Colegio de Postgraduados

Indicator microorganisms for quality in pulque from three producing municipalities in the state of Mexico

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ABSTRACT

Objective: To evaluate the physicochemical parameters and microbiological quality of pulque produced in three municipalities in the State of Mexico: Temoaya, Jiquipilco, and Ocoyoacac.

Design/methodology/approach: The physicochemical parameters evaluated included pH, total soluble solids (°BX) and alcohol content.

In addition, microorganism's indicative of microbiological quality was quantified, including: aerobic mesophilic bacteria, fungi, yeasts, and coliforms, complemented by the presence of lactic acid bacteria. Presumptive identification and antimicrobial susceptibility testing were performed on coliform isolates.

Results: The physicochemical results were within the typical ranges for pulque, with pH values 3.30 to 4.02, total soluble solids 4.62 to 6.37 °Bx and alcohol content 4.42 to 4.87%. Microbiological analyses revealed the presence of aerobic mesophiles, lactic acid bacteria, and yeasts in all samples. Coliform bacteria were detected only 20% of the samples. Biochemical test allowed the presumptive identification of *Escherichia coli* and *Shigella flexneri*. Antimicrobial susceptibility revealed, resistance of these isolates to antibiotic such as ampicillin, carbenicillin, cephalothin, cefotaxime and nitrofurantoin.

Limitations/implications: The study was limited to specific localities, which may affect the generalizability on the results. Nevertheless, it highlights the need to strengthen hygienic practices and microbiological control.

Findings/conclusions: The microbiological variability observed among municipalities highlights the influence of production and commercialization conditions. The detection of antibiotic-resistant bacteria represents a potential risk to food safety.

Keywords: Fermentation, bacteria, yeast, analyses, food safety.

INTRODUCTION

Pulque is one of the oldest fermented beverages in Mesoamerica and has traditionally been associated with its consumption during pre-Hispanic ceremonies (Escalante *et al.*, 2016; SADER, 2023). Following colonization, its production gained economic relevance with the establishment of pulque-producing haciendas; however, its consumption declined at the beginning of the twentieth century due to the introduction of industrialized beverages and discrediting campaigns (Trejo *et al.*, 2020; SADER, 2023). In recent years, this beverage has experienced a resurgence driven by its cultural, nutritional, and artisanal value, as well as by its promotion through regional fairs that highlight the importance of its production and consumption (Rojas *et al.*, 2016; Pérez & Cardoso, 2020).

In Mexico, pulque production is concentrated in the states of Hidalgo, Tlaxcala, Puebla, and the State of Mexico, the latter ranking third nationwide with a production close to 26,000 L, mainly in the municipalities of Jiquipilco, Ocoyoacac, and Temoaya (SECAMPO, 2023; SIAP, 2024). Pulque is obtained through the fermentation of aguamiel, the sap of agave plants, which provides a favorable environment for the development of microorganisms such as lactic acid bacteria and yeasts, responsible for acidification, ethanol production, and sensory characteristics (Escalante *et al.*, 2016; Rocha *et al.*, 2020; Álvarez *et al.*, 2020; NOM-199-SCFI-2017; Ruíz *et al.*, 2023). Its production is an artisanal process that does not involve distillation and, depending on the region, employs different agave species (Enríquez *et al.*, 2017; Villarreal *et al.*, 2019). Aguamiel is obtained by scraping the cavity formed in the center of the agave stem, where initial microbial development occurs during its accumulation in the cajete of the maguey plant. Subsequently, aguamiel undergoes the fermentation process, which involves not only autochthonous microorganisms but also those incorporated throughout different handling stages, including collection, transportation, inoculum (semilla) preparation, product handling, and storage (Tamang *et al.*, 2016; Amazará & Quintero, 2022; Colón *et al.*, 2025).

The microbiological quality of pulque depends directly on agricultural crop management and hygienic production practices (Cervantes & Pedroza, 2007; Gómez *et al.*, 2011). However, the process is not exempt from the risk of contamination by spoilage or pathogenic microorganisms, which may affect the quality and safety of the product, particularly when antimicrobial-resistant microorganisms are involved (Gómez *et al.*, 2011; Escalante, 2016; Lappe *et al.*, 2023).

Among the factors associated with contamination are the exposure of agave plants to domestic and farm animals, inadequate hygiene during aguamiel extraction and handling, poor sanitary conditions in production and storage areas, and inadequate distribution practices (Natera, 2005; Rojas *et al.*, 2016).

In this context, the quantification of indicator microorganisms of quality, such as aerobic mesophiles, total coliforms, fecal coliforms, *Escherichia coli*, molds, and yeasts, is a key factor in evaluating and ensuring the microbiological quality of various fermented beverages, including pulque (Ramos *et al.*, 2014; NOM-210-SSA1-2014).

Therefore, the objective of this study was to evaluate the microbiological quality of pulque produced in three municipalities of the State of Mexico (Temoaya, Jiquipilco, and Ocoyoacac) through the quantification of aerobic mesophiles, lactic acid bacteria, total coliforms, fecal coliforms, molds, and yeasts, with the aim of identifying microbiological parameters associated with this traditional beverage.

MATERIALS AND METHODS

Study Area

Pulque samples were obtained from points of sale in three producing municipalities of the State of Mexico: Temoaya (19° 24'-19° 35' N), Jiquipilco (19° 29'-19° 42' N), and Ocoyoacac (19° 12'-19° 18' N) (Figure 1) (INEGI, 2024).

Sampling

Physicochemical and microbiological evaluations were carried out at the Food Safety Laboratory of the Faculty of Agricultural Sciences at the Autonomous University of the State of Mexico. Sample collection was performed aseptically from products intended for sale. Duplicate samples of 100 mL of natural pulque were collected in sterile containers from five producers in each municipality. The samples were transported under refrigerated conditions (4±2 °C) to the laboratory in accordance with the guidelines established by the Mexican Official Standard NOM-109-SSA1-1994.

Physicochemical Analyses

pH was measured directly in the samples using a previously calibrated potentiometer (Conductronic PC45). Total soluble solids (TSS) were determined by measuring the refractive index in degrees Brix (°Bx) using a Milwaukee MA871 digital refractometer. Alcohol content was determined in accordance with the Mexican Official Standard NOM-199-SCFI-2017.

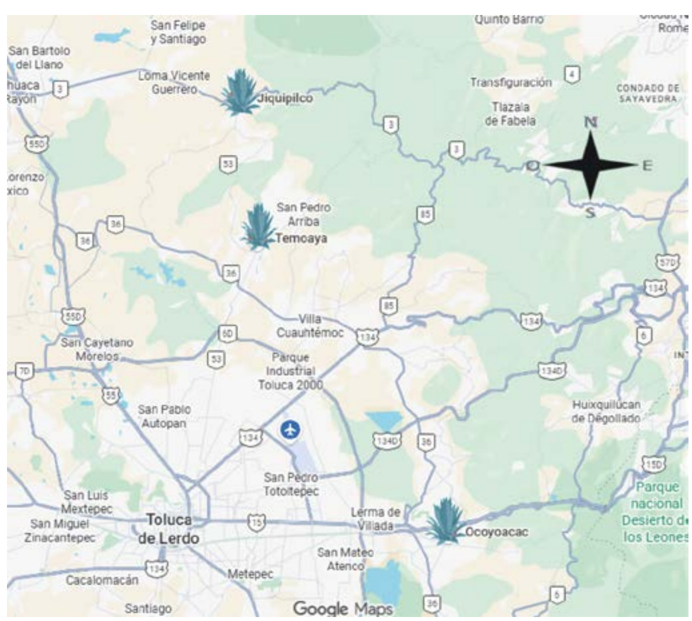

Figure 1. Geographic location of the municipalities of Jiquipilco, Temoaya and Ocoyoacac (Adapted from Google Maps, 2025).

Microbiological Analyses

Samples were analyzed through the preparation of serial dilutions (10^{-1} to 10^{-6}) using buffered peptone water (BD Difco). From these dilutions, viable counts of indicator microorganisms of microbiological quality were performed, including aerobic mesophiles according to NOM-092-SSA1-1994 and coliforms in accordance with the Mexican Official Standard NOM-210-SSA1-2014. The determination of lactic acid bacteria was carried out following the methodology established in NOM-110-SSA1-1994. Likewise, microbiological counts of molds and yeasts were performed according to the procedures described in NOM-111-SSA1-1994.

From the prepared dilutions, 100 μL aliquots were plated by surface spreading onto Petri dishes containing Standard Methods Agar (MCD LAB) for aerobic mesophiles; MRS agar (Millipore) for lactic acid bacteria; Violet Red Bile agar (MCD LAB) for coliforms; Potato Dextrose Agar (MCD LAB) for molds; and WL Nutrient Agar (MCD LAB) for yeasts associated with fermentation processes. Inoculated plates were incubated under the following conditions: aerobic mesophiles at 35 ± 2 °C for 24 h; lactic acid bacteria at 35 ± 2 °C for 5 days; total coliforms at 35 ± 2 °C for 24 h; molds at 25 ± 1 °C for 5 days; and yeasts at 30 °C for 48 h. After incubation, colony counts were performed for each microbial group evaluated, and results were expressed as Log CFU/mL.

The obtained results were analyzed using analysis of variance (ANOVA) for each microbial group evaluated. When statistically significant differences were detected ($p<0.05$), Fisher's exact test was applied. Statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA).

Obtaining Pure Cultures and Phenotypic Identification

Once colony counts were completed, colonies were selected based on their colonial morphology according to the microbial group evaluated, with the aim of obtaining axenic cultures, performing the corresponding tests, and preserving the isolates in storage vials.

Isolated BAL were subjected to Gram staining and catalase testing, while yeasts were stained with cotton blue (López *et al.*, 2014).

From the coliform isolates obtained (60), Gram staining and subculturing on selective media, including MacConkey agar (MCD LAB), Eosin Methylene Blue agar (MCD LAB), and *Salmonella-Shigella* agar (BD Bioxon), were performed. These tests were complemented by the determination of Indole, Methyl Red, Voges-Proskauer, and Citrate (IMViC) reactivity according to the procedures established in NOM-210-SSA1-2014, as well as complementary tests for Motility, Indole, and Ornithine (MIO), Catalase, Triple Sugar Iron (TSI), and Urease (Table 3) (Winn & Koneman, 2008; NOM-210-SSA1-2014).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was evaluated using the disk diffusion method (Kirby & Baüer), following the protocol described by the Clinical and Laboratory Standards Institute (CLSI, 2018). Susceptibility to 12 antimicrobials (Oxoid, UK) was evaluated: Amikacin (KA) 30 μg, Ampicillin (AMP) 10 μg, Carbenicillin (CAR) 100 μg, Cephalothin (KF) 30 μg, Cefotaxime (CTX) 30 μg, Ceftriaxone (CRO) 30 μg, Chloramphenicol (CL)

30 μg, Gentamicin (GM) 10 μg, Netilmicin (NET) 30 μg, Nitrofurantoin (F) 300 μg, Pefloxacin (PEF) 5 μg, and Trimethoprim-sulfamethoxazole (STX) 1.5/23.75 μg. Results were interpreted according to the inhibition zone diameter criteria established by CLSI (2018) for each antibiotic and classified as susceptible (S), intermediate (I), or resistant (R).

RESULTS AND DISCUSSION

Physicochemical Analyses

The results obtained for the physicochemical parameters evaluated are presented in Table 1. The samples showed an acidic profile with values ranging from 3.30 to 4.02. Statistically significant differences were observed among municipalities ($p \leq 0.05$). The highest pH values were obtained in samples from Ocoyoacac, whereas the most acidic values were recorded in Jiquipilco; however, all results were within the ranges reported for traditional pulque.

Regarding total soluble solids (°Bx), statistically significant differences were identified ($p<0.05$). The municipality with the highest values was Ocoyoacac, followed by Temoaya and Jiquipilco.

With respect to alcohol content, the samples presented values ranging from 4.42 to 4.87%, with no statistically significant differences among municipalities.

Microbiological Analyses

Figure 2 shows the counts of microbiological quality indicators obtained, including aerobic mesophilic bacteria, lactic acid bacteria, and yeasts, which were present in 100% of the samples analyzed. In contrast, coliforms were detected in only 20% of the samples corresponding to two municipalities, while fungi did not develop in any of the samples.

Within the aerobic mesophile group (Figure 2a), the municipalities of Jiquipilco and Temoaya presented the highest counts, with no statistically significant differences between them ($p>0.05$), whereas Ocoyoacac showed significantly lower values. Counts of lactic acid bacteria did not show statistically significant differences ($p>0.05$), with similar counts observed among the three municipalities (Figure 2b).

In the case of the coliform group (Figure 2c), significant differences were observed among the municipalities evaluated ($p<0.05$-0.001). No coliform growth was detected in Ocoyoacac, whereas Jiquipilco and Temoaya showed detectable counts, with the latter presenting the highest values.

For yeasts, highly significant statistical differences were identified among the three municipalities ($p<0.001$). Temoaya presented the highest values, followed by Jiquipilco, while Ocoyoacac showed the lowest counts (Figure 2d).

Table 1. Physicochemical Properties of Pulque.

Parameters	Municipalities		
	Ocoyoacac	Jiquipilco	Temoaya
pH	4.02±0.08	3.30±0.15	3.49±0.11
°Bx	6.37±0.23	4.62±1.38	5.43±1.33
Alcohol (%)	4.87±0.38	4.42±0.25	4.76±0.40

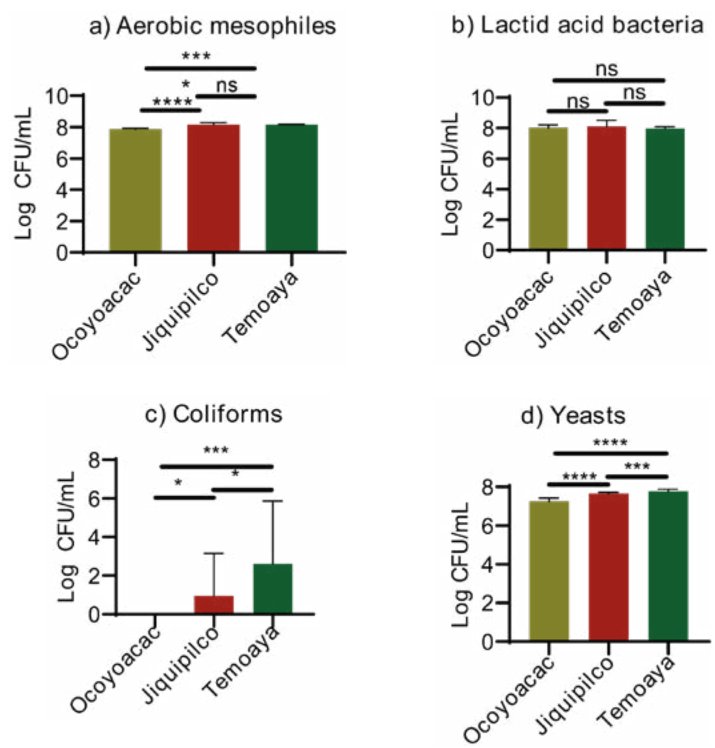

Figure 2. Counts of indicator microorganisms of microbiological quality in pulque. Fisher's exact test was performed: ns=not statistically significant ($p>0.05$), *=$p<0.05$, **=$p<0.001$, and ***=$p<0.0001$.

Phenotypic Identification

The results of the biochemical identification tests performed on strains isolated from pulque are shown in Table 2. The tests carried out allowed the evaluation of metabolic characteristics, which are important for the presumptive identification of enteric bacteria. The obtained profiles were consistent with the reactions described in the literature for *Escherichia coli* and *Shigella flexneri*.

Table 2. Biochemical Identification Tests of Strains Isolated from Pulque.

Bacteria	Reaction										
	M	I	O	H_2O_2	CS	RM	VP	U	TSI	GAS	H_2S
Escherichia coli	+	+	+	+	−	+	−	−	A/A	+	−
Shigella flexneri	−	+ (−)	+ (−)	+	−	+	−	−	K/A	−	−
*A.2	+	−	+	+	+	+	−	−	K/A	−	−
*A.4	+	−	+	+	+	+	−	−	K/A	−	−
*A.15	+	−	+	+	+	+	−	−	K/A	−	−
*M.22	+	+	+	+	−	+	−	−	A/A	+	−
*M.23	+	+	+	+	−	+	−	−	A/A	+	−
*M.25	+	+	+	+	−	+	−	−	A/A	+	−

* Isolates; M: Motility, I: Indole, O: Ornithine, H_2O_2: Catalase, CS: Simmons Citrate, MR: Methyl Red, VP: Voges-Proskauer, U: Urea, TSI: Triple Sugar Iron. K: Alkaline reaction (red color), A: Acid reaction (yellow color), Gas: Gas production, H_2S: Hydrogen sulfide production (black color).

Antimicrobial Susceptibility

The results obtained from the antimicrobial susceptibility tests of strains isolated from pulque, determined by the disk diffusion method, are shown in Table 3. The group of *E. coli* isolates showed 50% resistance to the antibiotics analyzed, 17% intermediate susceptibility, and 33% complete susceptibility. Resistance was mainly observed against ampicillin, carbenicillin, cephalothin, cefotaxime, nitrofurantoin, and trimethoprim-sulfamethoxazole, evidencing a multidrug resistance profile, particularly against β-lactam antibiotics.

In contrast, the strains identified as *Shigella* showed 50% susceptibility, 8% intermediate susceptibility, and 42% resistance. These results indicate greater overall susceptibility, although resistance to ampicillin, carbenicillin, cephalothin, cefotaxime, and nitrofurantoin was also observed.

The physicochemical and microbiological results obtained in the present study highlight the importance of pulque as a complex fermentative system whose physicochemical and microbiological quality is closely related to production conditions, autochthonous microbiota, fermentation conditions, and traditional production practices. This is consistent with other studies emphasizing the importance of the microbiological diversity of this beverage (SADER, 2019; Rodríguez *et al.*, 2021; Astudillo *et al.*, 2023).

The acidic pH observed in the samples complies with the standards established for traditional fermented beverages in NOM-199-SCFI-2017. Likewise, these values are consistent with the ranges reported for pulque by Álvarez *et al.* (2020) and Medina *et al.* (2023), who highlight that acidic pH occurs as a result of the production of organic acids, mainly lactic and acetic acids, which in some cases may inhibit pathogenic microorganisms (Cervantes & Pedroza, 2007; Medina *et al.*, 2023; Astudillo *et al.*, 2023).

Table 3. Antimicrobial Susceptibility of Strains Isolated from Pulque.

Antimicrobial	Inhibition (mm) *E. coli*	Interpretation	Inhibition (mm) *Shigella*	Interpretation
Amikacin	35	S	26	S
Ampicilin	0	R	0	R
Carbenicilin	0	R	0	R
Cephalothin	0	R	0	R
Cefotaxime	0	R	0	R
Ceftriaxone	20	I	30.2	S
Chloramphenicol	17.2	I	19.6	S
Gentamicin	27	S	21.6	S
Netilmicin	31	S	26.4	S
Nitrofurantoin	0	R	0	R
Pefloxacin	27	S	20.5	I
Trimethoprim Sulfamethoxazole	0	R	20.5	S

R: Resistant; I: Intermediate; S: Susceptible.

Regarding total soluble solids (°Bx), variations were observed that could be related to the type of maguey, the characteristics of the semilla or initial inoculum used, and the fermentation time (Enríquez *et al.*, 2017; Medina *et al.*, 2023; Liborio *et al.*, 2024). Likewise, the decrease in °Bx values in samples that presented higher microbial counts could indicate greater sugar consumption and increased fermentative activity by lactic acid bacteria and yeasts (Escalante *et al.*, 2016; Pérez & Cardoso, 2020).

The relationship between the presence of different microbial groups and the physicochemical parameters of pulque has been described as an important characteristic of fermentation-derived products, in which the microorganisms that develop play a fundamental role in the final quality of the product (Escalante *et al.*, 2016; Ruíz *et al.*, 2023).

The results obtained for alcohol content indicate that the samples comply with the parameters established for fresh pulque in NOM-199-SCFI-2017, which establishes an alcoholic content range of 4% to 7.5% Alc. Vol. These values are consistent with other studies, such as those by Gómez *et al.* (2011) and Medina *et al.* (2023), who indicate that these concentrations depend on fermentable sugars and the population dynamics of the yeasts present.

The presence of aerobic mesophiles is associated with the natural fermentative process of pulque, with significant counts linked to the nature of the product, as occurs in other beverages obtained through fermentation such as tuba and tepache (De la Fuente *et al.*, 2015; Amazará & Quintero, 2022; Liborio *et al.*, 2024). It is important to note that the bacterial counts obtained in the present study were higher than those reported by Escalante *et al.* (2008) and Liborio *et al.* (2024), who evaluated the bacterial community of pulque produced in the state of Morelos and in San Agustín Tlaxiaca, Hidalgo, Mexico, respectively.

The predominant presence of lactic acid bacteria is consistent with studies reporting a greater abundance of this microbial group in pulque production compared with other evaluated microbial groups (Lappe *et al.*, 2023; Escalante *et al.*, 2016; Tamang *et al.*, 2016). Likewise, these studies highlight their role in product acidification, the development of sensory characteristics, and their potential probiotic properties (Escalante *et al.*, 2008; Ruíz *et al.*, 2023; Giles *et al.*, 2024).

The yeast counts reported are consistent with Escalante *et al.* (2016) and Colón *et al.* (2025), who indicate that yeasts predominate during the intermediate and final stages of pulque fermentation. These microorganisms participate in the transformation of sugars present in aguamiel, thereby contributing to the production of ethanol, aromatic compounds, and secondary metabolites. In the same context, it has been reported that the metabolic activity of these microorganisms is related to changes in physicochemical parameters during the fermentation process (Chacón *et al.*, 2025).

In the present study, coliform bacteria were detected at low levels, which is consistent with other reports indicating that these bacteria, as they are not native to the fermentative process, may be introduced during the collection, storage, and commercialization of the product (Escalante *et al.*, 2008; Gómez *et al.*, 2011; Álvarez *et al.*, 2020; Medina *et al.*, 2023; Liborio *et al.*, 2024). Likewise, several authors have reported that, as the fermentation process progresses, factors such as changes in acidity, ethanol production, and microbial competition reduce the viability of these microorganisms (Gómez *et al.*, 2011; Jeon *et al.*, 2015).

The microbial communities present in pulque interact throughout the different stages of the production process, including crop development, collection, inoculation during fermentation, and transportation. Together, these microorganisms determine the microbiological quality and sensory characteristics of pulque (Álvarez *et al.*, 2020; Medina *et al.*, 2023).

On the other hand, coliform bacteria are not usually present in pulque, which may be attributed to its low pH and the metabolic activity of the microbial communities responsible for the fermentation process (Gómez *et al.*, 2011; Medina *et al.*, 2023). These factors create an adverse environment for enteropathogenic microorganisms, which may be inhibited by metabolites produced by bacteria and yeasts. Nevertheless, the introduction of undesirable microorganisms may affect the microbiological quality of the product, as they compete with beneficial microorganisms, alter the fermentation profile, and generate compounds with negative effects on the flavor, odor, and texture of the product (Escalante *et al.*, 2008; Jeon *et al.*, 2015). In this context, the detection of coliforms reinforces the need to improve hygiene practices during the production, handling, and commercialization of the product.

Phenotypic Identification

Biochemical tests showed profiles compatible with enterobacteria such as *Escherichia coli* and *Shigella flexneri*. These results are consistent with those reported by Cervantes and Pedroza (2007), who identified the presence of Gram-negative coccobacilli in their study and mentioned that the persistence of these microorganisms is associated with the lack of product standardization and inadequate hygiene practices during production. Similarly, Gómez *et al.* (2011) reported that *E. coli* O157:H7 may develop tolerance to acidic and alcoholic conditions in pulque, representing a potential health risk to consumers. In contrast, some studies suggest that the presence of pathogenic bacteria, particularly in aguamiel, is usually limited. An example is the work conducted by Gómez *et al.* (2011), who evaluated the fermentation process of aguamiel inoculated with five enteropathogenic strains (*Salmonella typhimurium*, *Escherichia coli*, *Shigella flexneri*, *Staphylococcus aureus*, and *Enterobacter aerogenes*) and observed that, under *in vitro* conditions, the development of these bacteria was inhibited. This inhibition has been attributed to the action of antimicrobial metabolites produced by lactic acid bacteria, which contribute to microbiological stability and reduce the risk of diseases associated with the consumption of this traditional beverage (Silva *et al.*, 2025). Conversely, Jeon *et al.* (2015) examined different fermented beverages and identified the survival of coliform bacteria. This highlights the importance of monitoring microbiological quality indicators together with traditional quality parameters.

Antimicrobial Susceptibility

The antimicrobial susceptibility profiles of strains isolated from pulque showed multidrug resistance in *E. coli* isolates and a lower proportion of resistance in *Shigella*.

This behavior is consistent with that reported by Oliveira *et al.* (2017), who, in their study on resistance to different antibiotics used to treat *E. coli* infections, highlighted the low effectiveness of ampicillin, amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole, nalidixic acid, and ciprofloxacin, with resistance rates higher than 50%. Sosa and Chapoñan (2022) also reported an evolutionary trend of resistance in *E. coli* to several antibiotics, including ampicillin, amoxicillin, cephalosporins, aminoglycosides (kanamycin), quinolones (ciprofloxacin), and sulfonamides (trimethoprim-sulfamethoxazole). Similarly, Duarte *et al.* (2021) identified comparable resistance patterns in *Shigella* spp., including resistance to trimethoprim-sulfamethoxazole, ciprofloxacin, and ampicillin.

The antimicrobial susceptibility patterns observed are of public health importance, since fermented foods may contain bacteria harboring resistance genes (Vinayamohan *et al.*, 2023). Likewise, several studies have indicated that antimicrobial-resistant bacteria may be transmitted from animals or plants to humans through agricultural production systems (Krahulcová *et al.*, 2022; Vinayamohan *et al.*, 2023; FAO, 2025).

The microbiological quality of pulque is determined by multiple factors, and currently there are no specific regulations in force regarding the safety of this beverage. This limits the establishment of standardized quality and safety parameters. Therefore, it is crucial to continue conducting studies of this type and to deepen microbiological analyses of pulque, evaluate production processes, and provide training to producers in order to generate information that may serve as a basis for establishing specific criteria.

CONCLUSIONS

The present study evaluated the physicochemical characteristics and microbiological quality of pulque produced in three municipalities of the State of Mexico: Temoaya, Jiquipilco, and Ocoyoacac. The results demonstrated that pulque complies with the physicochemical parameters established by Mexican regulations, as well as with the predominant presence of microorganisms characteristic of the fermentation process, such as lactic acid bacteria and yeasts. Nevertheless, the detection of coliforms with antibiotic resistance in some samples reveals potential deficiencies during collection, production, and commercialization processes. Therefore, it is necessary to implement good manufacturing and hygiene practices, as well as training programs directed toward producers and vendors. Further research addressing the microbiological diversity of pulque from the State of Mexico is recommended in order to strengthen its artisanal, cultural, and safety value as a traditional beverage.

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