± 2.65 ^{cdef}	**53.8** ± 4.22 ^{cdefg}	**1.64** ± 0.10 ^{cd}	**0.1** ± 0.01 ^{hijkl}	**3.6** ± 0.31
Cl4Y4	**0.0** ± 0.0 ^{a}	**7.55** ± 2.15 ^{fgh}	**36.0** ± 2.90 ^{a}	**56.6** ± 7.40 ^{bcd}	**0.82** ± 0.03 ^{jk}	**0.1** ± 0.02 ^{kl}	**3.1** ± 0.26
Cp1Y7	**1.3** ± 0.1 ^{efg}	**28.0** ± 0.69 ^{k}	**15.6** ± 1.07 ^{k}	**41.2** ± 0.74 ^{ijk}	**1.29** ± 0.03 ^{fg}	**0.2** ± 0.01 ^{defghij}	**1.9** ± 0.07
RmP12	**1.0** ± 0.3 ^{def}	**20.9** ± 3.72 ^{j}	**21.5** ± 4.07 ^{j}	**46.2** ± 1.91 ^{ghi}	**1.55** ± 0.09 ^{cde}	**0.2** ± 0.01 ^{defghi}	**2.6** ± 0.10

Data are presented showing average values (in bold) ± standard deviation. Different letters in the columns show significant differences according to ANOVA testing at a $p \leq 0.05$ (LSD test) except for dry weight.

of 20 mg/kg of 3-octanol was used as the internal standard. Electronic impact mode at 70 eV was used, with a gain factor of 1, with ionization source and quadrupole temperatures of 230 and 150°C, respectively, and a mass range between 33 and 500 μma. Identification of volatile compounds was obtained by comparing their mass spectra with those of the NIST/EPA/NIH Mass Spectra Library database version 1.7.

Statistical Analysis

Production differences of all the metabolites during fermentations were assessed by statistical analysis performed using the Analyze-it software for Microsoft Excel (version 2.20) and JMP routine of the SAS software for ANOVA analysis.

RESULTS

Pure Inoculum Fermentation Performance on Semi-synthetic (M2) Medium

Yeasts were firstly characterized in semi synthetic M2 medium. Results obtained showed a differential profile at 48 h (early stationary phase) in the consumption of sugars and fermentative metabolites (**Table 1**). All tested yeast species were able to produce ethanol, with *S. cerevisiae* strains being the most productive, ranging from 25 to 30 g/L as expected, but some of the non-*Saccharomyces* strains were also able to produce around 30 g/L such as the *K. marxianus* (Km1Y9), *T. delbrueckii* (Td1AN9) as well as *Z. bailii* (Zb3Y1) and *C. lusitaniae* (Cl4Y4) during this fermentation time. Regarding residual sugars, all the strains consumed first glucose present on the medium and still had residual fructose at this sampling time, being around 10 g/L in the case of the fructophilic strain control Fermichamp. In general, the non-*Saccharomyces* strains consumed at a lower rate the two hexoses than the *S. cerevisiae* strains. Some non-*Saccharomyces* strains such as *Kluyveromyces* (Km1Y9) and the *Torulaspora* (Td1AN91) also left < 10 g/L of fructose at this time. All yeasts were able to produce glycerol at this time, the *Torulaspora* strains being the ones that exhibited a higher average (>2 g/L), followed by the *Saccharomyces* strains. The *M. guilliermondii* strain (Pg1Y12) exhibits the lowest glycerol accumulation with 0.22 g/L. On the other hand, all the strains of non-*Saccharomyces* showed a lower concentration of acetic acid than the strains of *S. cerevisiae*, except *K. marxianus* (Km4D3) which showed

TABLE 2 | Volatile metabolite production for all yeast strains tested at 48 h of fermentation in semi synthetic medium M2.

Strain	2-methyl-1-butanol	Ethyl butyrate	Isoamyl acetate	Ethyl hexanoate	Hexyl acetate	Ethyl octanoate	Phenyl ethyl acetate	Ethyl dodecanoate
	μg/L							
Sc3D4	**250** ± 10	**2** ± 0.1	**7** ± 1	**8** ± 0.5	ND	**25** ± 1	**35** ± 4	**6** ± 2
Sc3D5	**260** ± 30	**2** ± 0.2	**4** ± 1	**4** ± 0.5	ND	**25** ± 2	**33** ± 3	**6** ± 1
Sc3Y2	**290** ± 0.2	**2** ± 0.5	**3** ± 1	**0.5** ± 0.2	ND	**9** ± 1	**46** ± 23	ND
Sc3D6	**230** ± 30	**3** ± 0.2	**7** ± 1	**6** ± 0.8	ND	**22** ± 2	**47** ± 28	**4** ± 0.3
ScMosca3	**260** ± 10	**2** ± 0.1	**5** ± 1	**4** ± 0.8	ND	**24** ± 1	**36** ± 6	**3** ± 1
Sc4Y3	**250** ± 40	**2** ± 0.3	**5** ± 1	**5** ± 0.7	ND	**22** ± 1	**33** ± 4	**4** ± 1
Sc3D2	**340** ± 40	**3** ± 0.5	**7** ± 2	**6** ± 0.8	ND	**38** ± 5	**31** ± 4	**21** ± 4
Sc3Y3	**400** ± 30	**3** ± 0.3	**14** ± 5	**7** ± 0.7	ND	**30** ± 4	**59** ± 30	**18** ± 1
Sc3Y5	**380** ± 20	**3** ± 0.2	**9** ± 1	**9** ± 1.2	ND	**24** ± 0.9	**36** ± 10	**4** ± 2
Sc3Y8	**290** ± 10	**2** ± 0.1	**4** ± 0.3	**4** ± 0.5	ND	**21** ± 4	**24** ± 3	**5** ± 0.9
Fermichamp	**280** ± 50	**2** ± 0.1	**9** ± 4	**4** ± 0.02	ND	**20** ± 3	**49** ± 20	**7** ± 1
Sc3Y4	**290** ± 10	**2** ± 0.1	**5** ± 0.4	**5** ± 0.5	ND	**29** ± 1	**33** ± 9	**5** ± 2
Km4D3	**460** ± 20	**1** ± 0.2	**15** ± 0.5	**1** ± 0.1	ND	**2** ± 0.1	**1425** ± 180	**2** ± 0.4
Km1D5	**510** ± 30	**1** ± 0.8	**378** ± 47	**0.2** ± 0.1	ND	**2** ± 0.5	**1412** ± 110	**2** ± 0.6
Km1Y9	**400** ± 30	**1** ± 0.2	**12** ± 3	**0.2** ± 0.03	ND	**2** ± 0.1	**1215** ± 150	ND
Td1AN9	**410** ± 20	**1** ± 0.3	**118** ± 69	**0.2** ± 0.05	ND	**2** ± 0.2	**1291** ± 320	**4** ± 0.6
Td1AN2	**260** ± 20	**0.4** ± 0.1	**291** ± 30	**1** ± 0.04	**0.3** ± 0.1	**3** ± 0.4	**1185** ± 120	**2** ± 0.4
Td1AN1	**390** ± 20	**1** ± 0.05	**2** ± 0.2	ND	ND	ND	ND	ND
Pk4D6	**180** ± 20	ND	**204** ± 20	ND	ND	**1** ± 0.2	**1229** ± 80	ND
PkP12g	**190** ± 10	**0.4** ± 0.1	**214** ± 20	**0.7** ± 0.02	ND	**9** ± 2	**1241** ± 290	**3** ± 1
Pm1AN3	**340** ± 70	**2** ± 0.3	**10** ± 4	**0.2** ± 0.1	ND	ND	**45** ± 30	**1** ± 0.03
Pg1Y12	**320** ± 20	**1** ± 0.1	**3** ± 1	**0.3** ± 0.2	ND	ND	**22** ± 10	ND
Zb3Y1	**280** ± 20	**1** ± 0.2	**1** ± 1	**0.1** ± 0.04	ND	**1** ± 0.1	**147** ± 10	**2** ± 0.1
Cl4Y4	**310** ± 60	**2** ± 0.3	**9** ± 10	**0.3** ± 0.2	ND	ND	**33** ± 30	ND
Cp1Y7	**230** ± 20	**0.3** ± 0.04	**167** ± 10	**0.1** ± 0.01	ND	**1** ± 0.04	**1345** ± 110	**1** ± 0.3
RmP12	**300** ± 3	**1** ± 0.1	**8** ± 4	**3** ± 0.2	ND	**16** ± 1	**491** ± 100	**5** ± 1

Data are presented as average values (in bold) ± standard deviation; ND, not detected.

an increased in the concentration (0.4 g/L) of acetic acid, a parameter that is important for the alcoholic beverage industry. Regarding volatile metabolite production on M2 (**Table 2**), all tested strains presented a similar production of some volatiles, such as 2-methyl-1-butanol (between 200 and 500 μg/L), a similar production of ethyl-butyrate (around 2 mg/L). In contrast, *Saccharomyces* strains produced higher ethyl-hexanoate concentrations and lower concentrations of isoamyl acetate and phenyl ethyl acetate, when compared with the non-*Saccharomyces* strains. Hexyl acetate was only detected for a *Torulaspora* (Td1AN2) strain. From all these data, plus previous works on tolerance to osmotic, oxidative, and temperature stresses (Oliva Hernández et al., 2013; De la Torre-González et al., 2016; Vergara-Álvarez et al., 2019), we selected a group of three *S. cerevisiae* mezcal strains (plus Fermichamp as fructophilic control) and the best performing strains of the *Kluyveromyces* (Km1Y9), *Torulaspora* (Td1AN9) and *Zygosaccharomyces* (Zb3Y1) genera, although it is worth mentioning that some other promising strains are still being investigated.

Pure Inoculum Fermentation Performance on Agave Medium

Fermentative profile of these selected yeasts was summarized at 96 h on *Agave* medium (**Table 3**) choosing the final stage of the fermentation. Mezcal *Saccharomyces* and non-*Saccharomyces* strains exhibited high fructose consumption (left around 3 g/L) and a high ethanol productivity (around 50 g/L). Notably, the control strain Fermichamp had a very poor growth and was unable to consume the hexoses, and consequently did not ferment the *Agave* medium, probably due to the presence of a high concentration of inhibitory Maillard compounds, saponins, and furfural (Díaz-Montaño et al., 2008). In general, Zb3Y1 produced the higher concentrations of glycerol and the lower concentration of acetic acid. In terms of dry weight, strain Km1Y9 is very promising, as it produced the lowest amount of biomass. Regarding the production of volatile metabolites for the individually inoculated strains on *Agave* medium, we can observe (**Table 5** upper panel) that, for the Fermichamp strain, the detected 2-methyl-1-butanol is part of the composition of the *Agave* medium, as this strain is unable to ferment

TABLE 3 | Residual hexoses and primary metabolite production for the selected yeast strains at 96 h fermenting as individual inoculum on *Agave* medium.

Strain	Glucose	Fructose	Ethanol	CO_2	Glycerol	Acetic ac.	Dry weight
				g/L			
Fermichamp	**11.7** ± 1.36 ^{b}	**93.6** ± 6.12 ^{c}	**0.00** ± 0.00 ^{d}	**0.50** ± 0.77 ^{f}	**0.00** ± 0.00 ^{c}	**0.00** ± 0.00 ^{c}	**3.3** ± 0.16
Sc3Y3	**0.00** ± 0.00 ^{a}	**3.26** ± 0.08 ^{a}	**50.2** ± 1.35 ^{bc}	**55.5** ± 1.96 ^{d}	**4.43** ± 0.23 ^{b}	**0.13** ± 0.05 ^{bc}	**8.9** ± 0.18
Sc3Y4	**0.00** ± 0.00 ^{a}	**2.97** ± 0.04 ^{a}	**47.6** ± 0.55 ^{bc}	**55.9** ± 0.89 ^{d}	**4.53** ± 0.17 ^{b}	**0.17** ± 0.02 ^{b}	**9.0** ± 0.20
Sc3Y8	**0.00** ± 0.00 ^{a}	**3.15** ± 0.03 ^{a}	**46.8** ± 1.21 ^{c}	**59.1** ± 0.98 ^{b}	**4.39** ± 0.23 ^{b}	**0.17** ± 0.07 ^{b}	**8.9** ± 0.22
Km1Y9	**0.00** ± 0.00 ^{a}	**2.19** ± 0.03 ^{a}	**55.8** ± 0.72 ^{a}	**61.4** ± 0.47 ^{a}	**4.23** ± 0.10 ^{b}	**0.82** ± 0.08 ^{a}	**4.8** ± 0.12
Td1AN9	**0.00** ± 0.00 ^{a}	**8.17** ± 1.58 ^{b}	**52.5** ± 0.68 ^{ab}	**57.3** ± 1.35 ^{c}	**4.06** ± 0.23 ^{b}	**0.71** ± 0.08 ^{a}	**5.7** ± 0.45
Zb3Y1	**1.75** ± 3.02 ^{a}	**3.33** ± 1.92 ^{a}	**49.3** ± 4.84 ^{bc}	**51.3** ± 1.42 ^{e}	**7.10** ± 0.31 ^{a}	**0.08** ± 0.05 ^{bc}	**9.2** ± 0.14

Data are presented showing average values (in bold) ± *standard deviation. Different letters in the columns show significant differences according to ANOVA testing at a* $p \leq 0.05$ *(LSD test) except for dry weight.*

TABLE 4 | Primary metabolite profiles and hexose consumption for the mixed cultures at 96 h in *Agave* medium.

Sacc-no Sacc	Glucose	Fructose	Ethanol	CO_2	Glycerol	Acetic ac	Dry weight
				g/L			
Sc3Y3-Td1AN9	**0.00** ± 0.00 ^{a}	**3.09** ± 0.20 ^{bc}	**52.8** ± 0.5 ^{ab}	**57.6** ± 1.18 ^{d}	**7.56** ± 2.29 ^{a}	**0.85** ± 0.54 ^{abc}	**6.1** ± 1.29
Sc3Y3-Km1Y9	**0.00** ± 0.00 ^{a}	**1.78** ± 0.03 ^{a}	**48.6**± 0.83 ^{c}	**75.6** ± 11.1 ^{a}	**5.06** ± 0.10 ^{ab}	**0.11** ± 0.04 ^{c}	**5.1** ± 1.36
Sc3Y3-Zb3Y1	**0.00** ± 0.00 ^{a}	**2.67** ± 0.06 ^{abc}	**52.3**± 4.12 ^{ab}	**50.5** ± 2.78 ^{g}	**4.53** ± 0.04 ^{b}	**0.10** ± 0.01 ^{c}	**9.3** ± 0.33
Sc3Y4-Td1AN9	**0.00** ± 0.00 ^{a}	**2.75** ± 0.13 ^{abc}	**52.3** ± 0.64 ^{a}	**55.5** ± 1.36 ^{e}	**5.85** ± 0.17 ^{ab}	**1.35** ± 0.24 ^{a}	**6.6** ± 0.17
Sc3Y4-Km1Y9	**0.00** ± 0.00 ^{a}	**1.79** ± 0.02 ^{ab}	**50.1** ± 1.02 ^{bc}	**59.8** ± 1.09 ^{c}	**5.05** ± 0.05 ^{ab}	**0.10** ± 0.06 ^{c}	**5.5** ± 0.80
Sc3Y4-Zb3Y1	**0.00** ± 0.00 ^{a}	**2.78** ± 0.16 ^{abc}	**52.3** ± 0.39 ^{a}	**54.5** ± 1.43 ^{e}	**4.55** ± 0.13 ^{b}	**0.09** ± 0.03 ^{c}	**9.0**± 0.43
Sc3Y8-Td1AN9	**0.00** ± 0.00 ^{a}	**3.13** ± 0.21 ^{c}	**52.8** ± 2.03 ^{a}	**64.2** ± 9.12 ^{b}	**6.22** ± 0.15 ^{ab}	**1.02** ± 0.47 ^{ab}	**6.3** ± 0.45
Sc3Y8-Km1Y9	**0.00** ± 0.00 ^{a}	**1.79** ± 0.02 ^{ab}	**49.9** ± 1.28 ^{bc}	**64.1** ± 1.04 ^{b}	**5.89** ± 1.89 ^{ab}	**0.24** ± 0.17 ^{bc}	**6.7** ± 1.43
Sc3Y8-Zb3Y1	**0.00** ± 0.00 ^{a}	**2.94** ± 0.18 ^{abc}	**52.8** ± 0.86 ^{a}	**52.0** ± 1.89 ^{f}	**4.58** ± 0.04 $^{b\ b}$	**0.11** ± 0.01 ^{c}	**9.2** ± 0.34

Data are presented showing average values (in bold) ± *standard deviation. Different letters in the columns show significant differences according to ANOVA testing at a* $p \leq 0.05$ *(LSD test) except for dry weight.*

it. The non-*Saccharomyces* strains Td1AN9 and Km1Y9 were characterized by their high production of phenyl ethyl acetate and no production of ethyl octanoate. From these results, we decide not to use the commercial strain Fermichamp for the mixed inoculum; hence, three mezcal *S. cerevisiae* strains in combination with the three previously selected non-*Saccharomyces* strains were further characterized.

Mixed Yeast Fermentation Performance on Agave Medium

For testing the performance of mixed inocula on *Agave* medium, three non-*Saccharomyces* strains (*K. marxianus* 1Y9, *T. delbrueckii* 1AN9, and *Z. bailli* 3Y1) were selected to be assayed in combination with each of the *S. cerevisiae* strains (3Y3, 3Y4, and 3Y8) inoculated at a ratio of 1:1 for mixed inoculum fermentations on *Agave* medium. The mixed cultures of the three *S. cerevisiae* with *K. marxianus*, *T. delbrueckii*, and *Z. bailli* were performed under semi-anaerobic growth conditions (1-hole vent caps) which allowed a longer carbon dioxide production and a high reproducibility of the fermentations. Hexose consumption, CO_2, dry weight, and ethanol production were analyzed at 96 h (**Table 4**). In general, the mixed cultures showed a moderate increase on ethanol, glycerol, and acetic acid concentrations as compared with their pure inoculum fermentations (**Table 3**). Accordingly, residual fructose in these mixed cultures was also lower than the pure cultures and having high yields of ethanol ($Y_{EtOH/S}$) ranging from 0.46 to 0.49. According to the statistical analysis on these data, fermentation performance for primary metabolites of the mixed inoculum of Sc3Y8 combined with either Km1Y9 or Td1AN9 was the best, followed by the mixed inoculum of Sc3Y3 with Km1Y9. Regarding volatile metabolite production for the selected strains fermenting in the *Agave* medium as mixed inoculum (**Table 5**, lower panel), we observed that, as expected from the individually inoculated experiments (**Table 5**, upper panel), the use of Td1AN9 and Km1Y9 produced a fermented product with a higher phenyl ethyl acetate concentration, being the highest for the combination with any of the *Saccharomyces* mezcal strains.

Population Quantification by Fish Analysis

To assess the population dynamics of the selected yeast species, a FISH analysis was performed, since it allows the reliable identification of two or more simultaneous strains in mixed cultures. From the previous results for the mixed yeast fermentations on *Agave* media, strains selected for this analysis were Sc3Y3 and the non-*Saccharomyces* strains Km1Y9 and

TABLE 5 | Volatile metabolite production for selected yeasts, fermenting as individual (upper panel) or mixed (lower panel) inocula fermentations, tested at 96 h of fermentation in *Agave* medium.

Strain	2-methyl-1-butanol	Ethyl butyrate	Isoamyl acetate	Ethyl hexanoate	Ethyl octanoate	Phenyl ethyl acetate	Ethyl dodecanoate
				μg/L			
Sc 3Y3	**200** ± 10	**4** ± 0.5	**6** ± 0.9	**6** ± 0.4	**15** ± 1	**14** ± 1	**2** ± 0.1
Sc 3Y4	**220** ± 3	**3** ± 0.2	**4** ± 0.1	**5** ± 0.01	**15** ± 2	**17** ± 0.1	**2** ± 0.1
Sc 3Y8	**230** ± 30	**3** ± 0.3	**5** ± 1	**6** ± 0.06	**16** ± 1	**18** ± 2	**2** ± 0.3
Fermichamp	**230** ± 60	ND	ND	ND	ND	**7** ± 0.3	ND
Td 1AN9	**180** ± 20	**1** ± 0.2	**24** ± 0.4	**1** ± 0.5	ND	**369** ± 30	**1** ± 0.4
Km 1Y9	**260** ± 80	**0.5** ± 0.1	**9** ± 1	**0.8** ± 0.3	ND	**329** ± 30	ND
Zb 3Y1	**260** ± 10	**2** ± 0.1	**1** ± 0.2	ND	ND	17 ± 1	**1** ± 0.1
Mixed inocula							
Sc3Y3-Td1AN9	**270** ± 10	**2** ± 0.2	**13** ± 2	**3** ± 1	**12** ± 0.4	**141** ± 4	**2** ± 1
Sc3Y3-Km1Y9	**250** ± 4	**2** ± 0.5	**14** ± 3	**2** ± 0.3	**8** ± 2	**283** ± 30	**1** ± 0.5
Sc3Y3-Zb3Y1	**220** ± 10	**2** ± 0.1	**3** ± 0.1	ND	ND	**21** ± 0.1	**1** ± 0.2
Sc3Y4-Td1AN9	**250** ± 30	**2** ± 0.2	**15** ± 2	**2** ± 0.1	**11** ± 0.2	**133** ± 10	**1** ± 1
Sc3Y4-Km1Y9	**260** ± 10	**2** ± 0.6	**14** ± 3	**2** ± 0.1	**9** ± 0.03	**315** ± 50	**2** ± 1
Sc3Y4-Zb3Y1	**220** ± 4	**2** ± 0.3	**3** ± 0.2	ND	ND	**19** ± 2	**2** ± 0.4
Sc3Y8-Td1AN9	**260** ± 10	**2** ± 0.4	**15** ± 4	**2** ± 0.02	**11** ± 2	**133** ± 3	**1** ± 0.1
Sc3Y8-Km1Y9	**240** ± 10	**1** ± 0.7	**14** ± 2	**2** ± 0.05	**8** ± 1	**273** ± 10	**1** ± 0.2
Sc3Y8-Zb3Y1	**200** ± 10	**2** ± 0.1	**3** ± 0.2	ND	ND	**21** ± 1	**2** ± 1

Data are presented as average values (in bold) ± standard deviation; ND, not detected.

Td1AN9, to be evaluated simultaneously in mixed cultures, to assess the influence of the presence of the three yeast populations. For this aim, ScX, KmX, and TdX probes allowed us to identify genera *Saccharomyces*, *Kluyveromyces*, and *Torulospora*, respectively. Formamide concentration was adjusted to 30% since this concentration allowed a good fluorescence signal and hybridization for all the probes selected (data not shown). Populations for each genus were inferred from the measured fluorescent area obtained by the different probes on these mixed fermentations on *Agave* medium. Two inoculation ratios were assayed for this FISH approach. The 0.1:1:1 (**Figure 1A**) and the 1:1:1 (**Figure 1B**) for Sc3Y3, Td1AN9, and Km1Y9 strains, respectively, visually show the population dynamics for each yeast in mixed fermentations. Quantitatively, **Figures 2A,B** present the fluorescent areas for each yeast species and the total area for both inoculation ratios (left axis) and compares them with the total cell concentration by using a Neubauer chamber (black dots, right axis). We can observe that DAPI (blue diamonds, all yeasts present) fluorescent areas had similar profiles to total cell count, as it was expected. Main changes were observed at the beginning of the fermentative process, probably linked to the yeast ratio differences. All yeasts showed a rapid increase on their areas during the first 24 h of culture, and in both inoculation ratios; *Saccharomyces* strain (red squares) had a higher final fluorescent area, whilst both *K. marxianus* (purple dots) and *T. delbrueckii* (green triangles) had the same profiles, being lower in area than the one for S. *cerevisiae*, nonetheless remaining present in the culture. To assess in more detail the growth and compatibility of the mixed inoculum, and to link it with the metabolites' profiles, we chose the combination of the three *S. cerevisiae* mezcal strains with *T. delbrueckii* to specifically follow by differential plate counts the evolution of their viable and culturable populations.

Population Quantification by Differential Agar Plate Counts

Solid WL Differential and Nutrient Agar (Sigma-Aldrich) were used for viable population differentiation during fermentation kinetics of the three mixed cultures tested, which comprised each selected *Saccharomyces cerevisiae* strain in combination with *T. delbrueckii* (Td1AN9), at the inoculation ratio of 0.1:1 (Sc:Td), to mimic naturally occurring conditions of mezcal fermentations. We observed (**Figure 3**) a rapid increase of *S. cerevisiae* populations during the first 24 h, but contrary to the behavior reported on other fermentations, for example, on grape juice (De la Torre-González et al., 2020), a drop of viability/culturability for the *Saccharomyces* strains was assessed at around 48 h after inoculation, while the *T. delbrueckii* strain remained highly viable up to the end of the fermentation. Depending on the specific *S. cerevisiae* strain tested, they were able to further increase their culturable population, but always remaining below of that of *T. delbrueckii*.

DISCUSSION

The use of non-conventional yeasts as inoculum for the traditional fermentative process has increased in the literature, since some strains can increase the aroma complexity of the fermented products (Pina et al., 2005; De la Torre-González

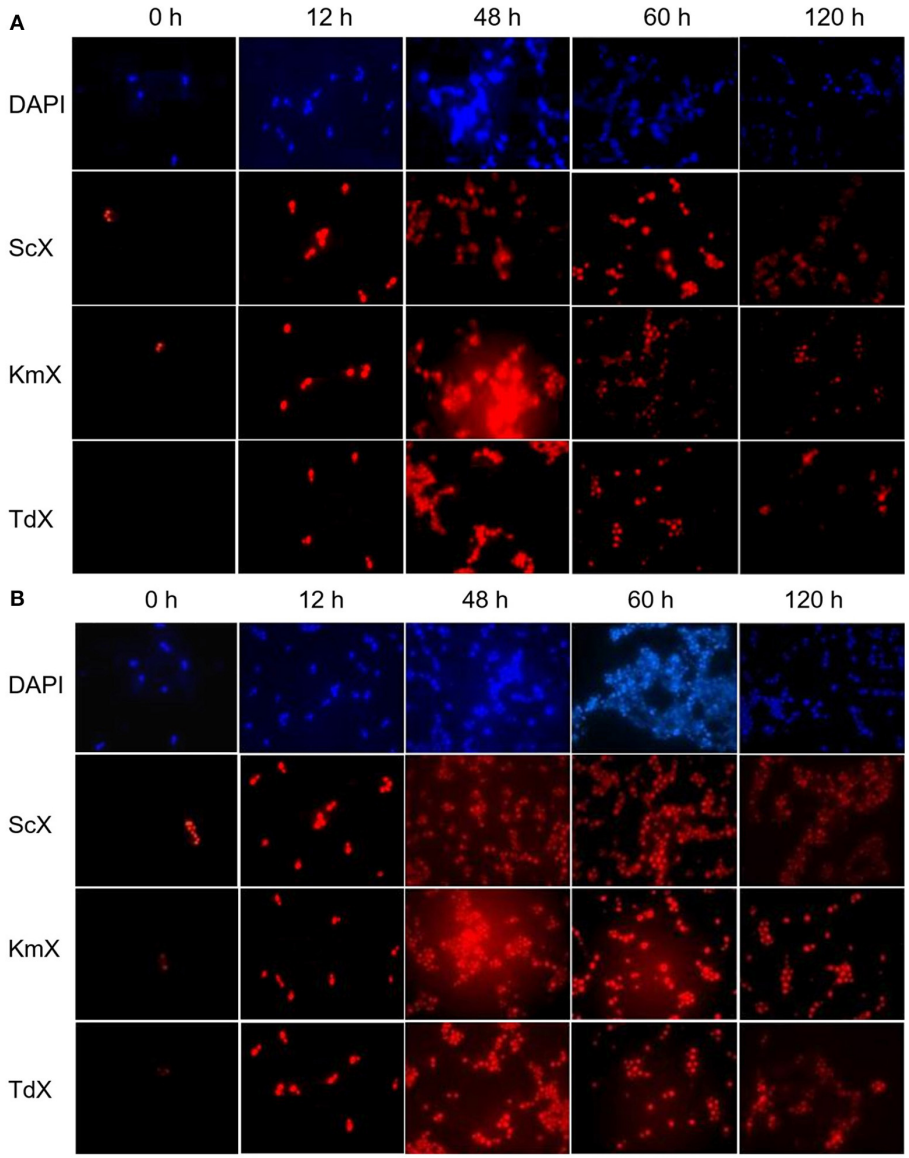

FIGURE 1 | Qualitative examples of FISH images obtained during fermentation of *Agave* medium using specific probes for *Saccharomyces cerevisiae* (Sc3Y3), *Torulaspora delbrueckii* (Td1AN9), and *Kluyveromyces marxianus* (Km1Y9) strains at inoculation ratios of **(A)** 0.1:1:1, and **(B)** 1.1:1, respectively. Fluorescent detection was carried out using an Olympus Microscope (Olympus BX51) at 100×.

et al., 2017; Tufariello et al., 2021). Tequila and mezcal liquors are produced from cooked *Agave* must fermentation, which usually depends on spontaneous or pure *S. cerevisiae* strain inoculation (Cedeño, 1995; Díaz-Montaño et al., 2008). The yeasts used in this work were obtained from mezcal musts and have been selected for having a higher tolerance to stress, particularly for osmotic, oxidative, and temperature unfavorable conditions (De la Torre-González et al., 2016; Vergara-Álvarez et al., 2019). In this study, we aimed to characterize the application potential of some of the yeasts found in mezcal from Tamaulipas (De la Torre-González et al., 2017), first by pure strain inoculation on a semi synthetic medium resembling *Agave* must hexose initial concentrations, and we found that all the strains, but mainly *Saccharomyces*, *Torulospora*, *Kluyveromyces*, *Clavispora*, and *Zygosaccharomyces* were the ones with the best fermentative performances, and also were good producers of volatile compounds, as previously observed for some of these genera (Arellano et al., 2008; Lopez-Alvarez et al., 2012; Páez-Lerma et al., 2013; Nolasco-Cancino et al., 2018). Particularly, *T. delbrueckii* had unique oenological characteristics such as production of specific aromas, as observed for Tondini et al. (2019) and Tufariello et al. (2021) for wine. Indeed, based on our results, these non-*Saccharomyces* strains could be used as pure inoculum to carry out to completion the *Agave* fermentation.

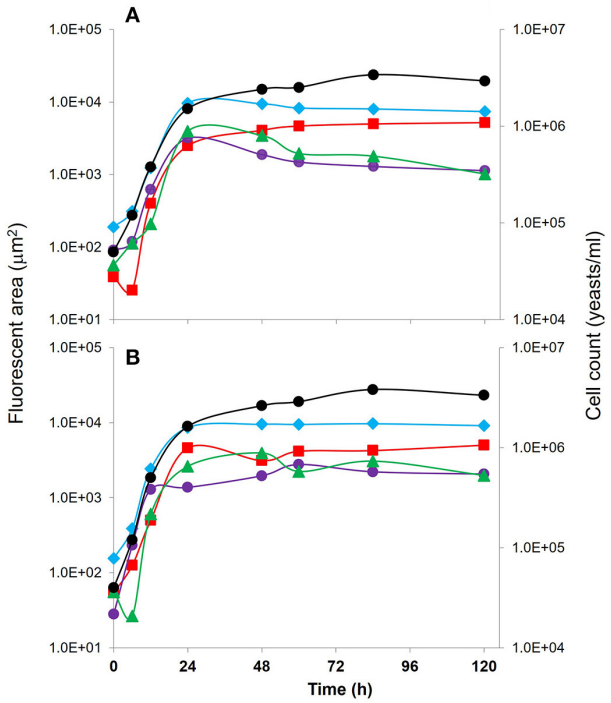

FIGURE 2 | Quantitative growth kinetics measured as total fluorescent areas (from FISH analysis) and as colony plate counts of mixed cultures (Sc:Td:Km) of Sc3Y3, Td1AN9, and Km1Y9 strains for two mixed inocula ratios: **(A)** 0.1:1:1, and **(B)** and 1:1:1, fermenting on *Agave* medium. Symbols: DAPI (all yeasts), Km, Sc, Td, cell count (yeasts/ml). Standard deviation was below 10% for all data.

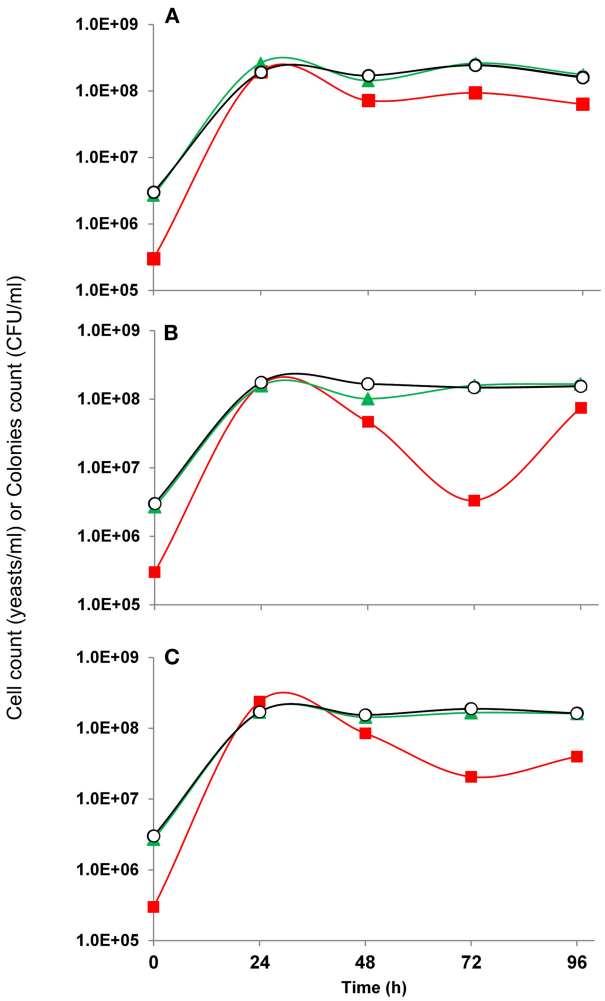

FIGURE 3 | Population growth kinetics of mixed cultures of mezcal *S. cerevisiae* and non-*Saccharomyces* (*Torulaspora delbrueckii*) strains fermenting *Agave* medium. **(A)** Sc3Y3-Td1AN9, **(B)** Sc3Y4-Td1AN9, **(C)** Sc3Y8-Td1AN9. The data shown were obtained from triplicates of two independent experiments. Markers are average values of: total cell count (yeasts/ml), Td (CFU/ml), Sc (CFU/ml), viable cell count (viable yeasts/ml). Standard deviation was below 10% for all data. Notice that total and viable cell count markers overlap.

Some authors have reported the use of few non-*Saccharomyces* strains as pure inoculum for *Agave tequilana* must fermentations, such as *K. marxianus*, *Pichia* spp., *Hanseniaspora* spp., and *Kloeckera* spp. to produce tequila (Arrizon et al., 2006; Díaz-Montaño et al., 2008; Amaya-Delgado et al., 2013; González-Robles et al., 2015; Segura-García et al., 2015) and mezcal from Oaxaca (Nolasco-Cancino et al., 2018).

Concerning *S. cerevisiae*, the used strains in this work were highly productive and readily adapted to *Agave* must conditions, whereas control Fermichamp was not able to ferment the *Agave* medium, as has been also observed for other wine *S. cerevisiae* strains by Arrizon et al. (2006). Another important factor that could have driven the selection of these yeasts, besides the toxic compounds found in the *Agave* musts, concerning the carbon source, is the presence of cellulose and the fructose polysaccharides (usually reported as ATF, *Agave tequilana* fructans, or as agavin), which is related to inulin but has a more branched structure, which implies that, besides the β-glucosidases reported for this genus, a battery of (endo and exo) fructanases and fructosyltransferases are probably active in these strains, as evidenced by Arrizon et al. (2012) and Vicente-Magueyal et al. (2020) for non-*Saccharomyces* yeasts, by Corona-González et al. (2015) for *S. cerevisiae*, and also observed by us for some of these strains (data not shown).

Among the mezcal *Saccharomyces* strains of this study, the main differences were found regarding their ability to ferment fructose, the accumulation/degradation of 2-methyl-1-butanol, and the levels of ethyl-decanoate and ethyl-hexanoate. In general, the volatile compounds produced by these pure mezcal strains on the *Agave* medium are of great aromatic value, particularly isoamyl acetate and phenyl ethyl acetate, compounds which could render (in the appropriate amounts) good organoleptic characteristics (fruity, sweet, pineapple) to a fermentative process (De la Torre-González et al., 2017). For other *S. cerevisiae* strains from *Agave* fermentations, there are also reports of

the differential production of methanol, 2-phenyl ethanol, n-propanol, acetoin, and amyl alcohols (Arrizon et al., 2006; Díaz-Montaño et al., 2008). In other fermented products, like wine, Romano et al. (2003) have reported that, for 30 *S. cerevisiae* wine strains fermenting Aglianico grape must, that a significant variability was observed amongst the strains in terms of the level of production of isoamyl alcohol, isobutanol, and acetic acid, whereas they all were very similar in terms of acetaldehyde, n-propanol, and ethyl acetate productions, which indicates that, even considering that *S. cerevisiae* is by far the most studied yeast, there is still a high variability in aromatic profiles that can be obtained with pure cultures of this species, and that is worth continuing the analysis of isolates from many different technological and natural environments.

Regarding the *K. marxianus* and *T. delbrueckii*, both strains used in the mixed cultures induced a higher phenyl ethyl acetate and isoamyl acetate in the fermented must, as evidenced by a fruiter aroma in the products. The yeast *T. delbrueckii* has been frequently reported to be important in wine production, as it has a high ethanol tolerance and a low acetic acid production when combined with *S. cerevisiae*, for the production of different types of wines, and where signature compounds were identified, such as linalool, 3-methyl-1-butanol, 2-phenylethanol, ethyl butanoate, ethyl decanoate, phenylacetaldehyde, ethyl propanoate, ethyl isobutanoate, and ethyl dihydrocinnamate, as well as benzyl alcohol (Tufariello et al., 2021). All this was linked to a higher β-glucosidase activity in the *T. delbrueckii* strains used than in the *S. cerevisiae* yeasts. Yeast *K. marxianus* (GRO6) has been proposed as a good candidate to be used as sole inoculum for tequila production, as it is also able to complete the fermentation with an adequate organoleptic profile (Amaya-Delgado et al., 2013).

Mixed cultures of non-*Saccharomyces* (Km1Y9, Td1AN9, and Zb3Y1) and *S. cerevisiae* strains (Sc3Y3, Sc3Y4 and Sc3Y8) showed an improved fermentation capacity as compared to pure cultures, having a higher ethanol, carbon dioxide, and glycerol production, and a lower residual fructose. Pure and mixed yeast populations on *Agave* medium, inoculated at two different radii, had similar profiles when assessed by FISH analysis (as demonstrated in **Figure 2**) where *S. cerevisiae* remained as the most abundant yeast, and the non-*Saccharomyces* strain (either *K. marxianus* or *T. delbrueckii*) had a drop in its population at the end of the fermentation. However, when viable and culturable populations were quantified by differential plating, it was evident that, contrary to what has been reported in other fermentation systems, the *S. cerevisiae* strain culturable concentrations decreased with time, while *T. delbrueckii* strain remained highly viable and culturable during the whole process (**Figure 3**), which is favorable in terms of a more extended period of production of volatile compounds on this medium. A similar behavior was also observed by Andorra et al. (2011) for mixed fermentations of *S. cerevisiae* and *Hanseniaspora guilliermondii* in a synthetic grape must, when comparing populations obtained by culture-independent (FISH probes and qPCR) and plating (using cycloheximide as differential factor in the media) techniques, observing that while the plating technique indicated that *S. cerevisiae* remained highly culturable throughout the fermentation, the *H. guilliermondii* strain sharply decreased its culturability with time, but when assessed by FISH probes and flow cytometry, they observed a species-specific intensity of hybridization, as *H. guilliermondii* signal (rRNA content) remained the same at a high, stable value during the whole fermentation, and even after boiling a sample of this yeast for 10 mins, which emphasize the importance to clearly assess the metabolic state of the strains and the specific stability/resistance of their rRNA, if culture-independent techniques are used to monitor yeast populations in alcoholic fermentations.

In terms of resistance to stress, data suggest that it is not only the presence of fructose as the main fermentable sugar but also that of toxic compounds present on the *Agave* must, as furfural and vanillin (data not shown), that affects the viability and metabolic performance of the strains, as has been also observed by Arrizon et al. (2006) for other *Agave* yeast strains. This clearly requires further investigation, but from the data obtained here and our previous work (Oliva Hernández et al., 2013; De la Torre-González et al., 2016, 2020; Vergara-Álvarez et al., 2019) with these mezcal strains, the selection of *Agave* must isolated strains (*Saccharomyces* and non-*Saccharomyces*) to be used as inoculum for tequila and mezcal (as the main liquors produced from *Agave* spp. plants, but there are others) would need to be tested at 30°C to assess that are be able to grow on agar plates containing 500 g/L of fructose, to be able to grow on yeast extract and peptone agar plates containing 8% ethanol and fructose as a carbon source. Finally, they should present a high viability/culturability on *Agave* must diluted at 10°Bx for 5 days. This could result in a robust selection of yeasts that have high fermentative performances that can be then tested in terms of their survival rates when cultured together, and then their aromatic profiles when fermented as pure inoculum or in co-culture, to select those with the desired, more complex bouquet. It is worth noting that there was not a formal organoleptic evaluation of the fermented musts obtained in this work, as there is still a step of distillation that needs to be performed to obtain the final products. As the focus of our work was the screening of a high number of strains, small scale fermentations were carried out, hence distillation was not carried-out. Certainly, the next stage would require us to produce pilot scale fermentations to be able to distill and obtain enough product to carry out the sensory evaluation.

CONCLUSION

Rational selection of yeast starters for tequila and mezcal productions must consider high fructose selective media (as the ones used in this work), since pure o mixed yeast starters may greatly influence the fermentative performance of the selected yeast. In this study, we selected three *Saccharomyces cerevisiae* and three non-*Saccharomyces* mezcal strains to be tested in a mixed starter. Specifically, *T. delbrueckii* (Td1AN9) and *K. marxianus* (Km1Y9) were the best candidates to be considered, either as pure inoculum, or even better, as part of a mixed inoculum with an *Agave*-isolated *Saccharomyces cerevisiae* strain, due to the high productivity of ethanol and glycerol; low acetic acid

production; and increased levels of phenyl ethyl acetate, isoamyl acetate, and hexyl acetate in the *Agave* medium. In addition, the high survival rates of these non-*Saccharomyces* strains during fermentation increase their technological feasibility as inoculum for *Agave* musts, with an extended metabolic activity during fermentation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FJD-G helped in planning and performed all the experiments, CPL-C financed and planned the whole thesis and supervised the metabolic and fermentation experiments, JAN-Z financed and planned the molecular biology experiments and helped analyze the data, DH planned and financed the FISH experiments, and PAV-L planned and supervised the GC-MS experiments. The manuscript was written, proofread, and approved by all authors. All authors contributed to the article and approved the submitted version.

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Mezcal Production in Mexico: Between Tradition and Commercial Exploitation

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Mezcal is a traditional iconic Mexican distilled beverage obtained from varied species of agaves. Regardless of the area of production, the process always consists of five stages: harvesting the agaves, cooking, crushing, fermentation, and distillation. It is produced in a large area of Mexican territory, a large part of which is protected by the Denomination of Origin mezcal (DOM). Over time, the word mezcal has evolved from a generic name to a more specific term used to describe the agave-distilled beverages produced in the territory protected by the DOM under the Mexican official standard NOM-070-SCFI-2016 which defined Mezcal as a "Mexican distilled alcoholic beverage, 100% from maguey or agave, obtained by distillation of fermented juices with spontaneous or cultivated microorganisms, extracted from mature heads of maguey or cooked agaves, harvested in the territory covered by the DOM." In the last 10 years, official production has increased, from $<$1 million liters in 2011 to almost 8 million liters. This substantial increase in production puts a lot of pressure on resources, in particular raw material, as part of the production is obtained from wild agave. On the other hand, it exposes tradition at risk by increasing production by modernizing production processes and sacrificing the artisanal aspect of this production. We consider appropriate to address the issue of sustainability in this context of great tradition and growing market demand. The article presents the relevant aspects of mezcal production, highlighting some particularities specific to certain production areas, it also addresses the problem of the official standard. A broad discussion is presented on the sustainability of artisanal processes, and the main points to be taken care of in this framework. Additionally, some elements considered as fundamental in the perspective of the design of a sustainable artisanal distillery are described. In summary, this article aims to review the current state of mezcal production, how sustainability may be addressed in a very artisanal process and what are the challenges of the production chain to satisfy an increase in demand without sacrificing the tradition and culture related to this iconic Mexican beverage.

Keywords: mezcal, agave, artisanal production process, sustainability, alcoholic beverage

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INTRODUCTION

Mexico is characterized as the country of agaves (also called magueyes), which grow in almost every state of the republic. In general, plants of the genus *Agave* are monocotyledonous, characterized by their rosette structure, long life span, with reproduction that can be sexual by seed (many of the species' flower only once in their life and then die) or propagate vegetatively by ground-level basal shoots or aerial bulbils (García-Mendoza et al., 2017). All *Agave* species are native from the American continent, and the central region of Mexico is considered the center of origin of this family (Eguiarte et al., 2000). These plants belong to the Mexican culture and have been used for multiple purposes since ancient times, like source of food, medicine, drink, construction material, living fences, fibers, vinegar, and ornaments, among others, and what remains mainly today, for the elaboration of beverages distilled or not (García-Mendoza, 1998; Delgado-Lemus, 2020). Agaves are notable for their high content of reserve sugars, fructans, which are highly branched polymers of fructose with a glucose molecule (Lopez et al., 2003).

The generic term for these distilled beverages obtained from agaves is mezcal, which derives from the Nahuatl mexcalli, from metl, maguey, and ixca, to cook or bake, i.e., cooked, or baked maguey (Blomberg, 2000; Bowen, 2015). In general, terms, mezcal can be defined as a distilled beverage obtained from the fermentation of the stems or the juices of the stems of different agave species, previously cooked and crushed or ground (NOM-070-SCFI, 2016). This definition includes all distilled beverages obtained from agaves, such as Tequila, Raicilla, Bacanora and mezcal itself (Álvarez-Ainza et al., 2017). A broad and entertaining description of the culture of mezcal can be found in Bullock's work (2017).

Over time, the word mezcal has evolved from a generic name to a more specific term used to describe the agave-distilled beverages produced in the territory protected by the Denomination of Origin mezcal (DOM) under the Mexican official standard NOM-070-SCFI-2016 (NOM) (https://mezcal.org/denominacion-de-origen-mezcal). Since its first publication in the Official Journal of the Federation (DOF) in 1994, as well as its subsequent amendments, mezcal is defined as a "Mexican distilled alcoholic beverage, 100% from maguey or agave, obtained by distillation of fermented juices with spontaneous or cultivated microorganisms, extracted from mature heads of maguey or cooked agaves, harvested in the territory covered by the DOM." **Figure 1** shows the DOM territory and its distribution in Mexico. For the first integrated states, the entire territory belongs to the DOM. In the later enlargements only certain municipalities of the new states were included in the DOM.

The same standard mentions that it is a liquid with aroma and flavor derived from the species of maguey or agave used, as well as from the elaboration process; diversifying its qualities by the type of soil, topography, climate, water, authorized producer, mezcal master, alcoholic graduation, microorganisms, among other factors that define the character and organoleptic sensations produced by each mezcal.

According to the specific process used for cooking the maguey or agave, crushing or grinding, fermentation and distillation, three categories of mezcal are obtained: mezcal; Artisanal mezcal and Ancestral mezcal (**Table 1**).

In addition to these three categories the NOM mentions six classes of mezcal: "Blanco" or "Joven" (colorless and translucent mezcal that is not subject to any further processing); "Madurado en Vidrio" (mezcal stabilized in glass containers for more than 12 months, underground or in a space with minimum variations in light, temperature and humidity); "Reposado" (mezcal that must remain between 2 and 12 months in wooden containers that guarantee its innocuousness, without restriction of size, shape, and volume capacity, in a space with minimum variations in light, temperature and humidity); "Añejo" (mezcal that must remain for more than 12 months in wooden containers that guarantee its innocuousness, with a capacity of <1,000 L, in a space with minimum variations in light, temperature and humidity); "Abocado con" (mezcal that must directly incorporate ingredients to add flavors, such as maguey worm, damiana, lemon, honey, orange, mango, among others) and finally "destilado con" (mezcal that must be distilled with ingredients to add flavors, such as turkey or chicken breast, rabbit, mole, plums, among others), in terms of the NOM.

Although the standard establishes a legal framework for the production of the beverage and provides a certain guarantee of quality for the consumer, both at national and international level, it is important to mention that several producers in the appellation of origin area do not certify under the NOM, partly because of the cost of this certification, partly because it is very complex to certify artisanal processes, and finally because the producer does not want to depend on a certifying body. Legally this prevents these producers from using the word mezcal on their labels (Gallardo-Valdez, 2016), in this case it is common to see mentioned for example the term "distilled agave beverage" or some already positioned brands do not mention anything specific. For this reason, it is difficult to have exact figures of the global annual mezcal production. **Figure 2** shows the production of certified mezcal between 2011 and 2020, which gives an idea of the production levels and the significant increase in production volumes since 2014. Oaxaca state is the principal mezcal producer, reflecting more than 90% of production in 2020 (COMERCAM, 2020). Based on personal communications we estimate that the real quantity of mezcal produced annually is twice the certified volume.

The past years also showed a significant increase in exports of both certified mezcal and certainly of non-certified products. In 2020, 66.6% of the certified mezcal was exported abroad, mainly to the United States (73.8%), followed by Spain, Canada, and Australia, among others. In total, mezcal has been exported to 72 countries, and since 2020, it is interesting to highlight some first sales in China (COMERCAM, 2020). In addition to this increase in exports, it is important to note that today there is a growing niche market for premium distilled agave beverages where consumers are looking for artisanal products with specific characteristics,

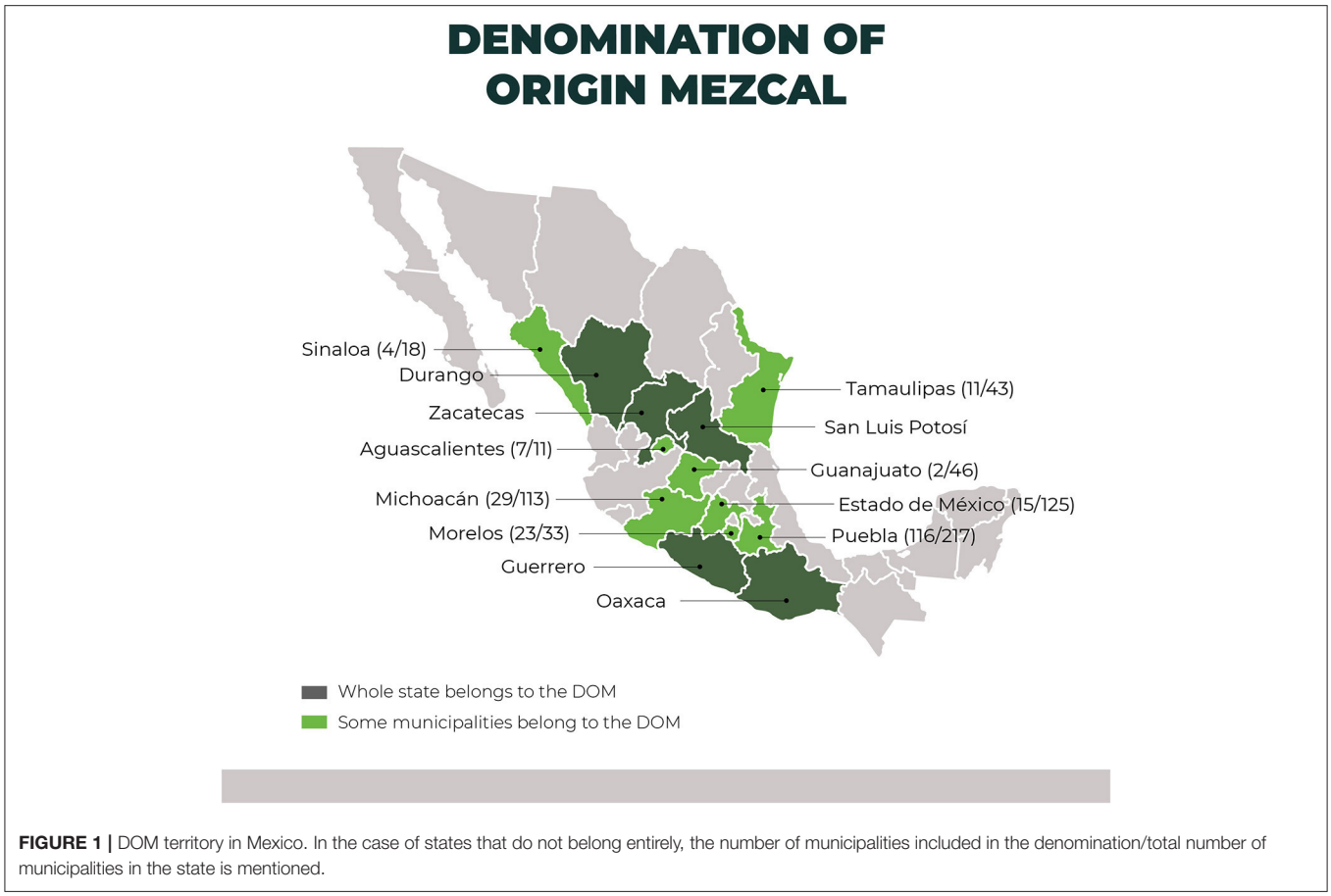

FIGURE 1 | DOM territory in Mexico. In the case of states that do not belong entirely, the number of municipalities included in the denomination/total number of municipalities in the state is mentioned.

TABLE 1 | Allowed process configurations of the different mezcal categories (NOM).

Categories of mescal	Mezcal	Artisanal mezcal	Ancestral mezcal
Main characteristics	(a) Cooking: pit, masonry, or autoclave ovens. (b) Milling: Chilean or Egyptian mill, grinder, mill train or diffuser. (c) Fermentation: wooden vats, masonry basins or stainless-steel tanks. (d) Distillation: stills, continuous distillers or copper or stainless-steel columns.	(a) Cooking: pit ovens or raised masonry ovens. (b) Grinding: with a mallet, Chilean or Egyptian mill, or grinder. (c) Fermentation: cavities in stone, soil or trunk, masonry basins, wooden or clay vessels, animal skins, which process may include the agave fibers (bagasse). (d) Distillation: with direct fire in copper pot stills or clay pots and, wooden, copper or stainless-steel pot stills; the process may include agave fibers (bagasse).	(a) Cooking: pit ovens. (b) Grinding: with a mallet, Chilean or Egyptian mill. (c) Fermentation: cavities in stone, soil or trunk, masonry basins, wooden or clay vessels, animal skins, which process may include the agave fibers (bagasse). (d) Distillation: with direct fire in clay or wooden pots; the process may include agave fibers (bagasse).

for example made of wild agaves, which puts an excessive pressure on the natural populations (Martínez Jiménez et al., 2019).

This article aims to review the current state of mezcal production in Mexico, how sustainability may be addressed in a very artisanal process and what are the challenges of the production chain to satisfy an increase in demand without sacrificing the tradition and culture related to this iconic Mexican beverage.

MATERIALS AND METHODS

The information analyzed was collected through a careful literature review in different databases.

Literature searches were conducted in Scopus, Google Scholar, and PubMed to detect the status of research on the sustainability of the mezcal production process. The search period was from 1990 to date. In addition, we collected information from technical reports, theses or books published mainly in Mexico and not available in the

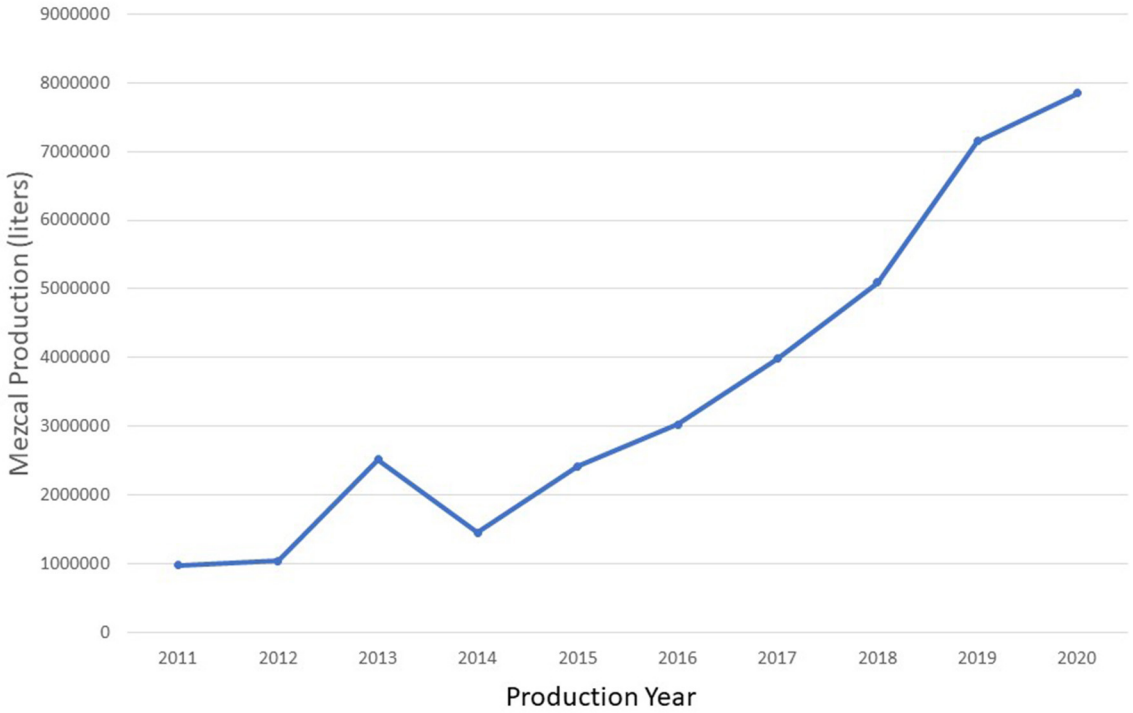

FIGURE 2 | Evolution of mezcal production between 2011 and 2020. Official production registered and certified by the mezcal regulatory council (COMERCAM, 2020).

databases. Several keywords or combinations of keywords were used for the search: [(1) mezcal, (2) mezcal and agave, (3) mezcal and sustainability, (4) mezcal and social, (5) sustainability and craft products, (6) sustainability and craft and product].

The database that yielded the most information was Scopus, with 208 published papers (search with the keyword mezcal). Many of them are directly related to the raw material, as the search combining the word mezcal with agave yielded 147 results. The combination of mezcal and sustainability allowed only nine papers to be found.

The analysis of the information found shows that the available data is very scarce, and few papers address the issue of sustainability of mezcal production as such.

MARKETING AND REGULATIONS

A decade ago, the mezcal production in most factories was a family business owning small and mid-sized enterprises. Their annual production was low because it did not represent the main economic activity. Moreover, mezcal production depended on factors such as the distance for agave harvesting, access to the factory, services such as water and electricity, number of families involved in the process, and the size of the factory. During a long time, the mezcal factories have managed to stay in the market operating under subsistence conditions, so most do not earn enough income to grow (Illsley Granich et al., 2009). They survived even in environments of economic, social and political crisis, trying to maintain their cultural identity (Vázquez Elorza et al., 2017).

In recent years, there has been an increase in the production and sale of mezcal, both in national and international markets, which has caused an excessive use of natural resources. The incorporation of national and international investors, politicians, entrepreneurs, professionals, distribution intermediaries, marketers, among others, have made their appearance interested in participating in the mezcal market, causing an increase not only in the production of mezcal, but also in the number of brands (Hernández López, 2020).

The participation of so many actors has caused that the producer is kept at a lower social and economic level, with low prices for their products, while the marketers increase the prices of the products, achieving high profits that do normally not benefit the economy of the producers (Gómez Jarquín et al., 2014). Despite this, some factories have managed not only to maintain their cultural identity, but also to grow, to produce high-quality products, to locate them in the best markets, and to achieve a better quality of life (Hernández López, 2020). Likewise, they are working to maintain the biodiversity of its natural environment, looking for the sustainability of their processes. They have had to propagate and replant agaves and timber trees to guarantee the quality and quantity of their raw materials, as well as to reduce and treat their waste to reduce the environmental impact.

It is possible to distinguish between mezcal producers: those that have certified mezcal brands and meet the requirements of NOM (Gómez Jarquín et al., 2014; Hernández López, 2020) and

those who fail to certify their products. Some producers cannot certify because of the cost of the certification process or because their products fail to comply with the provisions of the NOM.

In recent years, the DOM has incorporated other producing regions since its first version did not consider all the territories in Mexico where mezcal is produced (Gallardo-Valdez, 2016). Currently, the authorized areas include the entire states of Oaxaca, Zacatecas, Durango, Guerrero and San Luis Potosi and some municipalities of Michoacán, Tamaulipas, Guanajuato, Puebla, Morelos, Estado de Mexico and, very recently, Sinaloa. This continuous extension of the DOM has become controversial, especially in the original production states (NOM-070-SCFI, 2016). With the focus on the sustainability of production, an important question is how much does the DOM support small mezcal producers? Does this appellation really promote regional development and protect ancestral knowledge? The marketing of mezcal requires compliance with NOM standards. It is frequently seen as complex and even unattainable by a sector of producers. So, many of them decide to continue producing a spirit without certification, which is not necessarily of low quality, to sell it in their communities and maintain a tradition inherited from previous generations (Bullock, 2017).

However, not only by producing the distillate in the DOM territory, it can be called mezcal, but it is essential that it complies with the official Mexican standard of mezcal, which indicates the specifications that must be accomplished to grant the authorization for the packaging and sale of this spirit.

It is mandatory that each bottle describes the agave species used, the percentage of each species (in case of mixtures), the type of mezcal, the category of mezcal, the alcoholic content, the production region, among other concepts (NOM). **Table 2** lists the chemical parameters and their minimum and maximum permitted levels.

Among the compounds regulated by the standard, one of the most problematic is methanol, as it often does not comply with the standard (especially in the more artisanal processes) and creates a problem for the producer. This compound is found frequently in fruit-based drinks where pectin is hydrolyzed prior to or during fermentation by pectin methyl esterases (PME), which release the methoxy groups generating methanol (Bindler et al., 1988; Pineau et al., 2021). To reduce the production of methanol, processes such as the removal of the peel or skin of fruits (Hodson et al., 2017), use of very ripe fruit, heating and/or pasteurization to deactivate the PME enzyme (Hang and Woodams, 2010; Miljić et al., 2016), chemical pasteurization using dimethyl dicarbonate (Blumenthal et al., 2021) and even PME enzyme inhibitors such as the use of epigallocatechin gallate (EGCG) (Saelee et al., 2020) have been used.

Although the agaves used for mezcal production are not fruits, they contain pectin which affects the production of methanol. In the case of mezcal, there is no evidence that the activity of pectin methyl esterases (PME) is the main responsible for the methanol production, because, during the process, there is a cooking stage prior to fermentation, which may imply the thermal deactivation of the agave endogenous PME enzyme (Arellano et al., 2012). However, since the temperature is high for prolonged periods of time, the demethylation of the pectin is generated during this stage (Solís-García et al., 2017). There is evidence that during fermentation it is possible to observe an increase in methanol concentration (Vera Guzmán et al., 2009; Kirchmayr et al., 2017), however this variation can be attributed to a lack of homogenization of the must, due to the presence of bagasse (Solís-García et al., 2017). There is no evidence that methanol is produced during the distillation of mezcal, however, during the first distillation it is a widespread practice that agave bagasse is added to the distillation tank, with a high probability of generating methanol if pectins are still present. Distillation is also a stage in which various cuts of the distillate are made to separate the desired components from the unwanted ones, involving a separation of the streams that contain a higher concentration of methanol.

Finally, as in fruit distillates, the concentration of methanol in mezcal depends on the agave species (Vera Guzmán et al., 2009), the degree of maturity (Aguirre et al., 2001), thus as, the place where the agaves grow (Pinal et al., 2009; González Seguí et al., 2020). However, a main source of variation is distillation because the equipment used in the mezcal production processes is rustic or rudimentary; the heat is provided by wood combustion. Most of the stills do not have internal temperature measurement either in the pot, hat (chapeau) or even at the outlet of the condensates. Therefore, it is common to observe temperature variations during distillation, which implies that the volatile compounds are not separated based on their different physicochemical characteristics, causing that the methanol limits are not accomplished (Prado-Ramírez, 2014).

Methanol, as already mentioned, is a restricted compound in different beverages around the world. **Table 3** shows the maximum permitted levels as well as the raw material used. Methanol concentration in mezcal is not as high as compared to other distilled beverages, however, for many producers it is difficult to obtain their beverages below 300 mg/100 mL of anhydrous alcohol, mainly due to the distillation processes and equipment they use.

González Seguí et al. (2020) present an important discussion on the methanol concentration limit in the standard, based on a careful review of the origin of these limit values. The authors highlight that methanol concentration limits are set by the economy of each country for technological, but not toxicological, reasons. It is clear that mezcals exceed in some cases the amount of methanol set by the NOM, however, the values recorded are below European (European Commission, 2019), North American (Bindler et al., 1988), and World Health Organization standards (World Health Organization, 2014), where there is a history of higher tolerance to methanol than in current Mexican standards. Thus, the authors propose a thorough revision of these values, based on the particularities of the elaboration processes and not by taking data from other beverages. This would undoubtedly support the small producers who certify their products and could help to increase the volume of certified mezcal even more.

The NOM also regulates other compounds such as higher alcohols, furfural, aldehydes, lead, and arsenic. However,

TABLE 2 | Chemical compounds regulated by the NOM and their minimum and maximum permitted levels.

Parameters	Units	Low	High	Mandatory analytical technique
Alcohol Volume at 20°C	% ABV	35	55	NMX-V-013-NORMEX-2013
Dry extract	g/L of mezcal	0	10	NMX-V-017-NORMEX-2014
Higher alcohols	mg/100 mL of anhydrous alcohol	100	500	NMX-V-013-NORMEX-2013
Methanol	mg/100 mL of anhydrous alcohol	30	300	NMX-V-013-NORMEX-2013
Furfural	mg/100 mL of anhydrous alcohol	0	5	NMX-V-013-NORMEX-2013
Aldehydes	mg/100 mL of anhydrous alcohol	0	40	NMX-V-013-NORMEX-2013
Lead (Pb)	mg/L of mezcal	–	0.5	NMX-050-NORMEX-2010
Arsenic (As)	mg/L of mezcal	–	0.5	NMX-050-NORMEX-2010

TABLE 3 | Legal limits for methanol contents (in grams per liter of 100% vol. alcohol) in different spirits.

Country or organization	Maximum limit of methanol (mg/100 mL anhydrous alcohol)	Spirit beverage applied
European Union (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32019R0787&rid=6)	200	Wine spirit, brandy or Weinbrand
	1,000	Grape marc spirit or grape marc
	1,500	Fruit marc spirit
	1,000	Fruit spirits
	1,200	Fruits spirits produced from the following fruits: apricots (*Prunus armeniaca* L.), apple (*Malus domestica* Borkh.), plum (*Prunus domestica* L.), quetsch (*Prunus domestica* L.), peach [*Prunus persica* (L.) Batsch], mirabelle (*Prunus domestica* L. subsp. syriaca (Borkh.) Janch. ex Mansf.), pear (*Pyrus communis* L.), except for Williams pears (*Pyrus communis* L. cv "Williams"), raspberry (*Rubus idaeus* L.), blackberry (Rubus sect. Rubus)
	1,350	Fruit spirits produced from the following fruits: quince (*Cydonia oblonga* Mill.), blackcurrant (*Ribes nigrum* L.), juniper berry (*Juniperus communis* L. or *Juniperus oxicedrus* L.), Williams pear (*Pyrus communis* L. cv "Williams"), redcurrant (*Ribes rubrum* L.), elderberry (*Sambucus nigra* L.), rosehip (*Rosa canina* L.), sorb apple (*Sorbus domestica* L.), rowanberry (*Sorbus aucuparia* L.), wild service tree [*Sorbus torminalis* (L.) Crantz]
	1,000	Cider spirit, perry spirit and cider and perry spirit
	Not mentioned	Honey spirits, Hefebrand or lees spirit, Beer spirit, Topinambur or Jerusalem artichoke spirit
	10	Vodka
	Not mentioned	Spirit (supplemented by the name of the fruit, berries, or nuts) obtained by maceration and distillation
	Not mentioned	Geist (supplemented by the name of the fruit or the raw materials used)
	Not mentioned	Gentian, Juniper-flavored spirit drink, Gin, Distilled gin
	5	London gin
	Not mentioned	Others
United States (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-510200-brandy-containing-methyl-alcohol-food-additive)	350	Domestic brandies
	1,000	Foreign brandies
México (NOM-006-SCFI-2016; NOM-070-SCFI- 2016; NOM-142-SSA-1995)	300	Tequila, mezcal and other agave distilled beverages

these compounds generally comply with the marked levels if potable water and good manufacturing practices are used, so an extensive mention of these quality attributes is not necessary.

MEZCAL PRODUCTION AREAS AND PRACTICES

The production steps carried out during the production of mezcal show a high diversity of the agave species, operational conditions, production practices and the equipment used. In **Figure 3**, the principal steps of the process are presented with some particularities of each area of production. In the context of sustainability, several factors influence efficiency, the agave handling, and the conditions of every factory. Since the majority of mezcal producers are not measuring any process parameters and mostly do not record any data, it is difficult to know the average efficiency, for example, sugar hydrolysis during cooking or the conversion efficiency of sugars during the fermentation stage. In fact, the great diversity of *Agave* species and process conditions confers the products clear sensorial differences between Mexican states, regions, and factories (Arellano et al., 2012).

Agave Species and Harvest

Different agave species are used for mezcal production depending on the production region (**Table 4**). For example, mezcal production in Oaxaca (highest production volumes) uses mainly cultivated *A. angustifolia*, but several wild species are also used. *Agave cupreata*, *A. angustifolia*, and *A. inaequidens* are commonly used in Guerrero and Michoacán. The principal species used in Zacatecas and San Luis Potosí is *A. salmiana* meanwhile in Durango *A. duranguensis* (Bowen, 2015; Álvarez-Ainza et al., 2017; Bullock, 2017).

As a substantial number of the agave plants are recollected from the environment rather than being cultivated in fields, it is difficult to know when the plants reach their highest sugar content. Factors such as location, topology, climate, and soil composition may contribute to differences in size, weight, and composition (Vera-Guzmán et al., 2018). Most mezcal producers have learnt empirically which plants to harvest, and which leave to grow on. For some agave species, a widely used practice is waiting for the scape (quiote) to grow, cut it off and wait between 6 and 12 months to harvest the plants, since this moment implicates the highest sugar content (Aguirre et al., 2001). Since the agave plants need several years to grow and mature, depending on the agave species between 5 and 15 years (García-Mendoza et al., 2017), it is necessary to manage the proportion of plants to harvest and those to left on the "field/mountain." An

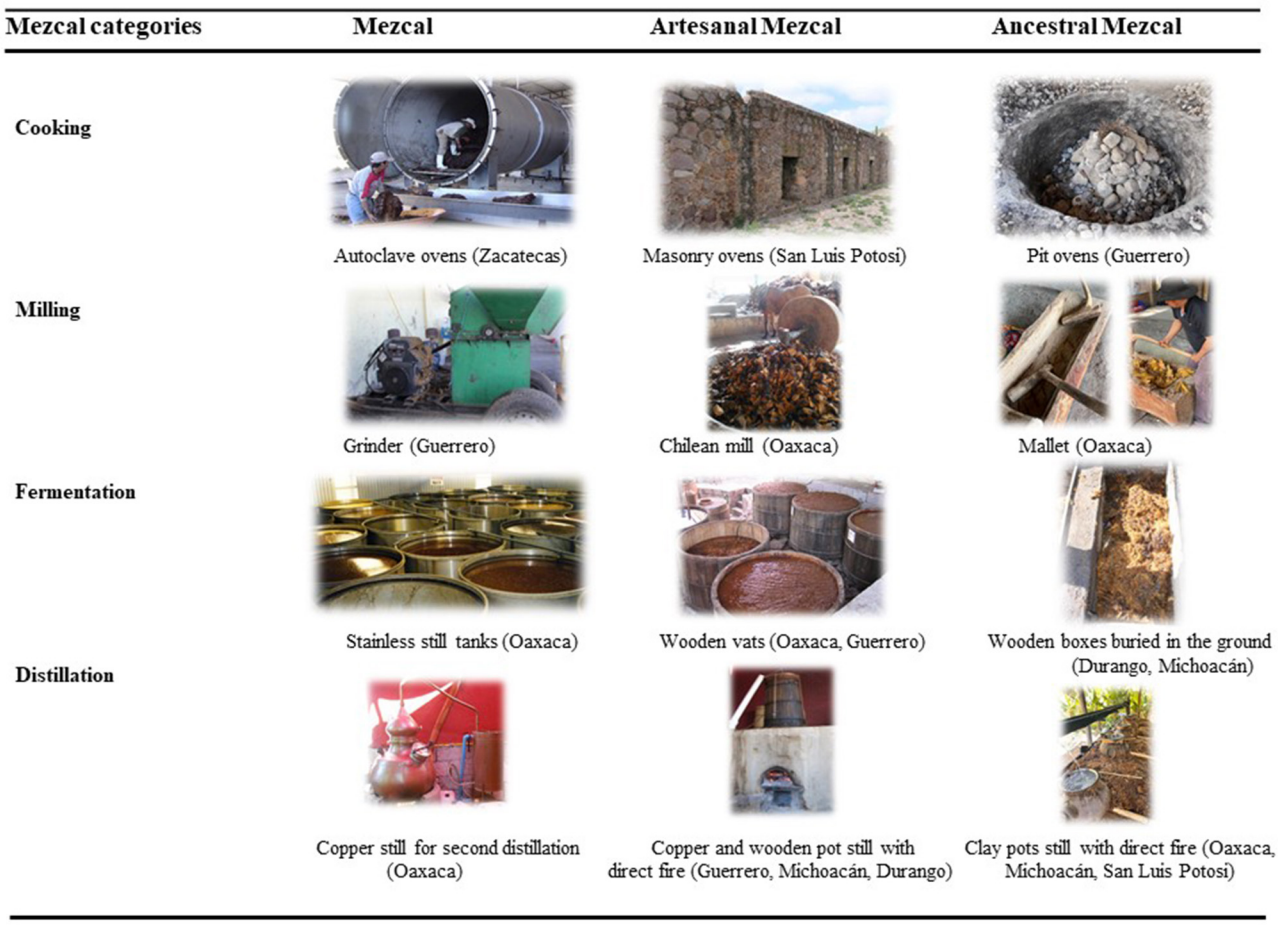

FIGURE 3 | Illustration of the principal steps of the Mezcal production. At each stage, an example of the equipment used for each type of mezcal is presented. The main geographical areas where they are used are mentioned too.

TABLE 4 | Agave species exploited in different production regions considered in the NOM.

State	Agave	References
Aguascalientes (seven municipalities)	*A. angustifolia* (C) *A. salmiana* (C)	Gallardo-Valdez and Solís-Medina, 2019
Durango	*A. durangensis* (C, W) *A. angustifolia* (W)	García-Mendoza, 2012; Bowen, 2015; Loera-Gallegos et al., 2018
Estado de México (15 municipalities)	*A. angustifolia* (C, W)	Sánchez-Jiménez et al., 2020
Guanajuato (two municipalities)	*A. salmiana* (W)	Molina-Guerrero et al., 2007
Guerrero	*A. cupreata* (W) *A. angustifolia* (C, W)	García-Mendoza, 2012; Kirchmayr et al., 2014; Bowen, 2015
Michoacán (29 municipalities)	*A. cupreata* (C) *A. inaequidens* (W) *A. tequilana* (C) *A. sahuayensis* (W)	García-Mendoza, 2012; Bowen, 2015; Cházaro-Basáñez et al., 2020; Delgado-Lemus, 2020
Oaxaca	*A. angustifolia* (C) *A. rhodacantha* (W) *A. potatorum* (C, W) *A. seemanniana* (W) *A. marmorata* (W) *A. karwinskii* (W) *A. americana* (W)	García-Mendoza, 2012; Bowen, 2015; Martínez Jiménez et al., 2019
Puebla (117 municipalities)	*A. potatorum* (C, W) *A. marmorata* (W) *A. angustifolia* (C, W) *A. salmiana* (C, W)	García-Mendoza, 2012; Bowen, 2015
Sinaloa (four municipalities)	*A. angustifolia* (C, W) *A. tequilana* (C)	Núñez-Hernández, 2011; Bowen, 2015; Salazar-Leyva et al., 2016
San Luis Potosí	*A. salmiana* (W)	García-Mendoza, 2012; Bowen, 2015
Tamaulipas (11 municipalities)	*A. americana* (W) *A. santicarolis* (W)	García-Mendoza, 2012; Zúñiga-Estrada et al., 2018
Zacatecas	*A. salmiana* (W) *A. durangensis* (W) *A. tequilana* (C) *A. guadalajarana* (W)	García-Mendoza, 2012; Bowen, 2015; Torres-García et al., 2019

C, cultivated; W, wild.

interesting way to manage agave is reported by Illsey et al. (2018) in the state of Guerrero, where the community implemented a controlled forest management of the agaves. In **Table 4**, some data of maturation age and mezcal production yields are mentioned, demonstrating the great differences between regions and agave species used.

In some mezcal factories, the distance to mature agave is long, also the time it takes to harvest enough plants and carry them to the factory are high, representing additional factors for sustainability since it requires longer transport distance probably inducing stress in harvested plants, which may increase the degree of methoxylation of pectins. This information, however, has been obtained from producer interviews and it is necessary to conduct additional work to obtain scientific data (**Table 5**).

TABLE 5 | Comparison of maturity age, production yields of different *Agave* species in different production areas.

Agave species	Maturity age (years)	Yield (agave kg/L of mezcal production at 55% ABV)	References
Agave angustifolia	6–10	8–14	Martínez Jiménez et al., 2019
Agave karwinskii	10–14	11–19	Martínez Jiménez et al., 2019
Agave americana	8–12	8–16	Martínez Jiménez et al., 2019
Agave potatorum	7–13	14–20	Martínez Jiménez et al., 2019
Agave tequilana	5–9	9–13	Interview with producers
Agave salmiana	7–10	25–35	Interview with producers
Agave guadalajarana	10–15	20–30	Interview with producers
Agave cupreata	7–12	12–17	Interview with producers
Agave sahuayensis	7–12	11–18	Interview with producers
Agave inaequidens	7–14	20–30	Interview with producers
Agave durangensis	6–10	12–18	Interview with producers

Agave tequilana plantations typically have between 2,500 and 5,000 agaves per hectare. Therefore, considering an average weight of 40 kg per agave head, between 100 and 200 tons can be produced per hectare (INEGI, 1997). However, this is not the case in mezcal production, where there is no massive cultivation. Therefore, it is recommended to carry out reforestation of wild agave and cultivation of the most used agaves to guarantee the production of the necessary material year after year, considering the time required to reach maturity.

Cooking

Compared to the tequila industry, where the agave stems are steam cooked (Cedeño, 1995), with or without applying pressure, or the fructans are acid hydrolyzed after their extraction from the plants, the application of heat is not well-standardized in most mezcal factories (Solís-García et al., 2017). The most common and traditional way to cook agave stems is under ground, in conical pit ovens lined with stone and heated by burning vast amounts of wood. The amount of firewood used is an average of a 1:1 ratio with the amount of agave that will be cooked, this is one ton of firewood per ton of agave. The type of stones used to line the oven and to separate the embers from the agave stems and the type of wood, the log size (size of the pieces) and for example the water content (green wood or dried/stored wood) are factors that determine the temperature and the time the agave stems need to be left to cook in the oven (Álvarez-Ainza et al., 2017). Although temperature and cooking time are the main factors that define the efficiency of fructan hydrolysis, some other factors can be logically inferred from the way the cooking is performed. The placement of bigger agave stems near to the heat source and smaller pieces on top of them surely contributes to a homogenous hydrolyses of the fructan polymers. In some factories, a bucket

of water is added through an orifice in the center, once the oven is tapped with jute sacks and soil, which favors a more homogeneous distribution of the heat inside the oven (Duran-Garcia et al., 2007). During the cooking stage, Maillard and oxidation reactions take place increasing the concentration of furfural, hydroxymethylfurfural and others compounds which may lead to fermentation inhibition, increasing fermentation time (Soto-García et al., 2009).

It is also necessary to carry out the transfer of the necessary firewood. Therefore, producers prefer to have large cooking ovens, to carry out the least number of cooking. The use of this type of oven does not allow the collection of sweet honeys that are exuded during cooking, which are lost in the bottom of the oven. Losses in general during this stage oscillate around 10%. Therefore, it would be advisable to design cooking equipment that, without losing its traditional vision, can recover sweet honeys and prevent the agave from overheating. A hybrid system between surface oven and masonry oven would be recommended, for example, connecting the still with just water to the oven to add vapor, maintain the temperature, humidity and collect the sweet honey in the bottom, could increase the efficiency process.

Grinding, Crushing, or Juice Extraction

To make the hydrolyzed sugars inside the cooked agave stems available for microorganisms during fermentation, they need to be grinded or crushed, or as in the tequila industry (Cedeño, 1995), the cooked agave is milled and washed out with warm/hot water. Then, the agave juice is obtained and subsequently fermented. In most cases of mezcal production, the crushed plant material is simply placed into fermentation vats and water is added. Regarding efficiency, the different equipment that can be used makes it difficult to compare this step between mezcal production regions. The most traditional way to achieve this is by cutting and crushing (with human force) the agave pieces with wooden mallets or by passing over a big millstone with animal force (Chilean mill) (Duran-Garcia et al., 2007). Each grinding equipment generates a different performance, while the wooden mallets generate between 100 and 150 kg/h, the blade mill and Chilean mill can grind up to 1,000 kg/h (Kirchmayr et al., 2014). Another difference between the equipment is the amount of large fragments of agave, the use of mallets to beat the agave is the system that does not completely crumble the agave, followed by the Chilean mill and finally the blade mill. Generating large fragments decreases fermentation performance, so guaranteeing homogeneous grinding with a high degree of defibration leads to better results (Duran-Garcia et al., 2007). It is even reported that including a motor in the milling pan can increase the milling performance by 20% (Caballero Caballero et al., 2013). There is no data on how many sugars are lost during bad grinding, however, they could range between 5 and 10%, according to the evaluation of the sugar content in the agave heads and the initial sugar content in the fermentation. Today, motored machinery adopted from other agricultural activities may achieve the goal in much less time, however, alters the image of an ancestral production process.

In addition to the above, although it is not a milling problem, the time that cooked agave is stored in some production areas is long, which causes the growth of desired and undesired microorganisms on the agaves surface, consuming sugars without ethanol production. This factor has not been evaluated either, but it can cause losses of up to more than 20% when the milling stage start 2 weeks after agave cooking (data obtained by interview with producers). It is recommended that the cooked agaves are not stored for more than 1 week, for that it is preferable to determine the size of the cooking oven should be no greater than the amount of agave that can be processed in a week.

Fermentation

The fermentation is a crucial step during mezcal production where a complex and variable microbiota intervenes (Kirchmayr et al., 2017). Since the fermentation is carried out with cooked agave parts and fibers it can be considered a semi solid-submerged fermentation process. It is not possible to directly calculate the yields or the sugar conversion efficiencies by measuring the sugar and ethanol content at the start and the end of the fermentation, respectively, since the sugars contained in the agave pieces and fibers slowly dissolve into the must (Soto-García et al., 2009). Usually, the producers calculate the amount of agave used to produce one liter of mezcal. Depending on the agave species and its maturity, up to 25% of its weight correspond to sugars (fructans before and fructose/glucose after cooking) (García-Mendoza et al., 2017) and it would theoretically be possible to obtain 1 liter of Mezcal (50% ABV) with 4 kg of agave. The reality, however, is far away from that. The efficiencies are normally between 10 and 20 kg agave for 1 liter of mezcal but sometimes may raise up to 25–35 kg of agave (**Table 4**).

The fermentation vessels are frequently packed first with the cooked agave pieces and fibers and afterwards just enough water is added to cover the fibers. In many regions, the plant material is compacted before water addition, which limits even more the possibility that the sugars diffuse into the liquid phase. Besides the known impact of high initial sugar content on the performance of the yeasts in a broad range of other alcoholic fermentations, it is noteworthy that several other compounds derived from the raw material (e.g., saponins) or generated during the cooking step (e.g., HMF, furfural) are reported growth inhibitors for the microorganisms (Soto-García et al., 2009).

Most fermentations are carried out as spontaneous processes, allowing the growth of the native microbiota. Yeast species as *Pichia kluyveri, Kluyveromyces marxianus, Candida ethanolica, Saccharomyces cerevisiae, Torulaspora delbrueckii*, *Hanseniaspora guillermondii* and others has been found during the fermentation; also, several bacteria as *Lactobacillus* spp., *Acetobacter* spp., *Weissella* spp. *Leuconostoc* spp., this diversity explains variations in volatile compound profiles of each batch (Verdugo-Valdez et al., 2011; Páez-Lerma et al., 2013; Kirchmayr et al., 2014; Kirchmayr et al., 2017).

During mezcal fermentation stage commercial yeasts are normally not used. The cooked agave contains yeast inhibitors such as HMF, furfural, and saponins, and commercial yeasts cannot growth (Garcia-Soto et al., 2011; Alcázar et al., 2017). The wild yeast is adapted producing better fermentation

behaviors. However, biodiversity must be conserved to warrant the native properties on each mezcal region process and avoid commercial yeasts.

Finally, the fermentations are frequently slow because the temperature is not controlled, the initial sugars generally are high (more than 150 g/L) and the initial yeast populations are low. Those factors trigger low ethanol production at the end of fermentation (25–50 g/L) (Vera Guzmán, 2012), This problem could be avoided if the mezcal producer increased the yeast at the beginning using an inoculum (with endogenous yeast) prepared before the fermentation. This activity let begin with higher yeast and low bacteria, increasing not only ethanol production, also, decreased the fermentation time and the quality could be homogenous (Nuñez-Guerrero et al., 2016).

Distillation

In the production of mezcal, two distillation steps are carried out. Only a few more technified factories achieve the desired alcohol content in one step by using distillation columns or through a special still design. The first step is carried out commonly by verting both the agave must and fibers into the still, obtaining an ∼5-fold increase in alcohol content in the distillate regarding the alcohol content in the must (between 20 and 30% ABV). The second step is achieved by distilling the obtained liquid again achieving a 2–3-fold increase in alcohol concentration (between 50 and 60% ABV). The still pots are frequently heated with wood where the amount and type of wood again defines the velocity of the temperature increase. To obtain a product which fulfills the specifications of the NOM standard the heads and tails are cut off during second distillation.

Distillation is the stage with greatest losses during the mezcal production process (15%), because some equipment used does not have a hermetic seal, which has repercussions in leaks during the process. In addition, the condensate does not have the correct temperature either in many mezcal factories the hot mezcal is recovered causing losses of aromas and ethanol ($>40^{\circ}$C). Finally, the process is carried out using firewood, there is no sensible control of the heat, causing variations in the separation of the compounds due to changes in temperature. As two distillations are carried out, normally, the same equipment is used, so the losses are accumulated. To improve this stage of the process, producers must add thermometers in the still head, in the cooling water for condensation and in the condensate, finally, based on the temperatures, design a distillation strategy that allows maintaining controlled distillation conditions. In addition, adapt systems that prevent leaks, although this part is a bit more complex, due to the diversity of equipment used to carry out this stage of the mezcal production process.

Technical Recommendations

The easiest way to control and assure the process efficiently is by measuring parameters such as temperature, sugar concentration, and alcohol content and by weighting the raw material before and after cooking as well as the volume of water added to the fermentation vessel. The temperature inside the pot, the distillation flow and the temperature of the distillate also are important parameters to determine. A continuous record of the different parameters recommended to measure during each step of the mezcal production process contributes to observing and understanding how these impact on the efficiency of the process and on the quality of the final product. Since the production process are highly diverse between states, regions and factories and may be affected also by the different seasons during the year, specific production schemes should be established which allow mezcal producers to obtain their products with constant efficiency and expected quality.

In **Table 6**, the main problems encountered in the different process steps during mezcal production and the parameters that should be determined are shown.

SUSTAINABILITY ASPECT IN ARTISANAL PRODUCTION

The United Nations recently defined the Sustainable Development Goals (SDGs), also known as the Global Goals (United Nations, 2021). The United Nations adopted them in 2015 as a universal call to action to end poverty, protect the planet, and ensure that by 2030 all people enjoy peace and prosperity. The United Nations also "recognize that action in one area will affect outcomes in others, and that development must balance social, economic and environmental sustainability." Following the above, the European Union views sustainable development as meeting current needs without compromising the ability of future generations to meet their own needs (European Union, 2021). The United States Environmental Protection Agency considers that sustainability means that humans and their environment can harmony produce what present and future generations need (United States Environmental Protection Agency, 2021). All definitions coincide in the necessity of interaction of economic, social, and environmental aspects that reinforce them. Thus, sustainability is a concept inherent in the modern world that must be applied to the productive chains that are economic entities, but with social and environmental dimensions that must be harmonious with each other. It has been mentioned the capacity of the origin denominations to produce positive impacts in the economic, social, and environmental dimensions (Pérez-Akaki et al., 2021).

An excellent review about the characteristics and multiple uses of agaves was recently published (Pérez-Zavala et al., 2020). Nevertheless, alcoholic beverages production is only a part of the agave producing chain, including food, fiber, and fodder, among other uses. Mezcal is a spirit experiencing a strong growing demand with production from 980,000 L to 7.85 million liters between 2011 and 2020 (Martínez et al., 2019; Pérez-Zavala et al., 2020). It also leads to a growing need for raw material and overexploitation since natural population's extraction is often practiced without programs to ensure avoiding the risk of species extinction. Certain efforts are in progress to culture some agave species but unfortunately using a model of substitution natural forest areas by vegetative propagation using a few clones. It leads to an impoverishment of the genetic diversity of agave populations (Delgado-Lemus et al., 2014b). Nevertheless, despite the risk of adopting unsustainable practices to meet the growing

TABLE 6 | Common problems encountered and recommended parameters to determine during mezcal production process.

Process step	Harvest	Cooking	Crushing/grinding	Fermentation	Distillation	Product handling
Natural resources	Agave	Wood		Water	Wood, cooling water	
Common problems	Harvest of not-mature agave, long distance to factory, long storage time until cooking	Sugar loss (agave carbonization), inhibitor production	Sugar unavailability (undergrinding), storage time of cooked agave	Residual sugars (slugish fermentations), acidification	Overheating, Product contamination (oxidized stills)	Product contamination (inapropriate product storage)
					Ethanol and VOC loss (inefficient condensation), lack of fractioning schemes	
Recomended parameters to determine	Sugar content (refractometer)	Temperature, weight (wood, crude agave)	Weight (cooked agave)	Weight (agave), Volume (water), sugar content (densimeter), temperature (water for prep and during fermentation)	Volume, alcohol content, temperature (still, condensator, cooling water)	Alcohol content, temperature
Waste/by-products	Agave leaves	Ashes, burned agave stems			Bagasse, stillage/vinasse	

market demand, mezcal is a DOM that still has time to plan a growth that will result in sustainable production.

Mezcal production begins with selecting appropriate wild, semi-cultivated, or cultivated agave plants (Maciel Martínez et al., 2020). Agaves are considered non-timber forest products, which are species of slow growth and maturation. Whether wild or cultured, agave plants must grow for at least 8 years to be suitable for mezcal production (Martínez et al., 2019). The exploitation of more than 50 agave species allows producing mezcal in the Mexican regions included in the DOM (Pérez-Zavala et al., 2020; Tetreault et al., 2021). Several agave species allow satisfying primary and secondary human needs been the central element in the economy of the communities that are maguey managers (Delgado-Lemus et al., 2014b; Torres et al., 2015a,b). Nevertheless, agave species used to produce mezcal are widely exploited in the regions considered into the DOM due to the growing demand for mezcal by the global markets. Consequently, it determines the establishment of monocultures for the most exploited species, generating many biological, environmental, and sociocultural losses (Torres-García et al., 2019). Poorly planned exploitation does not include actions that ensure the recovering of wild populations, putting them at risk of deforestation (Delgado-Lemus et al., 2014b; Félix-Valdez et al., 2016). The agave used for mezcal production needs to cut inflorescences early, interrupting the sexual reproduction cycle (Félix-Valdez et al., 2016). Plantations of agave species should follow adequate management to preserve genetic diversity (Delgado-Lemus et al., 2014b). Previously, it had been mentioned important aspects to adequate agave plantation management such as (Torres et al., 2015):

- Protection of young escapees by avoiding domestic animals,
- To allow that at least 30% of adult wild and cultured plants produce seeds for reforestation and genetic diversity assuring,
- Consider the impact of seasonal changes on natural and cultured populations,
- To introduce at least 20% of one- and 2-year-old cultured agave plants, and
- To consider both professional ecological research and local knowledge to develop effective management programs.

Increase in mezcal production has strongly pressed wild populations of *Agave potatorum* at a producing zone in Puebla-Oaxaca (Delgado-Lemus et al., 2014a). Considering this finding, Félix-Valdez et al. (2016) studied the effect of extraction of those wild populations on their genetic variability. They concluded that there is an evident environmental degradation, but population variability could not be seriously compromised. Nonetheless, strategies to reintroduce plants must be urgently implemented because of high structuring among populations and high dependence on pollinators. It is particularly important in areas of low plant density (1,000 m^2 with <6 reproductive individuals) to assure the pollinator's arrival. An additional recommendation is to design management plans without mixing seeds from different populations because adaptation to different environments may vary.

Mezcal production in Zacatecas was described focusing in to analyze the agro-industrial chain as well as to propose a tactical supply chain planning model (López Nava et al., 2014). Some of their findings are the following. Agave producers and some mezcal makers have poor socioeconomical conditions. There is deficient integration among agave producers and mezcal producers, which generates significant bottlenecks along the producing chain, causing that only 33% of the mezcal production capacity is used. Mezcal producers must upgrade the management of their factories, focusing on the marketing, sales, and outbound logistics activities which were the most susceptible to improvement. These authors recommended developing a marketing plan to enhance mezcal consumer appreciation and to implement a coordinated and collaborative process between micro, small, and medium-sized companies in the sector to strengthen the production chain.

Mezcal production from *Agave cupreata* was analyzed to determine the environmental impact and energy demand (Maciel Martínez et al., 2020). Those authors established that an industrialized process generates more CO_2 emissions than process carried out by hand, while processes using cultured agave also generates more CO_2 emissions than that using wild agave. They also determined an energy demand of 218.4 MJ per liter of mezcal which is equivalent to the energy obtained from the combustion of 6.16 L of gasoline.

Pérez-Zavala et al. (2020) pointed out that natural cross-pollination allows to maintain genetic diversity to agave species, while asexual reproduction by offshoots generates low genetic variability respect to mother plants. Then, wild plants extraction diminishes agave reproductive and demographic performance (Félix-Valdez et al., 2016).

It has been highlighted the efforts to culture non-timber forest products of high market demand such as agave. All efforts for culture non-timber forest products have the objective to diminish the pressures on wild populations. Nevertheless, it needs to use additional land as well as other resources not always available for rural communities, which can lead to a debt trap for those people. Additionally, these land-use changes can produce environmental problems such as deforestation, diminishing genetic diversity, and ecosystem degradation (Krishnakumar et al., 2012).

Sustainable practices in the mezcal production chain have focused mainly on raw material production (Maciel Martínez et al., 2020). In fact, it has been argued that mezcal culture would be protected preserving plants but also farming and production recipes passed from generation to generation (Bullock, 2017). Nevertheless, converting agave heads into mezcal needs to be improved to maximize the use of fermentable sugars. Moreover, the economy of the whole producing chain could be enhanced by working on the energy efficiency of the process and using subproducts such as agave leaves, vinasses, and bagasse. It would allow obtaining other valuable products and reduce the environmental impact of the mezcal production chain.

Mezcal production chain could be the point of support to create a sustainable economy based on the integral use of agave, extending the benefits so that they range from farmers to investors, preserving the environment and the lifestyle of the communities. Pérez-Zavala et al. (2020) pointed out that ubiquity of agaves in Mexico should be exploited to identify agave species probably unnoticed to promote investments that enhancing the life conditions, particularly for the Mexican population living in low socioeconomic communities.

Mezcal has had short chain involvement (proximity among producers and consumers) and small scales, but it could be changed by the recent accelerated growth of the product (Pérez-Akaki et al., 2021). This expansion of production and commercialization attracts the interest of international investors who simply add products to their products portfolios. Nevertheless, it could be unincentivized by Mexican normativity that recognizes mezcal's artisanal and ancestral categories (NOM-070-SCFI, 2016).

Bowen and Zapata (2009) argued that DOMs must have a legal framework that warrants sustainable production practices to really contribute to perdurable rural development and environmental preservation. Benefits to farmers have been limited although the growth in exportation of mezcal (Pérez-Akaki, 2016).

Strategies to reach sustainable production of mezcal should consider close interaction among the participants of the mezcal production chain (agave producers, distillery operators, bottlers, and sellers). It may ensure fair distribution of profits for all of them and should attract consumers for a high-quality product. Consumer's attraction actions can include alliances with:

- Organizations of restaurants and bars owners to promote mezcals from diverse origin (region and/or agave species).
- Research centers to study biology, physiology, and ecology of agave, to optimize their production processes and help to highlight the distinctive characteristics of each mezcal, as well as to help planning sustainable production.
- Government instances to fund programs and projects for wild populations preservation, assuring genetic diversity, and sustainable cultivation of agave. Mexican government should review and actualize the legislation concerning mezcal production to include a sustainability perspective.

Finally, it is mandatory to create geographic information system maps of wild and cultivated agave plantations and the location of distilleries, allowing everyone in the production chain to receive a fair profit and monitor levels of harvesting of wild agave. These maps will help to plan sustainable levels of extraction of wild agave populations. It must be understood that these are very current ideas, which means changing the way people do things. Mezcal chain producers face some constraints in reaching sustainable production, such as high fees and bureaucracy to certify the product, mainly small producers. They also face high cost of inputs, low efficiency of production processes, and poor linkage with researchers to help overcome such limitations. Pérez-Akaki et al. (2021) also highlighted the public institutions' responsibility to support and guide the development of productive chains to reach more equitable benefits for all participants.

Another proposal is that of Vázquez Elorza et al. (2020), who propose the establishment of technological route maps. This strategy seeks to improve the functionality of production chains, based on recognizing what is needed to develop better products for the market. Through workshops with agave and mezcal producers in Oaxaca and Aguascalientes, it was detected that changes in the markets force companies to innovate and look for alternative routes. However, in these workshops it was clearly detected that the lack of organization for the use of limited resources constitutes a very urgent problem to be solved, in addition to all the above-mentioned.

ADDITIONAL SUSTAINABILITY ASPECTS REGARDING MEZCAL FACTORIES

Despite the highly diverse practices and production processes that are carried out in the different mezcal production regions, exist some common features that all mezcal factories should consider. Those aspects include location, conception, construction, and area distribution of a mezcal factory.

Regarding the location, the mezcal production process requires high quantities of three natural resources: agave, firewood, and water. So then, it seems logical that any factory should be conceived and built a short distance to them. Unfortunately, due to historical reasons, e.g., the prohibition period during the 20th century, many factories are found in remote places, frequently in the mountains, where these resources are not always abundant. Remarkably, the water supply is limited in many factories, and the provision with this resource is accomplished with tank trucks, increasing fuel costs.

Since the four stages of mezcal production are sequential and lineal, an adequate space distribution is needed to enhance process efficiency and productivity. It is logical that the grinding area, for example, would be between the cooking and fermentation areas. As enormous amounts of material must be moved manually between areas, they should be adjacent. Ideally, they should be conceived in different heights/levels, with the cooking area at the highest point and the distillation and storage area at the lowest. Although, as mentioned, several factors regarding the storage of raw, cooked, and crushed agave may affect process efficiency, the space for each stage and the capacities of the machinery and equipment should be considered before constructing a mezcal factory. The load capacity of the pit oven, for example, should consider the time needed for processing the cooked agave. At the same time, the size of the still should contemplate the capacity of fermentation vessels.

Historical reasons forced mezcal production into secrecy, explaining why mezcal factories in the past had to be austere regarding equipment or construction. Still, nowadays, several aspects concerning good manufacturing practices should be implemented while preserving traditional processes. Some examples are the following:

1. Concrete floors and walls which separate the production areas from the surrounding environment would improve the cleanliness and the impact of weathering. The wood fire used to heat the stills during the distillation process generates smoke which directly affects the workers' health.
2. Installing chimneys is mandatory to assure the correct extraction of the smoke improving the worker's safety.
3. The utilization of by-products such as agave leaves, vinasses, and bagasse must be considered to conceive a new distillery.

Some calculations can be made to help establish a sustainable mezcal production plant that schedules a desired annual production. Consider a factory with the annual goal of producing 14,000 liters of mezcal at 55% ABV, a typical production in the mezcal industry. Considering a yield of 10 kg of agave per liter of mezcal, that factory will require ∼144 tons of agave. Since agave pineapples weigh 35 kg, around 4,100 agave plants will be harvested to achieve the production goal. Sustainable exploitation demands replanting at least that number of agave plants. Nonetheless, some plants would have a disease problem or damage by animals. It means that maintaining the wild or cultivated population will require planting 4,500 agave plants a year, considering around 10% of damaged plants.

Care about the factory operation; it must be considered that mezcal is usually produced for 9 months during the year because production is suspended during the rainy season. Then, near to 1,560 liters must be produced each month. It implies that the weekly agave processing must be close to 4,000 kg. Fermentation performs near 30–32°C, and warm water will require to start the fermentation in some cold areas from November to February. Inoculum is not usually employed to start fermentations.

Nevertheless, it has been proposed to add an inoculum based on selected wild yeasts. It should be prepared 1 day before starting the fermentation using agave juice and aeration in an appropriate volume (Kirchmayr et al., 2017). Regarding distillation, it is recommended to perform it by controlling the pot, the distillate, and the condensation water temperatures. An introductory remark about increasing the process yield is the need to recover sweet honey during cooking.

Producers want to preserve their traditional artisan processes but reach higher process yields. This change could take a few years, but it is likely never to happen. The producing areas are mainly located in highly marginalized municipalities that frequently do not have access to electricity, potable water, and access roads. Therefore, agave producers, mezcal producers, sellers, and governments must actively work to reach a better product quality and a higher price for the mezcal. This collaboration also would preserve agave species as well as the traditional knowledge and lifestyle of the producers.

Tequila was converted from a regional beverage to a distilled spirit with global success. However, it brought over-farming and technician processes needed to increase production and to satisfy the market demand. As a result, today's tequila has little in common with the traditional drink from a 100 years ago, while people of the producing communities have not received the benefits of the so-called tequila boom (Bowen, 2015). Bullock (2017) pointed out that it forgot the aim of a denomination of origin concerning protecting the product, but mainly the culture and people around the production process. This author questions whether it can be done better when taking mezcal to the global marketplace.

CONCLUDING REMARKS

This article presents elements of the current situation of mezcal, the production chain, as well as the need for sustainable development, considering that it is a traditional and artisanal process in a context of a substantial increase in demand. In this sense, the problems that this entails were mentioned, such as: the cultivation of agave, which in many production areas is wild and there is no established procedure for its cultivation or management to guarantee its commercial exploitation. The different agave species that can be used in mezcal production were also mentioned, which not only has an impact on the sensory properties of the product, but also on production yields and composition, including compounds such as methanol, furfural, and higher alcohols.

The review of the regulations that apply to mezcal suggests that a thorough and well-supported review of the parameters of the standard is urgently needed, particularly in the authorized levels of methanol, because wild agaves may contain methanol precursors in higher and variable concentrations between species, impacting on compliance with the physicochemical parameters of the official Mexican standard. Compliance with the current parameters sometimes becomes complex and can limit domestic marketing and definitely the export of the product.

Current artisanal and ancestral mezcal production processes require enormous quantities of agave to produce small volumes, due to losses at each stage of the production process. In the case of the use of wild agaves, as they are overexploited, their availability has gradually diminished in some regions, a situation that could, in a brief time, have an impact on mezcal production capacity. Currently, there is a fear that the adoption of technology will eliminate the characteristic of being a traditional and artisanal product, with its own regional characteristics. It is necessary and urgent to carry out studies to evaluate the impact of technological adoption to guarantee the homogeneous quality of the products obtained, which would make it possible to preserve and promote the recognition of mezcal and even the availability of agave in the required quantities. The article provides elements that demonstrate that it is possible to implement different technologies to avoid losses and standardize the process. These elements also contribute to a more sustainable process.

In general, strategies to achieve sustainability in the mezcal production chain must involve all actors in the chain, including consumers, so that they understand the consequences of consuming mezcal made from wild agave that does not come from a controlled and sustainable extraction process.

This work demonstrates that it is possible to move the mezcal production process toward a more sustainable process, without affecting its artisanal image, taking care of the cultural part of the process by applying appropriate technologies.

AUTHOR CONTRIBUTIONS

MK, NS-C, and AM contributed to conception of the review. MK, NS-C, MA-P, JP-L, and AM wrote sections of the manuscript. AM prepared the final version. All authors contributed to manuscript revision. All authors contributed to the article and approved the submitted verion.

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