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Deep microbial community profiling along the fermentation process of pulque, a biocultural resource of Mexico

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ABSTRACT

The biggest non-tree perennial plant species endemic to Mexico were called *metl* in the Nahua culture; during colonial times, renamed with the Antillean word *maguey*. Carl von Linné finally renamed them as *Agave*, a Greek-Latin root word meaning admirable. Since pre-Columbian times, one of the major products obtained from some *Agave* species is the fermented beverage called pulque or *octli*. This beverage represents an ancient biotechnological development obtained by the natural fermentation of mead from such plants. Pulque played a central role in Mexican pre-Columbian cultures, while in recent times, there has been a renewed interest in it, due to its high content in nutrients and probiotics. In this study, we used massive sequencing of the 16S rRNA gene and the ribosomal internal transcribed spacer (ITS) to profile the pulque microbiome. We identified 2,855 bacteria operational taxonomic units (OTUs) and 1,494 fungi species in the pulque fermentation. Our results provide the most diverse catalog of microbes during pulque production reported so far. These findings allowed us to identify previously unidentified and core microbes resilient during pulque production, with the potential to be used as fermentation stage biomarkers. We confirmed previous reports of pulque microbes and discovered new ones like the bacteria *Sphingomonas* and *Weisella*. Among fungi we found that *Saccharomyces cerevisiae* was second to *Candida zemplina* in the studied pulque samples.

1. Introduction

Pulque is a fermented alcoholic beverage originally from central Mexico. It represents an empirical biotechnological approach developed by ancient Mexicans hundreds of years before pre-Columbian times (Cruz-Ramírez et al., 2014; Escalante et al., 2016; Gonçalves de Lima, 1990; Loyola Montemayor, 1956). Pulque or *poliuhqui octli,* as initially named in some pre-Columbian cultures, is the final product of a process that starts with the collection of the mead (*aguamiel* in Spanish) from sexually-mature plants of specific *Agave* species such as *Agave atrovirens*, *A. mapisaga*, and *A. salmiana* (Escalante et al., 2016; Steinkraus, 2004). The mead is collected from cavities made after the cut of the floral stem from such species (Escalante et al., 2016; Steinkraus, 2004). This starting substrate is a transparent-yellowish liquid highly rich in sugars that also contains minerals, carbohydrates, proteins and which pH ranges from 4.5 to 7.5 (De León et al., 2005; Leal-Díaz et al., 2016; Massieu-Guzmán et al., 1949, 1959; Sanchez-Marroquin and Hope, 1953; Sánchez-Marroquín et al., 1957; Steinkraus, 1997). Several of the metabolites present in mead, mainly sugars, are essential for the fermentation process since they serve as substrates for the microbial consortia that converts mead into pulque (Sánchez-Marroquín et al., 1957; Ulloa and Herrera, 1976). After mead extraction, it is then collected and transported to fermentation basins (Fig. 1). Once in the basins, a microbial inoculum called *semilla* (seed), which results from a previous pulque preparation, is added. It takes about 18 h to arrive at an intermediate fermentation stage, named *contrapunta* in Spanish. Around 36 h after inoculation, the fermentation completes, and pulque is ready for consumption (Fig. 1**G**; Cervantes-Contreras, 2008; Sánchez-Marroquín et al., 1957; Ulloa and Herrera, 1976).

Previous studies have shown that pulque contains sugars and

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proteins, the reason why it has been historically considered as a beverage with nutritional value /) De León et al., 2005; Massieu-Guzmán et al., 1949, 1959). This nutritional composition is the result of a microbial community that carries out the fermentation process, by using the metabolites in mead as substrates to generate the unique organoleptic characteristics of the beverage (Ampe et al., 1999; Caplice and Fitzgerald, 1999; Kostinek et al., 2007; Snowdon et al., 2006). Multiple research groups had worked on pulque microbiology, from cultivable strains to clonal sequencing methods (Escalante et al., 2004, 2008; Escalante et al., 2016; Lappe-Oliveras et al., 2008). These studies have reported the occurrence of the Gram-negative bacteria such as *Zymomonas mobilis, Lactobacillus spp.*, and *Leuconostoc mesenteroides* as well as the yeast *Saccharomyces cerevisiae*, all considered as essential for pulque fermentation (Sanchez-Marroquin and Hope, 1953; Sánchez-Marroquín et al., 1957). *Zymomonas mobilis* can produce ethanol by using extracellular polysaccharides like dextrans, and produces fructans, while *S. cerevisiae* plays a crucial role in alcoholic fermentation (He et al., 2014; Torres-Maravilla et al., 2016). Other reported strains are *Lactobacillus acidophilus*, *L. kefir*, *L. acetotolerans*, *L. hilgardii*, *L. plantarum*, *Leuconostoc mesenteroides*, *L. pseudomesenteroides*, *Microbacterium arborescens*, *Flavobacterium johnsoniae*, *Acetobacter pomorum*, *Gluconobacter oxydans, Z. mobilis*, and *Hafnia alvei* (Escalante et al., 2004, 2008; Escalante et al., 2016; Lappe-Oliveras et al., 2008). A recent study explores bacteria diversity in mead and shows that independently of the season of the year when mead is collected, the primary identified microorganisms are *Lactococcus, Pediococcus, Trochococcus, Kazachstania zonata* and *Kluyveromyces marxianus* (Villarreal Morales et al., 2019). A summary with all the previously described microbes of pulque is available (Supplementary Table 1). With the use of massive sequencing technologies mainly by the 16S rRNA gene and the ribosomal internal transcribed spacer (ITS), the food microbiology field is entering a new era. Examples of the biodiversity prospect of fermented beverages and food are the microbial profiles for kefir, kimoto sake, makgeolli/nuruk, doenjang, kimchi, narezushi, dahi, khoormog and palm wine (Astudillo-Melgar et al., 2019; Bokulich et al., 2014; Jung et al., 2012; Kiyohara et al., 2012; Nalbantoglu et al., 2014; Nam et al., 2012; Oki et al., 2014; Shangpliang et al., 2017). This study represents the first attempt to assess the microbial diversity of pulque by profiling the three major fermentation stages of pulque preparation through massive amplicon sequencing of the 16S rRNA gene and ITS.

Fig. 1. Pulque production process. **A)** Adult (~ 8 years old) *Agave* plant used to obtain the Sap. **B)** The technique of scraping the basal floral stem of the plant. **C)** Mead (*aguamiel*) obtained after scraping. **D)** Traditional sap collecting using the *Acocote* (dried pumpkin). **F)** Fermentation basins. **G)** The starting pulque inoculum obtained from old pulque. In **A, B, D, and E,** the expert pulque brewer, traditionally called *Tlachiquero*.

2. Materials and methods

2.1. Sampling

Mead, contrapunta and pulque samples collected from three different locations in Hidalgo State, Mexico (Fig. 2A): Epazoyucan (20° 01' 03" north latitude; 98° 38' 11" west longitude and altitude of 2456 m above sea level), Tepeapulco (19° 47' 06" N; 99° 33' 11" W and altitude of 2508 masl) and Zempoala (19° 48' and 20° 03' N; 98° 50' W and altitude of 2400–2900 masl). Mead (0 h fermentation) samples were collected directly from the *Agave* plant, contrapunta (~12 h fermentation), and pulque (~24 h fermentation) samples were collected from traditional fermentation containers after liquid homogenization. All the samples were frozen immediately after collection and until the DNA extraction.

2.2. Physico-chemical analyses

Mead and pulque samples from the three locations were used for a triplicate physicochemical analysis by a certified laboratory (ASAP laboratory SA de CV) according to the norms NMX-V-037−1972, NMX-V-022−1972 and NMX-V-40−1972 (D.O.F, 1972). The average and the standard deviation were calculated for each physicochemical property (Fig. 2B).

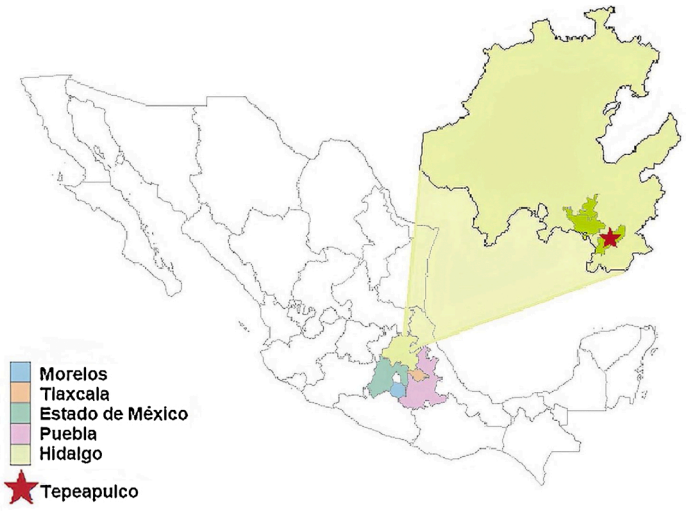
2.3. Metagenomic DNA extraction, library preparation, and sequencing

Mead, contrapunta, and pulque samples from Tepeapulco were used for metagenomic DNA extraction. All the samples were processed in the same manner by using the DNAzol® Reagent following the manufacturer's protocol (Chomczynski et al., 1997). The libraries were prepared at Genomic Services in LANGEBIO CINVESTAV using an amplicon-based approach according to the Illumina MiSeq protocols. For bacteria, 16S rRNA gene amplicons using the V3 and V4 regions were sequenced using 357 F (5'-CTCCTACGGGAGGCAGCAG-3') and 939R (5'-CTTGTGCGGGCCCCCGTCAATTC-3') primers. Fungal internal transcribed spacer (ITS1) were amplified using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCGTAGGTGAACCTGCGG3') primers. Illumina overhang adapter sequences were added and then sequenced on the Illumina MiSeq platform (2 × 300 bp).

2.4. Sequence processing and statistical analyses

We are using a previously reported pipeline for 16S rRNA gene amplicon sequences (Alcaraz et al., 2016, 2018). The pipeline involves sequence quality control through FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), FASTX tools (http://hannonlab.cshl.edu/fastx_ toolkit/) using Phred > 20 as the minimum cut-off for base-calling and length minimum cut-offs of 470 bp length of merged

A)



B)

Properties	Tepeapulco (Mead/Pulque)	Zempoala (Mead/Pulque)	Epazoyucan (Mead/Pulque)
pH	**4.37**±0.005 / **3.97** ±0.00	**6.19** ±0.01 /**4.05**±0.00	**3.95**±0.00/**4.2**±0.00
Density	**7°**±0.00 /**1°**±0.00	**6°**±0.00 / **1°**±0.00	**6°**±0.00/**1°**±0.00
Total solids	**13.61**±0.16/ **1.97**±0.08	**11.36**±0.15/**1.70**±0.06	**8.33**±0.22/**1.75**±0.05
Total reduced sugars	**11.12***±0.01 /**0.41*** ±0.3	**10.33***±0.01/**0.15***±0.00	11.2*±0.01/0.49*±0.00
Direct reduced sugars	**2.91***±0.00/**0.05***±0.00	**2.19***±0.00/ **0.11***±0.00	**2.01***±1.64/**0.12***±0.00
Gums	**0.28***±0.02 / **0.28***±0.00	**0.22***±0.01/**0.13***±0.00	**0.67***±0.02/**0.11***±0.00
Proteins	**0.40***±0.01/**0.28***±0.00	**0.43***±0.02/**0.39***±0.02	**0.29***±0.03/**0.31***±0.02
Ashes	**0.59***±0.06/**0.25***±0.00	**0.37***±0.14/**0.24***±0.01	**0.17***±0.00/**0.20***±0.01

°= °Be, *=g/100ml, ± standard deviation of the average from a triplicate analysed per location.

Fig. 2. (A) Map showing the three pulque collected locations from the State of Hidalgo, Mexico. (B) Physicochemical analyses of the mead and pulque samples.
First, samples of mead and pulque from the three locations were subjected to physicochemical analyses by a certified laboratory according to the Mexican norm NMX-V-037−1972 (See methods). The results obtained (Fig. 2B) showed that most of the physicochemical characteristics analyzed, such as pH, density, and gum content, did not show evident variation. Properties such as total solids, total reduced sugars, the total content of proteins, and ashes showed a slight variance among samples from the three different sampling locations (Fig. 2B).

amplicons. Pair-ends were merged using either PANDASEQ (Masella et al., 2012) or CASPER (Kwon et al., 2014). Identified chimeric sequences were cleaned from the dataset through blast-fragments and *Chimeraslayer* with the QIIME's *parallel_identify_chimeric_seqs.py* script (Caporaso et al., 2012). Operational Taxonomic Units (OTUs) are open OTUs at a $\geq$ 97 % identity clustering, we used cd-hit-est (Huang et al., 2010), also an option in the QIIME pipeline. We have previously benchmarked cd-hit-est, as a robust option for open OTU-picking, and depending on the OTU picking strategy, differences can be as high as two-fold (Alcaraz et al., 2018). Then, we conducted taxonomy assignment of 16S rRNA gene OTUs with the Greengenes database (v13.8) (DeSantis et al., 2006) and the UNITE database (its_12_11_otus) (Nilsson et al., 2018) for the ITS phylotyping, using an *e-value* cut-off of $1e^{-10}$ for the BLAST alignments. Complete sequence procedures are available at GitHub (https://github.com/genomica-fciencias-unam/pulque).

Biodiversity indexes and calculations were calculated with R (v 3.5.1) (Team, RC, 2014), and the following libraries: phyloseq (McMurdie and Holmes, 2013), vegan (Dixon, 2003), ape (Paradis and Schliep, 2018), dplyr (Wickham, 2009), DESeq2 (Love et al., 2014), plotted with ggplot2 (Wickham, 2009) and RColorBrewer (www.ColorBrewer.org). Multiple normalization procedures were used like log2 transformations, relative frequency, and normalized logarithmic transformation (rlog) for describing community profiles, community distances, and calculation of differential OTU enrichments. In the differential OTU enrichments, all the comparisons were corrected for multiple testing false discovery rate (FDR), using Benjamini-Hochberg correction, and using *p* adjusted (p-adj) values $< 1e^{-4}$. Full statistical analysis is available at GitHub (https://github.com/genomica-fcienciasunam/pulque).

3. Results

3.1. Sampling and physicochemical analyses

Samples from the three different stages of the pulque production process (mead, contrapunta, and pulque) were collected in three different locations of the State of Hidalgo (Fig. 2A), in the central Mexican plateau. This region has been considered, since pre-Columbian times, as the center of origin of this ancient fermented beverage (Evans T. S., 1990).

The physicochemical analyses showed high similarity among pulque samples from the three locations. To determine microbial diversity, we extracted DNA from mead, contrapunta, and pulque from the Tepeapulco samples. DNA was then used to generate sequencing libraries to screen both bacterial (V3 and V4 regions) and fungi (ITS) diversity through Illumina® MiSeq™ sequencing (See Methods).

3.2. Bacteria diversity along with pulque production

We were able to identify a total of 2,855 bacteria operative taxonomic units (OTUs; 97 % sequence identity) using the 16S rRNA gene. Within the pulque production, the bacteria had the most considerable abundance in mead with an average of 662 OTUs, followed by a decrease in contrapunta with an average of 472 OTUs and a slight average increase 483 bacteria OTUs in pulque. Our sampling estimated a vast diversity from the non-parametric Chao1 richness estimator with up to 3,077 expected OTUs in mead, 2,042 in contrapunta, and 2,764 in pulque. However, the Shannon diversity index (H') stated that the least diverse environment in the pulque production is the mead (H' = 1.47), then an increase in the richness and species abundance in the intermediate stage of contrapunta (H' = 2.5), with decrease in pulque (H' = 1.58). The inverse Simpson diversity index was congruent with the richness and diversity described with the Shannon diversity index, where the highest value (D = 0.61) corresponds to the contrapunta sample with lower dominance in that of mead (D = 0.39) or pulque (D = 0.45). The Proteobacteria phylum dominated all the phases of pulque production (94.95 % average), with a predominance of *α-Proteobacteria* and *γ-Proteobacteria*. The rest of bacteria diversity belongs to *Firmicutes* (*Bacilli* class; 4.7 %), *Actinobacteria* (0.09 %), *Cyanobacteria* (0.09 %), TM7 (0.04 %), albeit with lower abundances (>1e-02 %) the following phyla: *Chloroflexi*, *Bacteroidetes*, *Tenericutes, Planctomycetes, Thermi,* and *Acidobacteria*.

The most abundant OTUs (228,685 reads) we identified in all stages of pulque production were OTU_312, a member of the *Sphingomonas* genus (*Alphaproteobacteria*). The second one is OTU_286 (38,331 reads) belonging to *Acetobacter* (*Alphaproteobacteria*) and ubiquitous in pulque production. The third in total abundance and present during pulque production was OTU_101 (9,603 reads) identified as *Lactobacillus* (*Bacilli*). We identified 57 different bacteria genera, at some point of the pulque production (Fig. 1). A total of 12 different genera were shared between the three phases of pulque production: *Sphingomonas*, *Acetobacter, Lactobacillus, Acinetobacter, Enterobacter, Gluconobacter, Halomicronema, Lactococcus, Leuconostoc, Marivitia, Serratia,* and *Weissella* (Fig. 3**; Suppl.** Fig. 1).

We also identified the exclusive bacteria OTUs for mead, contrapunta, and pulque. The pulque samples host unique OTUs (708), belonging to 15 different genera, mainly *Sphingomonas, Lactobacillus,* and *Acetobacter*. But with lower abundances of *Lactococcus, Leuconostoc, Serratia, Acinetobacter, Arcobacter, Dysgonomonas, Euzebia, Gluconobacter, Sphingopyxis, Streptomyces, Synechococcus,* and *Weissella* (**Suppl.** Fig. 2).

The broader set of unique bacteria OTUs belongs to mead (1,010 OTUs) clustered into 30 genera (Fig. 3**)**. The most considerable abundance is again for *Sphingomonas, Lactobacillus,* and *Acinetobacter*. However, there are multiple genera with low abundances like *Acholeplasma, Arcobacter, Bdellovibrio, Campylobacter, Clostridium, Coprococcus, Enterobacter, Erwinia, Glaciecola, Gluconacetobacter, Gluconobacter, Kaistobacter, KSA1, Lactococcus, Leuconostoc, Mesorhizobium, Microbulbifer, Neoasaia, Podophyllum, Prevotella, Pseudoclavibacter, Serratia, Sphingobium, Sphingopyxis, Synechococcus,* and *Trichococcus* (**Suppl.** Fig. 3).

The contrapunta phase harbors 696 exclusive OTUs distributed in 23 genera (Fig. 3**)**. The most abundant genera were: *Acetobacter, Sphingomonas,* and *Lactobacillus*, which were dominant of the exclusive OTUs. The remaining 20 genera had low abundances: *Acinetobacter, Adlercreutzia, Ardenscatena, Chelativorans, Clostridium, Commensalibacter, Devosia, Gluconacetobacter, Gluconobacter, Klebsiella, Lactococcus, Leuconostoc, Luteimicrobium, Mesorhizobium, Pseudomonas, Ruminococcus, Salinibacterium, Sphingobium, Sulfurospirillum,* and *Weissella* (**Suppl.** Fig. 4).

Comparing the three phases of pulque fermentation, we used log2 fold changes and their *p-value* adjusted for multiple testing (*p-adj* < 1e-4; Bonferroni*)* to identify significant differential OTUs. Between pulque and mead, we found 17 differential OTUs, only five over-represented in the pulque belonging to *Sphingomonas, Lactobacillus*, and *Acetobacter*. Mead has 12 over-represented OTUs belonging to *Gluconacetobacter, Arcobacter, Sphingomonas, Lactobacillus, Serratia,* and an unidentified *Enterobacteriaceae* genus (**Suppl. Fig. 5; Supplemental Data 2**).

The log2 fold change contrast between mead and contrapunta identified 71 differential OTUs (*p-adj* < 1e-4; Bonferroni). We identified 37 differential OTUs in mead distributed in *Acetobacter, Lactobacillus, Sphingomonas, Leuconostoc, Acinetobacter, Lactococcus,* and unidentified *Acetobacteraceae*. Contrapunta harbors *Sphingomonas* OTUs, *Lactobacillus, Acinetobacter, Serratia, Lactococcus, Leuconostoc,* and unidentified genera of *Enterobacteriaceae, Bifidobacteriaceae,* and *Lactobacillales* (**Suppl. Fig. 6; Supplemental Data 2**).

The last microbial contrast diversity was performed between pulque and contrapunta for differential bacteria OTU abundances, and pulque has 29 differential OTUs: *Acetobacter, Lactobacillus, Sphingomonas, Acinetobacter, Gluconobacter, Lactococcus,* and three unidentified genera from *Bifidobacteriaceae, Lactobacillales*, and *Acetobacteraceae*. Then, contrapunta has 18 differential OTUs than those of Pulque:

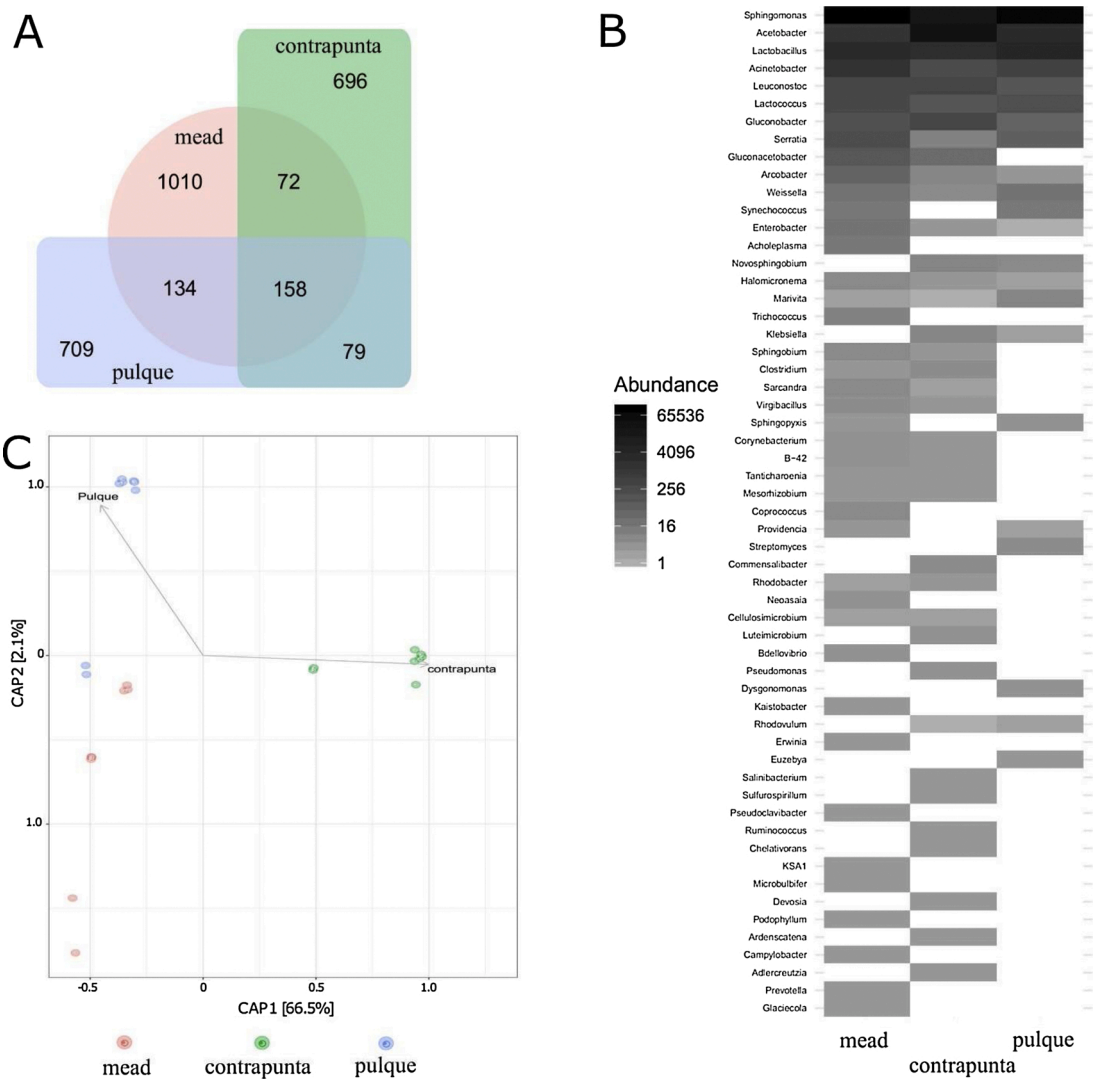


Fig. 3. Bacteria diversity (16S rRNA amplicons) in pulque production. A) Shared Bacterial OTUs from the different stages in pulque production, the 158 core OTUs are grouped in 12 genera. B) A total of 57 different genera were identified in the pulque production. Notice the ubiquitous presence of some genera like *Sphingomonas, Acetobacter, Lactobacillus*, which are part of the pulque core bacterial genera. C) Constrained analyses of principal coordinates (CAP) of the pulque production bacterial communities. Each dot represents a bacterial community in different stages of pulque preparation. There are significant differences between pulque production stages ($p < 1e\text{-}4$; PERMANOVA 9,999 replicates), with the contrapunta stage explaining 66.5 % of the observed variance.

Sphingomonas, Lactobacillus, and *Acinetobacter* (**Suppl. Fig. 7; Supplemental Data 3**).

The β-diversity analyses are used to understand the species composition from local to the regional level. In pulque, we used a constrained analysis of principal coordinates (CAP) from the bacteria OTU diversity in each fermentation stage, using *Bray-Curtis* dissimilarities to calculate the ecological distance. We found significant differences ($p < 1e\text{-}04$; 9,999 ANOVA permutations) between the three stages. The ordination explained 66.5 % of the variance (CAP1) by the bacterial communities' difference in contrapunta compared to mead and pulque (Fig. 4). Differences between mead and pulque are represented in CAP2, and they only explained the variance of 2.1 %.

3.3. Fungal diversity along with pulque production

A total of 1,494 fungi OTUs described by the internal transcribed spacer (ITS 97 % sequence identity) were identified. The most considerable observed richness was found in mead with an average of 776 OTUs, followed by pulque with 130 OTUs, and contrapunta with just 66 OTUs. In the fungi, we found equal observed and estimated OTU numbers (Chao1 = 776 ± 224), which means that the diversity was entirely covered with our sampling. The Shannon diversity index was quite similar for fungi across the fermentation process (H' = 2.6). Inverse Simpson's index was slightly higher in pulque (D = 0.872) than contrapunta (D = 0.855), and mead (D = 0.854). The pulque process phyla composition was *Ascomycota* (1227 OTUs; 82.12 %), *Basidiomycota* (58 OTUs), 206 OTUs were not identified up to phylum level and described as uncultured fungi, Zygomycota (2 OTUs), and 1 *Glomeromycota* OTU.

The most abundant species was *Candida zemplinina*, which was highly abundant in mead (25.15 %), depleted in contrapunta (1.7 %), and recovered in pulque (17.98 %). Second *Clavispora lusitaniae* (23 % in mead; 16 % in contrapunta; and 9% in pulque), *Candida stellata* as third in contrapunta (32.96 %), barely detected in mead (0.6 %), and observed in pulque (6.19 %). Interestingly, *S. cerevisiae* appears as 9th in abundance, with low abundance in mead (0.74 %), rising in contrapunta (13.31 %), and decreasing in pulque again (8.49 %). The 1,494 OTUs were distributed in 94 identified species, and 27 species annotated as "uncultured" and "fungal sp". Some of the unidentified species are identified as fungi but not even classified to Phylum level like the 12th

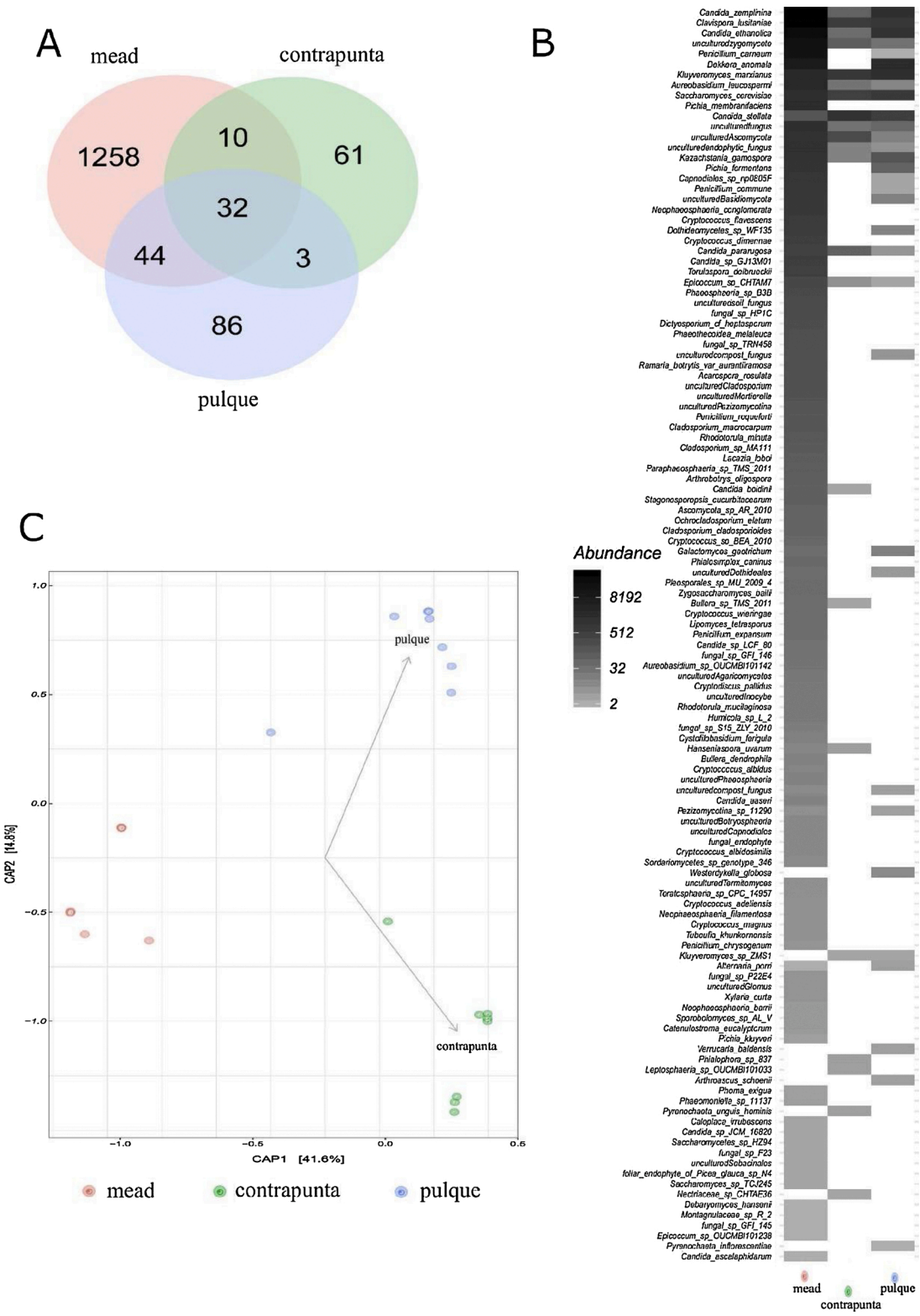


Fig. 4. Fungi diversity in pulque production. A) A total of 1,494 OTUs belonging to 121 different species classified in the pulque production stages. B) Heatmap showing the prevalence of the 121 identified fungi species C) Constrained analyses of principal coordinates (CAP) of the fungi communities' pulque production. Each dot represents a fungi community in different stages of pulque preparation. There are significant differences between the mead production stage (p < 1e-4; PERMANOVA 9,999 replicates), with the contrapunta and pulque stages explaining 41.6 % of the observed variance.

most abundant OTU (Fig. 4).

We identified diverse fungi across the pulque fermentation represented by 32 OTUs, clustered into 20 core set species (Fig. 4**).** The most abundant species in the core was *Kluyveromyces marxianus,* followed by *S. cerevisiae, Dekkera anomala, Kazachstania gamospora,* an uncultured compost fungus, and *Westerdykella globosa* as the most abundant within the core. Mead-exclusive fungi (Fig. 4) are the largest ones with 1,258 identified OTUs grouped into 63 genera with a large abundance of

Penicillium, Pichia, Candida, Cryptococcus, and *Clavispora* (**Suppl. Fig. 8**). There were just 61 contrapunta-exclusive OTUs (Fig. 4). They grouped in 11 genera with a dominance of *Kluyveromyces*, *Saccharomyces*, and unidentified fungi (**Suppl. Fig. 9**). Then, pulque-exclusive fungi OTUs are just 86 (Fig. 4), where its composition is similar to the one described to the core.

There were significant changes (p-adj < 1e-4) in the fungi community of the mead-contrapunta transition, with only three OTUs being over-represented (OR) in mead: an uncultured *Ascomycota* (id = 1286), *Kluyveromyces marxianus* (id = 703), and *Candida stellata* (id = 1149). *Contrapunta* has 64 OR OTUs in this comparison, clustered into 32 species, with *Clavispora lusitaniae, Kluyveromyces marxianus, Saccharomyces cerevisiae,* and *Kazachstania gamospora* leading the enriched fungi list in contrapunta stage (**Supplemental Fig. 11; Supplemental Data 4**).

The fungi community changes in the pulque-contrapunta transition were moderate, with only one species being significantly OR (p<1e-4) in pulque: *Dekkera anomala*. In contrapunta, there were only four species being OR than pulque*: S. cerevisiae, Candida etanolica, Candida zemplinina,* and two OTUs of uncultured *Ascomycota* (**Supplemental Fig. 12**).

The changes are noticeable when comparing the final product, pulque, and starting source (mead). There are only two OTUs significantly (p<1e-4) enriched in pulque*: S. cerevisiae* and *Candida stellata.* In mead, there are 62 OR OTUs, engulfed into 32 fungi species, which persisted along the fermentation process but were significantly richer at the beginning of it (**Suppl. Fig. 13; Supplementary Data 5**).

We found significant differences ($p<1e^{-4}$; 9,999 ANOVA permutations) between the three pulque production stages in fungi diversity, particularly between pulque and contrapunta. The ordination explained 41.6 % of the variance (CAP1) by the difference of the fungi communities in mead, compared to contrapunta and pulque (Fig. 4). Differences between mead and pulque are represented in CAP2, and they roughly explain 14.8 % of the variance.

4. Discussion

The overall microbial diversity found in this study, along three pulque fermentation stages, was astounding, with 2,855 bacteria OTUs and 1,494 fungi species identified. The bacterial diversity is fluctuant along pulque production with the most considerable bacterial diversity reached in the intermediate contrapunta phase (H' = 2.5), but lower diversity in the start (H' = 1.47), and the pulque (H' = 1.58). On the other hand, the fungi held more stable diversity indexes ranges in the production (H'=[2.65–2.70]).

In this study, *Sphingomonas* was the most abundant genera of bacteria found in large abundance (~ 50 %) in all pulque production phases, with 3,410 OTUs (considering singletons), representing considerable intra-specific biodiversity of *Sphingomonas*. Multiple strains of *Sphingomonas* are well known historically for their ability degrading organic aromatic compounds (PAH) (Gibson et al., 1967), and it is common to isolate species from this genus from soils (Leys et al., 2004). *Sphingomonas* species have chitinolytic activity and produce a biosurfactant that can act like a gelling agent, associated with the characteristic viscosity of pulque. Recently, a new plant-isolated species *Sphingomonas pokkalii* had been described with some phenotypic traits that would explain the prevalence of *Sphingomonas* in the pulque production: a full-battery of reactive oxygen species coding genes, including several redundant superoxide dismutase and catalases; over-representation of carbohydrate metabolism and degradation genes along with the genus ability to degrade PAH; and the possibility to play with the plant development by IAA pathway plant-hormone production (Menon et al., 2019). It is the first report *of Sphingomonas* as the dominant bacteria during the whole pulque production. In particular, we have evidence that classifies the 16S rRNA gene (470 bp) fragment as *Sphingomonas wittichii.* The *S. wittichii* RW1 is known for their metabolic diversity, capable of degrading dioxins (dibenzo-*p*-dioxin), chlorinated contaminants, and aromatic compounds from decaying plants. Some of the most abundant *S. wittichii* RW1 genome genes are TonB-dependent receptors (TBDR), general dehydrogenases, and ring-related phenylpropionate dioxygenases (Miller et al., 2010; Moreno-Forero and Van Der Meer, 2015; Chai et al., 2016). TBDR is part of a carbohydrate scavenging response of plant carbohydrates (Blanvillain et al., 2007).

The second most abundant bacteria in this study was *Acetobacter,* with 877 OTUs. The maximum abundance of *Acetobacter* was during the contrapunta stage, then decreasing its presence in pulque. *Acetobacter,* along with *Gluconacetobacter and Gluconobacter*, belongs to the core pulque microbiome, and it has been previously reported as responsible for acetic acid production *via* the carbohydrate oxidation and dehydrogenation (Escalante et al., 2016). The *Acetobacter* strains are probably already bacterial colonizers of the *Agave* plants, in high sugar content niches. A high sugar accumulation previous to flower development in the semelparous (reproducing sexually once in a life) of *Agave* species is known to happen (Rocha et al., 2005). *Acetobacter's presence* has also been documented in other plant species like the grape berries, being ubiquitous and increasing their presence in some conditions, like the sour-rot disease, which strongly affects wine production. However, it represents an advantage for acetic acid bacteria in accessing rich sugar environments (Hall et al., 2019).

With 494 different OTUs in this study, Lactobacillus is described as an essential genus for pulque production due to its fermentative abilities (Escalante et al., 2004, 2016). Lactobacilli were the second most abundant genus in this study. *Lactobacillus* species have also been isolated from ricotta cheese, vegetables, fermented milk, meat, fish, bread, wine, fermented olives, yogurt and cacao (Felis and Dellaglio, 2007; De Vos et al., 1993; Gomez-Gil et al., 1998; Hirsch, 1952; Roh et al., 2010; Takeuchi et al., 1995). Our study results are consistent with previously reported abundance of 3.2×10^9 CFU/mL units for lactic acid bacteria (LAB), including *Leuconostoc* (Escalante et al., 2016). With an estimate of up to 85 % of previously reported 16S rRNA gene clones belonging to *Lactobacillus acidophilus,* thus consistently indicating the dominance of some bacteria in the pulque production and introducing the idea of geographical variability of the microbes producing pulque (Escalante et al., 2016). *Leuconostoc* was identified as a core member for all the stages of pulque production in this study, along with other LAB genera like *Lactobacillus, Lactococcus, and Leuconostoc,* and *Weisella*. Their tolerance explains the prevalence of LAB bacteria, which has large ranges from mead (pH = 7.5) to acid pulque (pH = 3.5) (Escalante et al., 2016). Species of the *Leuconostoc* genus have been associated with gums' production, which may be associated with pulque consistency; some of them have been isolated from cheese, distilled spirits production, and fermented sausages and vegetables. Furthermore, some *Leuconostoc* produce some bacteriocins like mesentericin, leucocin, and nisin that inhibit Gram-positive bacteria (Caplice and Fitzgerald, 1999; Choi et al., 1999; Cogan and Jordan, 1994; Bhadra et al., 2007; ; Hechard et al., 1999; Hemme and Foucaud-Scheunemann, 2004; Steinkraus, 1997). *Lactococcus* species have been observed in the early stage of milk fermentation.

Weissella spp is another ubiquitous pulque bacteria, hosting multiple species isolated from fermented foods, obligate hetero-fermenters, CO_2 producers, and thus acid producers (lactic and acetic). Some *Weissella spp* are used as probiotics, but there is a word of caution for using them without virulence genes screenings as some could be opportunistic human pathogens (Abriouel et al., 2015; Fusco et al., 2015). The *Weissella* genus includes species associated with fermentation such as those isolated from pozol (a Mexican fermented drink), Jeotgal (Korean fermented food), Boza, Suusac (milk of camel), Togwa (fermented Tanzania drink), Nushera, Khuleanito, cacao grains fermentation, Jian-gun (fermented cucumbers) and Gari (cassava fermentation) (Ampe et al., 1999; Gonçalves de Lima, 1990; Jans et al., 2012; Kostinek et al., 2007; Lopez-Dıaz et al., 2001; Mathara et al., 2004; Mugula et al., 2003; Muyanja et al., 2003; Neve et al., 1988; Van Oevelen et al., 1977; Osimani et al., 2015).

Regarding the fungal diversity, in this study, the most abundant species was *Candida zemplina,* which is resilient across all fermentation stages. This yeast has been reported previously in spontaneous fermentation in beer and wines production and thought to enhance organoleptic properties of the products, interacting with *Saccharomyces cerevisiae* (Estela-Escalante et al., 2016; Sipiczki, 2003). Another enduring yeast during the *pulque* production was *Clavispora lusitaniae*, also known as *Candida lusitaniae*, an opportunistic pathogen involved in < 5% candidiasis haploid and phylogenetic outgroup of the most known *Candida albicans* related species (Butler et al., 2009). *Candida lusitaniae* is a previously known fermenter species to produce bioethanol from soybean residues, with a unique capability in the total yield of ethanol, second to galactose adapted *S. cerevisiae* strains (Tran et al., 2017). *C. lusitaniae* has been reported in the fermentation of other traditional fermentation like Dolo, produced from sweet wort fermentations, from Burkina Faso, with up to 13 % in a fermentation dominated by *S. cerevisiae* (Sanata et al., 2017). Some initial strains like *Penicillium carneum,* previously classified as *P. roqueforti* which is responsible for blue mold in apples (Peter et al., 2012) and mycotoxin (patulin) production (Boysen et al., 1996, 2000) are in large abundance in mead, not detected in contrapunta and drastically reduced in pulque. *Kluyveromyces marxianus* was found within pulque core, is capable of fermentation at high temperatures (45 °C), also able to ferment complex sugars such as inulins and hemicellulose, and been used to produce bioethanol with a broader substrate range and higher temperature tolerance than *S. cerevisiae* (Fonseca et al., 2008; Lertwattanasakul et al., 2015). *Kluyveromyces* can release fructose from inulins as many microorganisms. Some other microbes found in this study (*Gluconacetobacter, Weisella, Leuconostoc, Serratia* and *Candida)* that use fructose as a carbon source (Estrada-Godina et al., 2001; Grimont and Grimont, 2006; Silva--Santisteban et al., 2006; Simoncini et al., 2007; Villarreal Morales et al., 2019; Wang et al., 2005; Wickham, 2009; Yamada et al., 2012).

We identified 14 mead restricted bacterial genus: *Archoleplasma, Trichococcus, Coprococcus, Neoasia, Bdellovibrio, Kaistobacter, Erwinia, Pseudoclavibacter, Microbulbifer, KSA1, Podophyllum, Campylobacter, Prevotella, and Glaciecola* (Fig. 3). Some species of *Archoleplasma* are capable of growth at alkaline pH and ferment carbohydrates to acetate, lactate, and alcohol (Lelong et al., 1989). Some *Trichococcus* include aerotolerant and fermentative members that grow in glucose and produce lactate, acetate, formate, and ethanol anoxically (Jian-Rong et al., 2002; Seviour et al., 2015). Some *Coprococcus* ferment mucins, plant-derived carbohydrates into butyric, acetic, formic, propionic, and lactic acids (Holdeman and Moore, 1974; Salyers et al., 1977). It has been reported *that Neoasia* participates in rice beer production (Das et al., 2019). On the other hand, *Bdellovibrio* can predate Gram-negative bacteria, suggesting that it could regulate pathogenic bacteria in pulque. *Bdellovibrio* has also been isolated from the fermentation of Chinese rice wine and reported to be plant-associated (Martínez et al., 2016; Jialiang et al., 2018; Alcaraz et al., 2018). *Kaistobacter* strains were also found in pulque and are reported in Chinese grape wine (Yu-ie et al., 2018). The potential *Erwinia* role in pulque could be the metabolic conversion, from D-glucose to ketogluconate and the probable production of exopolysaccharides, which could contribute to pulque drink consistency (Bellemann et al., 1994; Truesdell et al., 1991). *Pseudoclavibacter* has been reported in raw milk and traditional fermented dairy products (Hurood cheese and Jueke) from Inner Mongolia, China, and smear-ripened cheese (Gao et al., 2017; Mounier et al., 2006). Certain *Pseudoclavibacter* produces acid phosphatases, alkaline phosphatase, arginine dihydrolase, cystine arylamidase, leucine arylamidase, and lipases (Kim and Jung, 2009). *Microbulbifer* members can produce polyhydroxyalkanoates, secondary metabolites such as 4-hydroxybenzoic acid, esters, and parabens and have the ability to use chitin, cellulose, xylan, and alginate (Tian et al., 2018). Some species like *M. hydrolyticus* have been reported as involved in the degradation of *Agave* fibers by the action of lignocellulosic enzymes (González et al., 2005).

From the 81 fungal species found as mead-exclusive, like some of the most abundant are *Pichia membranifaciens, Neophaeosphaeria conglomerate, Cryptococcus flavescens, Cryptococcus dimennae, Torulaspora delbrueckii, Candida sp GJ13M01, Phaeosphaeria b3b, Dictyosporium cf heptasporum, Phaeothecoidea melaleuca, Acarospora rosulata, Penicillium roqueforti*. *Pichia membranifaciens* produce killer toxins, ranging from pre- and post-harvest biocontrol of plant pathogens to applications during wine fermentation and aging, PMKT and PMKT2 are toxins are lethal for sensitive yeast cells and filamentous fungi (Belda et al., 2017). *Cryptococcus flavescens* produce Xylanase which degrades the linear polysaccharide xylan into xylose, thus breaking down hemicellulose (Andrade et al., 2015). *Torulaspora delbrueckii* has been isolated from the mezcal fermentation of *Agave salmiana* (Verdugo Valdez et al., 2011). It has also been used as starting co-culture in red wine to improve its aromatic profile (Zhang et al., 2018). Mixed *T. delbrueckii/S. cerevisiae* inoculation can also increase the total ester concentration, such as isoamyl acetate, ethyl hexanoate and 3-hydroxybutanoate (Zhang et al., 2018). While *Penicillium roqueforti,* known for cheese production, has been reported to generate methyl ketones also can produce alcohol (Frisvad and Samson, 2004; Kinsella and Hwang, 1976; Yeluri-onnala et al., 2018).

We hypothesize that the great fungal and bacterial diversity found in mead might be, in part, enriched with endospheres of the adult *Agave* pineapple. We supported this hypothesis by recent results showing that the root and leaf endosphere of diverse *Agave* species, including *A. salmiana* ecotypes, harbor abundant microorganisms (Coleman-Derr et al., 2016). However, further studies and data comparisons are needed to test this assumption.

This study provides the most diverse catalog of bacteria (2,855 OTUs) and fungi (1,494 species), reported so far and associated with the three stages of pulque production. Compared to previous work, different species dominance could be explained by the geographical variation of the pulque production and its spontaneous fermentation inocula (Escalante et al., 2016). This work provides new resources in pulque fermentation research. It expands the definition of essential microbiota responsible for pulque fermentation (Escalante et al., 2016). The described 158 OTUs grouped into 12 core bacteria genus, and 20 fungi core species found in all the pulque fermentation stages. The main bacterial community difference was the contrapunta (Fig. 3C), while the mead had more important fungi community differences than those in pulque or contrapunta (Fig. 4C). New critical players for pulque fermentation described here were identified like the bacteria *Sphingomonas* and *Weisella*, and the most abundant fungal species in this pulque was *Candida zemplina.* With the enriched or exclusive OTUs in each fermentation step, we provide possible biomarkers for each pulque production stage.

5. Conclusions

There are specific microbial communities for each pulque fermentation stage, the bacterial communities of pulque and mead are closer to each other than contrapunta (the higher bacterial diversity); fungal communities of pulque and contrapunta clustered apart from the source mead, and the overall diversity is quite similar when measured with the Shannon diversity index. We identified resilient bacteria in large abundance along the fermentation process like *Sphingomonas*, *Acetobacter, Lactobacillus, Acinetobacter, Enterobacter, Gluconobacter, Halomicronema, Lactococcus, Leuconostoc, Marivitia, Serratia,* and *Weissella.* The most relevant yeast identified before this work in pulque fermentation was *Saccharomyces cerevisiae,* and we found it too. However, in the 9th position, the most abundant fungi were *Candida zemplina, Clavispora lusitaniae*, and *Candida stellata*. There is yet a vast bacterial and fungal diversity to explore yet in the pulque production. Further experimental validation will require the complementation of classical microbiology and other omics techniques like RNAseq and metabolomic analyses in each stage of the pulque fermentation. Current and future biotechnology findings and potential applications, related to pulque and mead

microorganisms, will allow the re-launching of pulque production beyond its use as an alcoholic beverage and for the rescue of *pulque* producing Agave as one main crop for Mexican communities in central Mexico.

Data availability

Raw data for the pulque microbiome was deposited in the NCBI BioProject and Sequence Read Archive database under accession code PRJNA556980. OTU tables and sequences are available at GitHub (https://github.com/genomica-fciencias-unam/pulque).

CRediT authorship contribution statement

Carolina Rocha-Arriaga: Conceptualization, Investigation, Formal analysis, Data curation, Writing - original draft. **Annie Espinal-Centeno:** Formal analysis, Methodology, Project administration. **Shamayim Martinez-Sánchez:** Formal analysis, Data curation. **Juan Caballero-Pérez:** Formal analysis, Data curation. **Luis D. Alcaraz:** Conceptualization, Investigation, Resources, Writing - review & editing. **Alfredo Cruz-Ramírez:** Conceptualization, Supervision, Resources, Writing - original draft, Writing - review & editing, Funding acquisition.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126593.

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