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RESEARCH PAPER

Bacterial population monitoring during alcoholic fermentation of mezcal in Durango by DGGE

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Abstract

D.S. Torres-Velázquez, J.A. Rojas-Contreras, N.O. Soto-Cruz, N. Urtiz-Estrada, J. López-Miranda, M.R. Kirchmayr, and J.B. Páez-Lerma. 2022. Bacterial population monitoring during alcoholic fermentation of mezcal in Durango by DGGE. Int. J. Agric. Nat. Resour. 112-122. Mezcal is the second most important alcoholic beverage produced in Mexico, and the microorganisms present during the fermentation process are primarily responsible for its organoleptic characteristics. Among these, the bacterial populations have been less studied, mainly due to the difficulty of recovering them. The aim was to apply a culture-independent molecular technique to detect the bacterial populations involved in spontaneous mezcal fermentation in Durango State. Samples were obtained from several stages in the mezcal production line. Denaturing gradient gel electrophoresis (DGGE) analysis was performed using DNA extracted from isolated microorganisms and metagenomes obtained from samples directly taken from the fermentation process. This study indicated that non-isolated bacteria were significant in the fermentation process and indicated that the lactic acid and acetic acid bacterial populations present in the monitored process are similar to those present in other regions during fermentation, although they differ in some species that may play essential roles in the development of the typical organoleptic properties of mezcal produced in the State of Durango. Furthermore, this study indicates that culture-independent analysis by DGGE can reveal the bacterial diversity during the mezcal fermentation process without the use of complex isolation procedures and can be a useful tool for the analysis, monitoring and control of mezcal fermentation processes.

Keywords: Acetic acid bacteria, agave, culture-independent methods, lactic acid bacteria.

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Introduction

Agave is a genus of monocots that remains among the most widely used plants in Mexico. It is fermented with its natural microbiota to obtain a variety of products, including mezcal and tequila (Escalante-Minkata et al., 2012). Mezcal is produced from several species in the territory that are protected by the Appellation of Origin Mezcal, and different regions of the country belong to the denomination of origin (NOM-070-SCFI-2016, 2017). The biochemical process that determines the main sensorial characteristics of mezcal takes place during agave juice fermentation, which lasts several days and is carried out spontaneously by diverse microorganisms in production processes that differ from one region to another. The biodiversity involved varies depending on the agave species, production practices, and regional climatic conditions. Accurate descriptions of the fermentation processes are useful to conserve microbial biodiversity and to evaluate their biotechnological potentials, for example, as starter cultures as well as to produce compounds of commercial importance (Lappe-Oliveras et al., 2008; Escalante-Minakata et al., 2012; Nolasco-Cancino et al. 2018).

Agave-associated bacteria and their role in fermentation are less studied than yeasts. This is partially due to the use of culture-dependent techniques that hinder their recovery. Isolation of bacteria present in extreme environments, such as alcoholic fermentation, includes complex and time-consuming methodologies, implying the loss of several species in the analyzed system and limiting proper study of these microorganisms. (König & Frölich, 2009; Guillamón & Mas, 2009; Torija et al., 2010). Culture-independent analysis techniques avoid biases that are inherent in culture-dependent analysis methods that distort the correct assemblage in microbial communities (Dolci et al., 2015).

Only a few studies have focused on bacterial detection during mezcal fermentation. Two culture-dependent studies of the microorganisms involved in mezcal production revealed microbial diversities that were rich in bacteria; Escalante-Minakata et al. (2008) detected bacterial populations in San Luis Potosí that were dominated by lactic acid bacteria (LAB), and Kirchmayr et al. (2017) reported the presence of diverse bacteria, including LAB and acetic acid bacteria (AAB), for mezcal fermentations in Oaxaca. Furthermore, based on the first culture-independent analysis of LAB for mezcal, which was previously undescribed, only by Narváez-Zapata et al. (2010) proposed the malolactic fermentation stage in mezcal production from Tamaulipas State. These studies strongly suggest that LAB, AAB and other bacteria vary from one region to another and may play an important role in the typical final product quality by generating substances such as ethanol and organic acids, which are significant components of mezcal, although some of them are undesirable at high concentrations and could act as precursors of desirable substances in the unique organoleptic characteristic development the artisanal beverage, such as esters (De León-Rodríguez et al., 2006; Páez et al., 2011; Kirchmayr et al., 2017). Despite this, no prior similar studies have been conducted in Durango.

Denaturing gradient gel electrophoresis (DGGE) is a culture-independent analysis that enables microbial population estimations directly from analyzed samples; it consists of the analysis of amplified DNA fragments by polymerase chain reaction (PCR) and subsequent electrophoresis in a polyacrylamide gel with a gradient of denaturing chemical agents (Cocolin et al., 2013). The methodology is versatile with broad applications and has been successfully used to provide descriptions of microbial populations present in diverse environments (Ercolini, 2004).

This study aims to identify the bacterial populations in spontaneous mezcal fermentation in the state of Durango, Mexico by applying a culture-independent technique, DGGE, using isolated strains as a reference marker.

Materials and Methods

Sampling

Sampling was conducted during the artisanal agave fermentation process in the municipality of Nombre de Dios, Durango, Mexico. There were five sampled points: M0, MA, M1, MF, and F0; 1 g of solid samples or 1 mL for liquid samples diluted in 9 mL of sterile water. Sample M0 was taken after crushing the cooked plant. Sample MA was obtained after 1 d of spontaneous fermentation of the milled plant within the crushing pile. This prefermented agave is then placed in a fermentation vessel (fermentation pile) and diluted with water. Sample M1 was obtained from this fermentation vessel after 1 d of fermentation, while sample MF was taken from the fermented agave material mixture after approximately 3 d of fermentation. The liquid remaining after removal of the fermented plant material at the end of the fermentation, which is called residual must, was the source for the F0 sample. Specific MRS broth (Sigma–Aldrich®, Merck KGaA, Darmstadt, Germany) solid medium was used for LAB isolation, and GYC (5% D-glucose, 1% yeast extract, 0.5% $CaCO_3$ and 2% agar (w/v) supplemented with 2% (v/v) ethanol solid medium was used for AAB isolation. Cycloheximide (CHX, 50 μg mL^{-1}) was added to inhibit growth of fungi. Plates were incubated for 2-4 days at 28 °C as a sterility test.

Isolation

Dilutions of the samples ranging from 10^{-3} to 10^{-6} in sterile water were plated on nutrient agar (pH 6.5) as mentioned above and incubated at 30 °C for 24 h. To isolate the microorganisms, the dilution plate (per sample), which presented approximately 200 CFU, was selected, and to obtain statistical and representative isolation, 10% of the colonies were taken according to the Harrison disc technique and inoculated in new plates (approximately 20 for each sample). Isolated microorganisms whose micromorphologies corresponded to bacteria were preserved at -20°C in growth medium supplemented with 10% glycerol.

DNA extraction

From the isolated bacteria, each was inoculated and incubated in 1 mL of nutritive liquid media as described above, without agar to obtain $1x10^6$ cells/mL (~24 h). The tubes were centrifuged at 20000 x g for 5 min, and the pellets obtained were washed in 1 mL of sterile deionized water. DNA was extracted by the method described by Har Ju et al. (2004). Harvested DNA samples were suspended in 40 μL of sterile TE 1X (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 10 mM NaCl).

A commercial MOBIO PowerSoil® DNA Isolation kit was used for direct DNA extraction from must samples according to the manufacturer's instructions. Genomic and metagenomic DNA integrity were analyzed by 1% agarose gel electrophoresis for 1 h at 95 V in TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, and 1 mM EDTA pH 8.0).

PCR amplification of a partial sequence of the 16S ribosomal gene

A partial sequence (V7-V8) of the 16S ribosomal gene (located between nucleotides 1069–1090 and 1374–1394 of *Lactobacillus plantarum*) was amplified using the specific primers for LAB and AAB: WBAC1 (5'-GTC GTC AGC TCG TGT CGT GAG A-3') and WBAC2-GC (5-CCG GGA ACG TAT TCA CCG CGC GCC CGC CGC GCC CCG CGC CCG GCC CGC CGC CCC CGC CCC-3'), respectively; 100 ng samples of extracted DNA were amplified in a SureCycler 8800 thermocycler, mod. 68800A, (Agilent Technologies, Serial No. MY122303899) at a final volume of 50 μl containing 10 mM Tris-HCl; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; 0.2 M primers; 1.25 IU of Taq DNA polymerase (Promega Corp, Madison, Wis.) ac-

cording to the procedure proposed by López et al. (2003). Amplified fragments were separated and observed in 1% agarose gels.

Bacterial DGGE analysis

DGGE analysis was performed with a DCode™ Universal Mutation Detection System (BIO-RAD) using previously reported electrophoresis conditions (López et al., 2003). The gels were stained with ethidium bromide and photographed in a digital transilluminator (Gel Doc XR + ™ System, BIO-RAD). The quantities of amplified DNA solution used for the DGGE analysis were 5 µL (~2 µg) and 20 µL (~8 µg) for isolated bacteria and metagenomic DNA, respectively.

Sequence analysis and submission to the GenBank public database

DNA from isolated microorganisms was sequenced and used as a reference for metagenomic analysis; bands with different migratory patterns obtained from direct DNA extraction from the samples were also sequenced.

DGGE bands were excised from the gel, placed in sterile deionized water (20 µL per band) and reamplified using previously reported conditions (López et al., 2003). The resultant PCR products were purified in low melting point agarose gel and quantified by Nanodrop, 100-120 ng were mixed with 10 pmol of the primer, WBAC 1, in a final 16 µL volume. These fragments were sequenced using the Sanger dideoxy sequencing method by the Synthesis and Sequencing Unit, Institute of Biotechnology (UNAM). Sequences obtained for cultured and uncultured bacteria were corrected using ClustalW of the Bioedit Sequence Alignment Editor (version 7.2.5) and subjected to a standard nucleotide search for highly similar 16S gene sequences in the National Center for Biotechnology Information Database Nucleotide Collection using the Basic Alignment Search Tool (BLAST) (Bethesda, MD, USA). The sequences were submitted to the GenBank public database.

Chromatographic analysis

Glucose, fructose, and ethanol present in the M0 and MF samples were quantified by high-performance liquid chromatography (Agilent Technologies 1260 Infinity Series, Serial No. DE90378449, 76337 Waldbronn, Germany). Samples were filtered through a 0.45-µm nylon membrane and injected (5 µL) onto an anionic exchange column (Aminex® HPX-87H Column 300 × 7.8 mm) with a mobile phase consisting of 5 mM H_2SO_4 advanced at a rate of 0.6 mL min^{-1}. The column and oven temperatures were 65 °C and 50 °C, respectively. The final product was also analyzed. The ethanol and methanol concentrations in the mezcal samples were quantified by gas chromatography with direct injection. An HP-Innowax column was used with an Agilent 6890N (G1530N, Serial No. US10124045, U.S. A) (30 m length × 0.25 mm inner diameter × 0.25 µm thick). Nitrogen was used as the carrier gas and was delivered at a rate of 1.5 mL min^{-1}. The temperatures of the injector and the detector were set to 220 °C and 250 °C, respectively, in a 10:1 split in the injector. The oven temperature was programmed as follows: 35 °C for 2 min, increased by 10 °C min^{-1} to 210 °C and held for 1 min (De-León-Rodríguez et al., 2006). Methanol and ethanol obtained from Sigma™ were used as standards for quantification.

Results

Isolation

Isolation of bacteria from the samples was performed to obtain the migratory patterns of known species present during the fermentation process and to construct a reference ladder for DGGE metagenomic analysis. After micromorphological

analysis, a total of 17 bacteria were isolated, eleven from the crushing pile and six from the fermentation pile, and visible bands were obtained using the previously mentioned primers in agarose gel electrophoresis (1%) after amplification by PCR of extracted DNA.

Bacterial DGGE analysis

Figure 1 shows the migratory patterns obtained after PCR-DGGE of DNA. Although 58% of the bands exhibited the same migration patterns (f), it was possible to observe six different patterns, which presumably meant that there were six different species of bacteria. This was confirmed after sequencing of DNA obtained from the excised bands.

According to BLAST sequence analysis, the bacteria isolated from the crushing pile are highly related to *Acetobacter malorum* (e), *Bacillus subtilis* (f), *Lactobacillus casei* (d) and *Leuconostoc mesenteroides* (b). Bacteria from the fermentation pile are closely related to *Acetobacter malorum* (e), *Bacillus subtilis* (f), *Bacillus cereus* (a) and *Weissella paramesenteroides* (c). DNA from the bacteria was used in the DGGE metagenome analysis as a reference marker.

The PCR-DGGE results of DNA samples extracted directly from the system are shown in Figure 2. A comparison between species that were detected by culture-dependent and culture-independent methods is presented in Table 1. Some patterns correspond with those of isolated microorganisms (b, c, d, e), and at least three additional patterns (g, h, i) can be observed, indicating the presence of bacteria did not recover after isolation. The band intensities suggest that non-isolated bacteria are significant microorganisms in the fermentation process, considering that they were found in the mature must (MA), residual must (F0), and final must (MF), which emphasizes the importance of culture-independent analysis to detect species in complex environments. Moreover, some bands intensified (d, g), while others faded (h, i) from one sample to another, indicating that microbial population ratios evolve as agave fermentation proceeds. Bands corresponding to non-isolated bacteria were cut, sequenced and BLAST analyzed and yielded the following highly related species: *Lactobacillus amylovorus* (g), *Acetobacter pasteurianus* (h), and *Lactobacillus fermentum* (i).

The percentage of identity of the nearest neighbors and accession numbers are shown in Table 2. The sequence data were submitted to GenBank, and the accession numbers MK182816 to MK182820 for the nucleotide sequences are also included in Table 2.

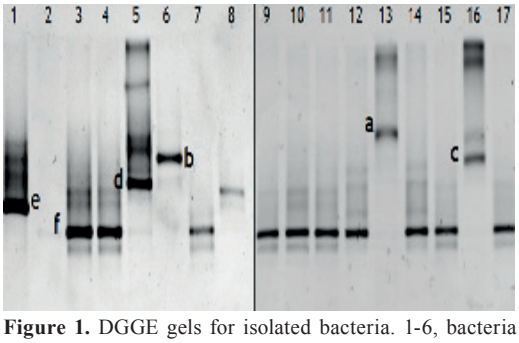

Figure 1. DGGE gels for isolated bacteria. 1-6, bacteria isolated from the fermentation pile, 1, 3 (M1), 4-5 (MF), 6 (F0); 7-17, bacteria isolated from the crushing pile (MA). Representative bands with characteristic migratory patterns (a-f) are marked.

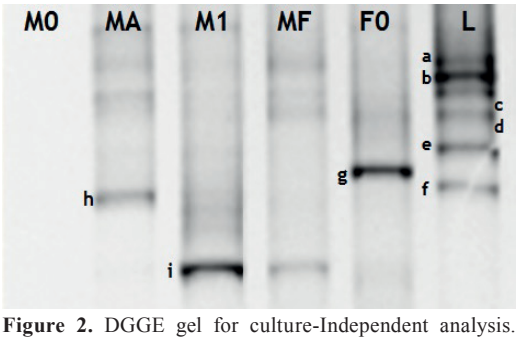

Figure 2. DGGE gel for culture-Independent analysis. Initial must (M0), "winey" must in the crushing pile (MA), must after 1 day of the process in the fermentation pile (M1), final must in the fermentation pile before being distilled (MF), and residual must (F0). Representative bands with characteristic migratory patterns are marked (a-g).

Table 1. Species detected by culture-dependent methods (D) and cultured independent methods (I). (X) Indicates detection of a species in the named sample.

Sample Bacteria	M0		MA		M1		MF		F0	
	D	I	D	I	D	I	D	I	D	I
Bacillus cereus (a)			X	X						
Lactobacillus mesenteroides (b)									X	
Weissella paramesenteroides (c)			X	X		X		X		X
Lactobacillus casei (d)						X	X	X		X
Acetobacter malorum (e)			X	X	X	X				
Bacillus subtilis (f)			X		X		X			
Lactobacillus amylovorus (g)										X
Acetobacter pasteurianus (h)				X		X		X		
Lactobacillus fermentum (i)						X		X		

Table 2. Nearest neighbor species after sequencing of bands obtained by DGGE analysis of extracted DNA and GenBank accession numbers.

Band	Nearest neighbor	Similarity (%)	Accession number GenBank
E	*A. malorum* (KX131150.1)	99	MK182816
F	*B. subtilis* (EU304922.1)	99	MK182817
D	*L. casei* (HQ644230.1)	99	MK182815
B	*L. mesenteroides* (MG755393.1)	99	MK182813
A	*B. cereus* (KF624703.1)	100	MK182812
C	*W. paramesenteroides* (LC094433.1)	99	MK182814
G	*L. amylovorus* (KU612254.1)	99	MK182818
H	*A. pasteurianus* (KT935504.1)	98	MK182819
I	*L. fermentum* (LC150759.1)	99	MK182820

Chromatographic analysis

Fructose was initially the dominant sugar (M0), representing approximately 93% (122.2 g L^{-1}) of total sugar content (132.4 g/L). Glucose was completely consumed during the fermentation process, whereas the residual fructose in the MF sample was approximately 3% of the initial fructose concentration in the M0 sample. The ethanol content in this sample was approximately 36.02 g L^{-1}, indicating an ethanol yield of 27 g consumed sugar/g of ethanol produced. The ethanol content in the final product after distillation was 47% v/v, with a methanol concentration of 202.04 mg per 100 mL.

Discussion

Metagenomic analysis indicated the presence during fermentation of most isolated bacteria, except for *Bacillus* species. Furthermore, the analysis revealed bands corresponding to bacteria that were not isolated but were dominant in specific stages of the process by considering the band sizes (bacteria closely related to *A. pasteurianus*, *L. amylovorus* and *L. fermentum*); these microorganisms belong to genera known for their capacity to metabolize and generate compounds that influence the sensorial characteristics of fermented beverages and thus the quality and acceptance of such products, such as acetic acid, lactic acid,

and ethanol (König & Frölich, 2009; Guillamón & Mas, 2009). The migratory patterns observed across the different sampled points indicate that most detected bacteria remained throughout the process but varied in their proportions as the process progressed.

It was not possible to detect bacteria in the initial must (M0). The absence of bands indicates low or no bacterial populations in the M0 sample. This can be explained by considering the previous stage of agave cooking, which eliminates microorganisms since they are exposed to elevated temperatures (Lappe-Oliveras et al., 2008; Escalante-Minakata et al., 2012).

The genus Bacillus was previously observed in the residual must of mezcal production in Tamaulipas (Narvaez et al., 2010). In this study, *Bacillus* was detected in both the crushing and fermentation piles. Our work found that a bacterium closely related to *B. subtilis* (f) was the most abundant isolated microorganism. Another bacterium detected was related to *B. cereus* (a), which is common in soil and water and is considered essential for the final taste of several Asian products (Tamang et al., 2016). Most bacterial isolates obtained by Kichmayr et al. (2017) from mezcal fermentations in Oaxaca corresponded to spore-forming microorganisms, and their presence was explained by contact of cooked agave with biological vectors, soil, water, and air; however, no specific impact of these bacteria on the alcoholic fermentation process were attributed. Similarly, in the present study, because these species could not be detected by culture-independent analysis, their role in the mezcal fermentation process could not be determined. Their presence within isolates could also be due to the ability of this genus to form highly tolerant spores under harsh conditions, including the presence of ethanol, which may also account for their detection in many fermented foods and beverages (Cocolin et al., 2013; Lappe-Oliveras et al., 2008). Most likely, *Bacillus* population abundances were quite low in comparison with other bacteria present or were maintained only as dormant spores in the original samples, which avoided their detection by culture-independent analysis, but rebounded after inoculation on nutrient-rich medium, enabling isolation over species with higher nutrient demands, such as LAB and AAB (Guillamón & Mas, 2009; König & Frölich, 2009).

Two isolated bacteria belong to the AAB group, which is of great importance in the food industry for its ability to oxidize sugars and alcohols to organic acids and high tolerance to product accumulation during alcoholic fermentation, showing ethanol tolerance levels from 5 to 10% v/v (Guillamón & Mas, 2009; König & Frölich, 2009). The highest ethanol concentration observed at the end of the studied fermentation process was approximately 4.5% v/v. The main application of AAB is acetate production, the major component of vinegar (Sengun & Karabiyikli, 2011), which is also a volatile compound present in mezcal (Martell Nevárez et al., 2011; Páez Lerma et al., 2011). *A. malorum*, the most closely related species to the bacterium corresponding to band "e", has previously been detected in rotting fruit (Torija et al., 2010) but not in mezcal fermentations. The culture-independent analysis showed that this bacterium was introduced in the crushing pile process, persisted in the fermentation pile and remained throughout the process. Bacteria closely related to *A. pasteurianus* (h) were isolated from the prefermented material and remained at lower proportions for the rest of the fermentation process; this species has been previously observed in the production of wine, beer, vinegar (Torija et al., 2010) and more recently in mezcal from Oaxaca (Kirchmayr et al., 2017). Detection of these species at several sampling points during the mezcal fermentation process suggests that they could have essential contributions to the transformation of ethanol into acetate, an undesirable metabolite in mezcal, at high concentrations.

LAB are highly common bacteria in dairy products and are of great interest as probiotics (Omar et al.,

2012), and they have been observed in alcoholic fermentations such as wine production (König & Fröhlich et al., 2009). LAB were present in other agave fermentations at the beginning of the process (Lachance, 1995), and Narvaez et al. (2010) detected them throughout fermentation by culture-independent methods, suggesting that malolactic fermentation performed by these bacteria may affect the quality of Tamaulipas mezcal. This theory was supported by Kirchmayr et al. (2012), who also detected eleven different species of LAB in mezcal from Oaxaca. The present study is consistent with that notion since it detected five LAB species from different genera during the fermentation process.

Three bacteria from the *Lactobacillus* genus were detected. A bacterium closely related to *Lactobacillus casei* (band d) was isolated from the fermentation pile. This species is a homofermentative microorganism that can produce up to 1.5% lactic acid (Jay, 1998), which was previously detected at the end of fermentation by culture-independent methods (Narvaez et al. 2010) and by culture-dependent methods by Kirchmayr et al. (2017). Another detected bacterium (band i) was closely related to *Lactobacillus fermentum*, which is a heterofermentative LAB capable of metabolizing a wide variety of substrates and is common in many dairy products and other fermented foods (Cocolin et al., 2013). It was detected in the fermentation tank on day 1 and at the end of the process by metagenomic analysis, suggesting its active participation in generating compounds of sensorial interest, such as lactic acid, ethanol, acetate, and CO_2. This species has not previously been reported in mezcal fermentations. Finally, a bacteria highly related (band g) to *Lactobacillus amylovorus* was present in the metagenomic analysis as the main band in residual must and as a band that was almost imperceptible during the fermentation process, suggesting a minor role.

The genus *Leuconostoc* is an obligate heterofermentative wine-related BAL of importance in compound production (König & Frölich, 2009). *Leuconostoc* bacteria have been detected by DGGE throughout the mezcal production process in Tamaulipas (Narváez-Zapata et al., 2010), and one isolate from this genus was obtained from mezcal in Oaxaca (Kirchmayr et al*.,* 2017). In the present study, a bacterium closely related to *L. mesenteroides* was isolated from the crushing pile and was also observed in the metagenomic analysis as a faint band, which suggests its probable contribution to the process.

A bacterium from the genus *Weissella* was also detected, and it was closely related to *W. paramesenteroides*, an obligate heterofermentative observed by both isolation and metagenomic analysis. This genus was previously detected in mezcal production in San Luis Potosi (Escalante-Minakata et al., 2008) and in Oaxaca (Kirchmayr et al*.,* 2017). Diverse carbohydrates can be metabolized by this species, which has been isolated from a wide range of European, Asian, and African fermented foods (Fusco et al., 2015). Another species from the genus *W. cibaria* was observed in residual must from Mezcal produced in Tamaulipas (Narváez-Zapata et al., 2010).

Sugar depletion throughout the fermentation process may be attributed to the presence of yeast but also to the bacteria detected in the present study. The ethanol production efficiency at the end of the fermentation was 0.27 g per g of sugar consumed, which is a low rate, probably due to multiple factors related to nonideal manufacturing practices during the artisanal process, such as fermentation carried out in an open pile, no temperature control leading to a longer fermentation process and non-immediate distillation after the alcoholic fermentation stage ended, thus allowing ethanol loss by volatilization and by the action of ethanol-consuming microorganisms, including AAB, detected in this study. The methanol and ethanol contents in the final product were within the allowed ranges (NOM-070-SCFI-2016).

Conclusions

The most abundant detected bacteria in the study were closely related to *L. fermentum, A. pasteurianus, A. malorum* and *L. casei*. The DGGE analysis performed can reveal the bacterial diversity during the mezcal fermentation process without the use of complex isolation procedures; this underscores the importance of using culture-independent techniques in analyses of complex environments.

Bacterial populations play an essential role in mezcal production since some of them were present throughout fermentation. It is important to point out that the significant bacteria observed in this study are different from those obtained in other mezcal-producing regions, which suggests their importance in the development of unique organoleptic characteristics in products typical of each region. This work opens the possibility for further rapid analyses using DGGE that must be conducted to obtain a more complete picture of the bacterial populations involved in mezcal. Additionally, using different sets of primers may enable detection of other bacterial genera not yet detected.

Resumen

D.S. Torres-Velázquez, J.A. Rojas-Contreras, N.O. Soto-Cruz, N. Urtiz-Estrada, J. López-Miranda, M.R. Kirchmayr, y J.B. Páez-Lerma. 2022. Monitoreo de poblaciones bacterianas durante la fermentación alcohólica de mezcal en Durango por DGGE. Int. J. Agric. Nat. Resour. 112-122. El mezcal es una de las principales bebidas alcohólicas de México, los microorganismos presentes durante la fermentación son los principales responsables de sus características organolépticas, dentro de ellos, las poblaciones bacterianas son las menos estudiadas debido a la dificultad que implica recuperarlas. El objetivo fue aplicar una técnica molecular independiente de cultivo para la detección de poblaciones bacterianas involucradas en el proceso de fermentación espontánea de agave en el Estado de Durango para lo que se obtuvieron muestras de varias etapas en la línea de producción. El análisis por DGGE se realizó a partir de ADN extraído de microorganismos aislados y de muestras tomadas directamente del proceso. Este análisis señaló a bacterias no aisladas como significativas durante la fermentación. Además, sugiere que las poblaciones de bacterias ácido-lácticas y ácido-acéticas son similares a las aisladas en otras regiones productoras de mezcal, pero difieren en algunas que pudieran ser importantes en el desarrollo de características sensoriales típicas del Mezcal de Durango. Además, este estudio indica que el análisis por DGGE puede revelar la diversidad bacteriana durante el proceso sin necesidad de aislamiento y puede ser una herramienta útil para el análisis, monitoreo y control de los procesos de fermentación del Mezcal.

Palabras clave: Agave, bacterias ácido-acéticas, bacterias ácido-lácticas, métodos independientes del cultivo.

References

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., & Rantsiou, K. (2013). Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *International Journal of Food Microbiology, 167*(1), 29–43. https://doi.org/10.1016/j.ijfoodmicro.2013.05.008

De León-Rodríguez, A., González-Hernández, L., Barba de la Rosa, A. P., Escalante-Minakata, P., & López, M. G. (2006). Characterization of Volatile Compounds of Mezcal, an Ethnic Alcoholic Beverage Obtained from Agave salmiana. *Journal of Agricultural and Food Chemistry, 54*(4), 1337–1341. https://doi.org/10.1021/jf052154

Dolci, P., Alessandria, V., Rantsiou, K., & Cocolin, L. (2015). Advanced methods for the identification, enumeration, and characterization of microorganisms in fermented foods. In W. H. Holzapfel (Ed), *Advances in Fermented Foods and Beverages : Improving Quality, Technologies, and Health Benefits* (pp. 157–176). Elsevier Woodhead Publishing. https://doi.org/10.1016/b978-1-78242-015-6.00007-4

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods, 56*(3), 297–314. https://doi.org/10.1016/j.mimet.2003.11.006

Escalante-Minakata, P., Barba de la Rosa, A. P., Santos, L., & De León-Rodriguez, A. (2012). Aspectos químicos y moleculares del proceso de producción del mezcal. *BioTecnología, 16*(1), 57–70.

Escalante-Minakata, P., Blaschek, H. P., Barba de la Rosa, A. P., Santos, L., & De León-Rodríguez, A. (2008). Identification of yeast and bacteria involved in the mezcal fermentation of Agave salmiana. *Letters in Applied Microbiology, 46*(6), 626–630. https://doi.org/10.1111/j.1472-765x.2008.02359.x

Fusco, V., Quero, G. M., Cho, G.-S., Kabisch, J., Meske, D., Neve, H., Bockelmann, W., & Franz, C. M. A. P. (2015). The genus Weissella: taxonomy, ecology, and biotechnological potential. *Frontiers in Microbiology, 6.* https://doi.org/10.3389/fmicb.2015.00155

Guillamón, J. M., & Mas, A. (2009). Acetic Acid Bacteria. In H. König, et al. (Eds.), *Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 43–64). Heidelberg Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-319-60021-5_2

Jay, J. M. (1998). Fermentation and Fermented Dairy Products. In J. M. Jay et al. (Eds.), *Modern Food Microbiology* (5th ed. pp. 130-137) . Food Science Texts Series. Springer. https://doi.org/10.1007/978-1-4615-7476-7_7

Kirchmayr, M. R., Segura-García, L. E., Lappe-Oliveras, P., Moreno-Terrazas, R., de la Rosa, M., & Mathis, A. G. (2017). Impact of environmental conditions and process modifications on microbial diversity, fermentation efficiency and chemical profile during the fermentation of mezcal in Oaxaca. *LWT - Food Science and Technology*, *79*, 160–169. https://doi.org/10.1016/j.lwt.2016.12.052

König, H., & Fröhlich. J. (2009). Lactic Acid Bacteria. In H. König et al. (Eds.), *Biology of Microorganisms on Grapes, in Must, and in Wine* (pp. 3–30). Heidelberg Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-85463-0_1

Lachance M. A. (1995). Yeast communities in a natural tequila fermentation. *Antonie VanLeeuwenhoek, 68*: 151–160.

Lappe-Oliveras, P., Moreno-Terrazas, R., Arrizon-Gaviño, J., Herrera-Suarez, T., García-Mendoza, A., & Gschaedler-Mathis, A. (2008). Yeasts associated with the production of Mexican alcoholic non-distilled and distilled Agave beverages. *FEMS Yeast Research. 8*, 1037–1052

López, I., Ruiz-Larrea, F., Cocolin, L., Orr, E. Phister, T., Marshall, M., Vander Gheynst, J., & Mills, D. (2003). Design and Evaluation of PCR Primers for Analysis of Bacterial Populations in Wine by Denaturing Gradient Gel Electrophoresis. *Applied and Environmental Microbiology, 69*(11), 6801–6807.

Martell Nevárez, M. A., Córdova Gurrola, E. E., López Miranda, J. L., Soto Cruz, N. O., López Pérez, M. G., & Rutiaga Quiñones, O. M. (2011). Effect of

fermentation temperature on the chemical composition of mescals made from Agave duranguensis juice with different native yeast genera. *African Journal of Microbiology Research, 4*(22), 3669–3676. https://doi.org/10.5897/AJMR11.467

Narváez-Zapata, J. A., Rojas-Herrera, I. C., Rodríguez-Luna, R. A., & Larralde-Corona, C. P. (2010). Culture-Independent Analysis of Lactic Acid Bacteria Diversity Associated with Mezcal Fermentation. *Current Microbiology, 61,* 444–450.

Nolasco-Cancino, H., Santiago-Urbina, J. A., Wacher, C., & Ruíz-Terán, F. (2018). Predominant Yeasts During Artisanal Mezcal Fermentation and Their Capacity to Ferment Maguey Juice. *Front. Microbiol. 9*(2900), 1–12. https://doi.org/10.3389/fmicb.2018.02900

NOM-070-SCFI-2016 (2017). Alcoholic Beverages-Mezcal-Specifications. Official Mexican Standard. (In Spanish). *Diario Oficial de la Federación.* http://www.dof.gob.mx/normasOficiales/6437/seeco11_C/seeco11_C.html

Omar, J., Chan, Y., Jones, M., Prakash, S., & Jones, P. (2012). Lactobacillus fermentum and Lactobacillus amylovorus as probiotics alter body adiposity and gut microflora in healthy persons. *Journal of Functional Foods, 5*(1), 116–123. http://dx.doi.org/10.1016/j.jff.2012.09.001

Páez-Lerma, J., Soto Cruz, O., Botello-Alvarez, E., & Rutiaga-Quiñones, M. (2011). Representative Volatile Compounds of the Mescal Produced from Agave duranguensis. (In Spanish, with English abstract.) *Revista Científica de la Universidad Autónoma de Coahuila, 3*(6).

Sengun, I. Y., & Karabiyikli, S. (2011). Importance of acetic acid bacteria in food industry. *Food Control. 22*, 647–656.

Tamang, J. P., Watanabe, K., & Holzapfel, W. H. (2016). Diversity of Microorganisms in Global Fermented Foods and Beverages. *Front. Microbiol. 7*(377), 1–28. https://doi.org/10.3389/fmicb.2016.00377

Torija, M. J., Mateo, E., Guillamón, J. M., & Mas, A. (2010). Identification and quantification of acetic acid bacteria in wine and vinegar by TaqMan–MGB probes. *Food Microbiology. 27*(2), 257–265. https://doi.org/10.1016/j.fm.2009.10.001