DOI: https://doi.org/10.20870/oeno-one.2024.58.4.8104

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ORIGINAL RESEARCH ARTICLE

# Characterisation of the metagenome of Peruvian Pisco grapes from Ica and Arequipa for the development of their regional identity

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*Associate editor:*
Antonio Morata

*Received:*
27 April 2024

*Accepted:*
5 September 2024

*Published:*
28 October 2024

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## ABSTRACT

Pisco grapes and their associated microbiome are the fundamental inputs for the production of Pisco; thus, knowledge of the varietal and regional differences between microbiota is of high value due to contribute to the distinctiveness and quality of the Pisco. To this end, the metagenomics can be applied for an in-depth exploration of the berry microbiome. The aim of this work was to characterise the metagenome of Peruvian Pisco grapes from Ica and Arequipa, thereby contributing to their regional identities. Sixteen composite samples of berries from the protected designation of origin regions of Ica and Arequipa were analysed for both bacteria and fungi *via* massive sequencing. *Ralstonia, Gluconobacter, Pantoea, Tatumella, Bradyrhizobium, Komagataeibacter* and some unclassified species from the Orbaceae family were identified as the predominant genera of bacteria, and *Cladosporium, Starmerella, Pichia, Hanseniaspora, Wallemia, Claviceps, Alternaria* and some unclassified species from the Saccharomycetales order or other orders within the Ascomycota division as the predominant fungi as well as its richness and diversity. As has been shown elsewhere in the literature regarding wine, we have proven here that the terroir of Pisco depends on the composition of the microbiome and is affected by edaphoclimatic factors and cultivation practices. These exploratory results will contribute to a better understanding of the terroir of Pisco, thus providing fundamental knowledge for its regional identity.

**KEYWORDS:** metagenome, Pisco grapes, terroir, regional identity, geographic areas, richness, biodiversity

## INTRODUCTION

Grape cultivation is one of the most important fruit growing activities in Peru due to its extent, production value and crucial contribution to the national wine industry. Pisco grapes are highly appreciated since they constitute the main raw material for the production of the emblematic Peruvian beverage, Pisco.

Several studies have shown the composition of the microbial communities of fermenting grapes or musts to vary depending on the region of grape vine cultivation (Bokulich *et al*., 2014; Compant *et al*., 2019; Gao *et al*., 2019; Belda *et al*., 2017; Nanetti *et al*., 2023). The reasons for these geographical differences are yet to be fully understood; however, some studies have shown that microbial populations found in musts may originate from the native environment surrounding the vineyard (Morrison-Whittle & Goddard, 2018; García-Izquierdo *et al*., 2024), and others have found geographic differences between populations to be more evident for fungi than for bacteria (Miura *et al*., 2017; Cachón *et al*., 2019; Kioroglou *et al*., 2019; Gobbi *et al*., 2022). In this context, characterising the microbiota of the different Pisco varieties according to their regions of origin will contribute to defining their identities by revealing characteristics unique to each region.

The limitations that were identified when developing conventional microbiology and the need for rapid, precise and exhaustive descriptions of microbial populations led to the development of culture-independent techniques (Fabres *et al*., 2017; Morrison-Whittle *et al.*, 2017). The use of massive or next-generation sequencing (NGS) technology has proven suitable for understanding the microbial communities of a complex sample without the need for prior culture, as well as fast, low in cost and accessible, with abundant and freely available data banks (Knief, 2014; Wang *et al*., 2015; Barbero, 2017; Morgan *et al*., 2017; Sanz Heras, 2018; Berbegal *et al*., 2019; Fernandes *et al*., 2023). Studies at this level contribute to knowledge regarding different aspects of the grape microbiome; for example, the characterisation of the metagenome in botrytised wine in order to explore the bacterial communities of wine fermentation to better understand its biodiversity (Bokulich *et al*., 2012; Englezos *et al*., 2022). Other studies have focused on the characterisation of grapevine microbiomes in relation to biotic and abiotic factors (Oyuela, 2019; Rivas *et al*., 2021), as well as vineyard microbiome monitoring trials, using metagenomic data from vineyard soils and data on diseases to control microorganisms (Ghiță *et al*., 2022).

There are a large number of studies that have used metagenomics for the analysis of microbial communities with a focus on wine grapes; for example, these studies aimed to characterise the microorganisms associated with vine diseases (Bubeck *et al*., 2020), soil microorganisms that influence the microbiota of wine (Zarraonaindia *et al*., 2015; Yang *et al*., 2020; Zhou *et al*., 2020), epiphytic microbiome in grape berries (Hall *et al*., 2019; Ranade *et al*., 2023), endophytic microbiome in wine grapes (Pacífico *et al*., 2019), endophytic bacteria from wine grapes and their diversity (Aleynova *et al*., 2023), fungal diversity in grape must (Mandakovic *et al*., 2020; Martiniuk *et al*., 2023), microbial diversity during wine fermentation (Pinto *et al*., 2015; Shimizu *et al.*, 2023), and the influence of geographical

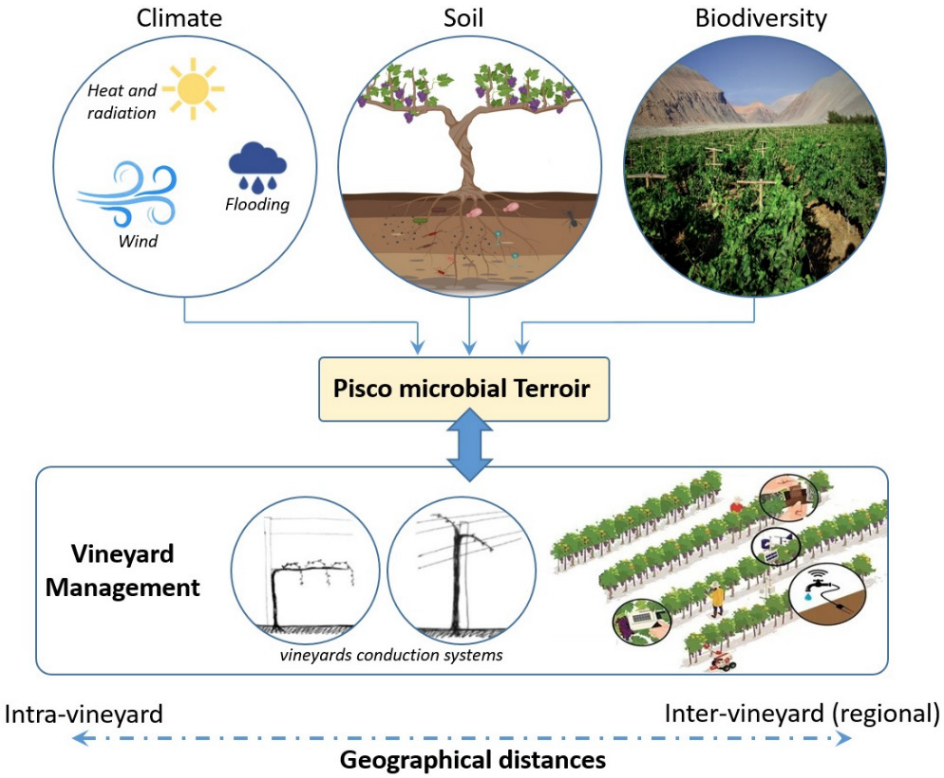



**FIGURE 1.** Interaction of the factors that contribute to the microbial terroir of Pisco in each vineyard [adapted from García-Izquierdo *et al.* (2024)].

patterns and agronomic management on bacterial diversity of grape (Vitulo *et al*., 2019; Blanco *et al*., 2024), among others. However, knowledge on Pisco grapes at the metagenomic level is still limited, therefore, NGS was the method of choice for the simultaneous detection of multiple microorganisms present in Pisco berries.

All the growing conditions associated with a given geographical location constitute the terroir, which plays a key role in the identity and valorisation of a product (Tonietto and Carbonneau, 2004; Cadot et al., 2012; Johnston-Monje *et al*., 2023). Terroir is currently understood as being the result of complex interactions between climate, soil, topography, biodiversity, landscapes, biotic interactions and agricultural management (Belda *et al*., 2020; Leal *et al*., 2024; Onetto *et al*., 2024), which defines microbial diversity. Geographical distance, whether between Pisco grape varieties (intra-vineyard) belonging to the same vineyard or between vineyards (inter-vineyard) in different Protected Designation of Origin (PDO) regions, is particularly important in the case of Pisco, because together with other factors such as climate, soil and biodiversity they describe the microbial patterns of a region (terroir) [Figure 1].

This work focuses on the characterisation of the metagenome of Pisco grapes from two important regions, Ica and Arequipa, in order to help develop their regional identities. To this end, the diversity and abundance of bacteria and fungi in eight varieties of Pisco grapes from both regions were determined (Figure 2).

## MATERIALS AND METHODS

### 1. Details on vineyards

The vineyards from which the Pisco grape berries were sampled correspond to two regions of Peru with protected designation of origin for Pisco: Arequipa and Ica. In the Arequipa region, samples were taken from the Majes Tradición Vineyard (I): 16° 18’ 00’ ‘S, 72° 29’ 42’ ‘W, elevation: 618 masl; in the case of Ica, samples were obtained from the CITEagroindustrial Ica (II): 13° 59’ 51’ ‘S, 75° 46’ 03’ ‘W, elevation: 407 masl, and Vineyard Álvarez (III): 14° 01’ 17’’ S, 72° 44’ 44’ ‘W, elevation: 407 masl (Figure 2).

Pisco grapes of the eight existing varieties (Albilla, Italia, Mollar, Moscatel, Negra Criolla, Quebranta, Torontel and Uvina) were obtained from both regions Arequipa and Ica (Figure 3).

### 2. Sample collection

Sampling was carried out during the pre-harvest stage in February 2020 and at a minimum distance of 7 m from the edges of each plot on 9 plants in a 49 m$^2$ delimited quadrant. Samples were grouped in composites in order to have replicates by site (Oyuela, 2019) [Figure 4]. Berry samples were collected aseptically using flamed scissors and gloves. All samples were preserved in sterile bags, labelled with their respective nomenclature, and transported in a cold chain to the laboratory, keeping them –20 ºC until DNA extraction was carried out.

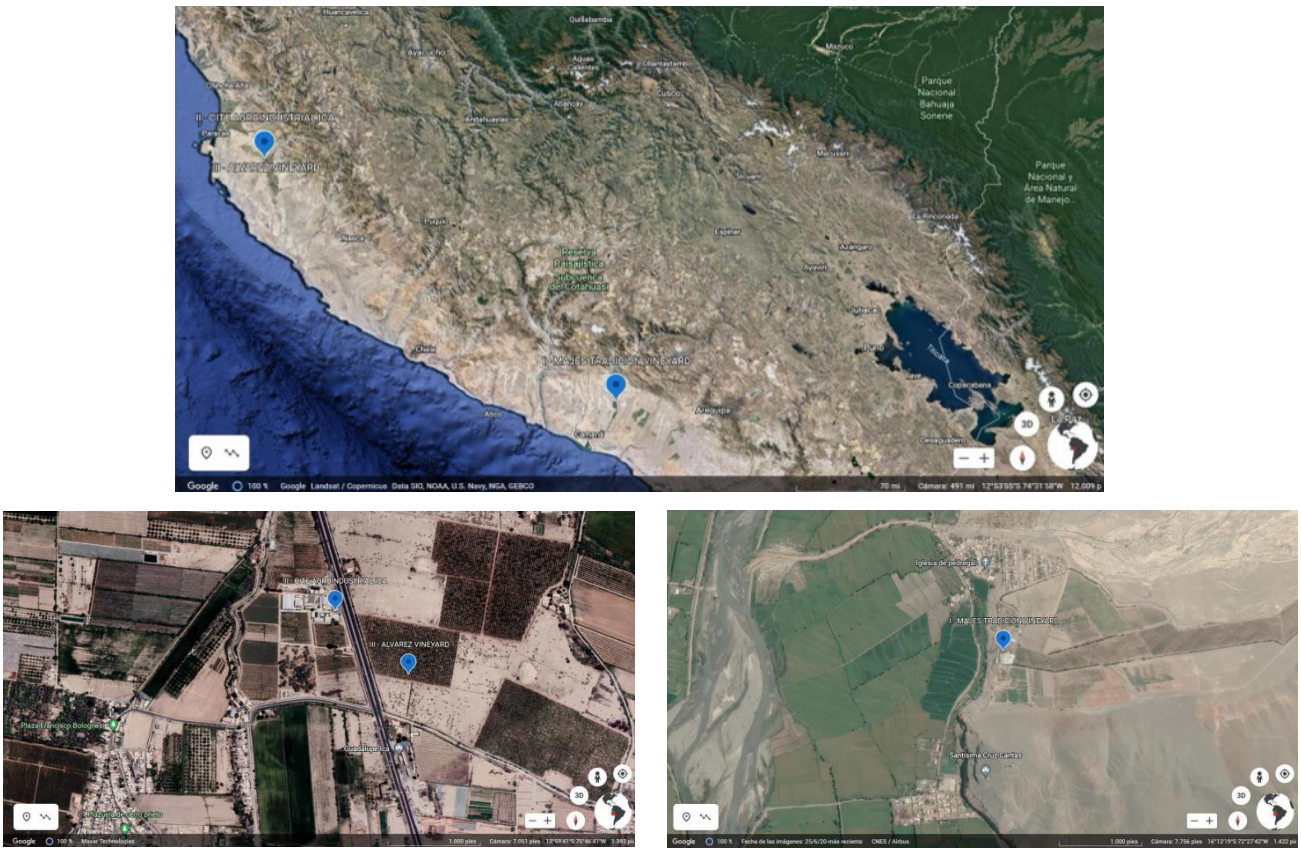



**FIGURE 2.** Sampling locations: A) Topographic view of the sampling area; B) CITEagroindustrial Ica and Vineyard Álvarez, Ica; and C) Majes Tradición Vineyard, Arequipa.

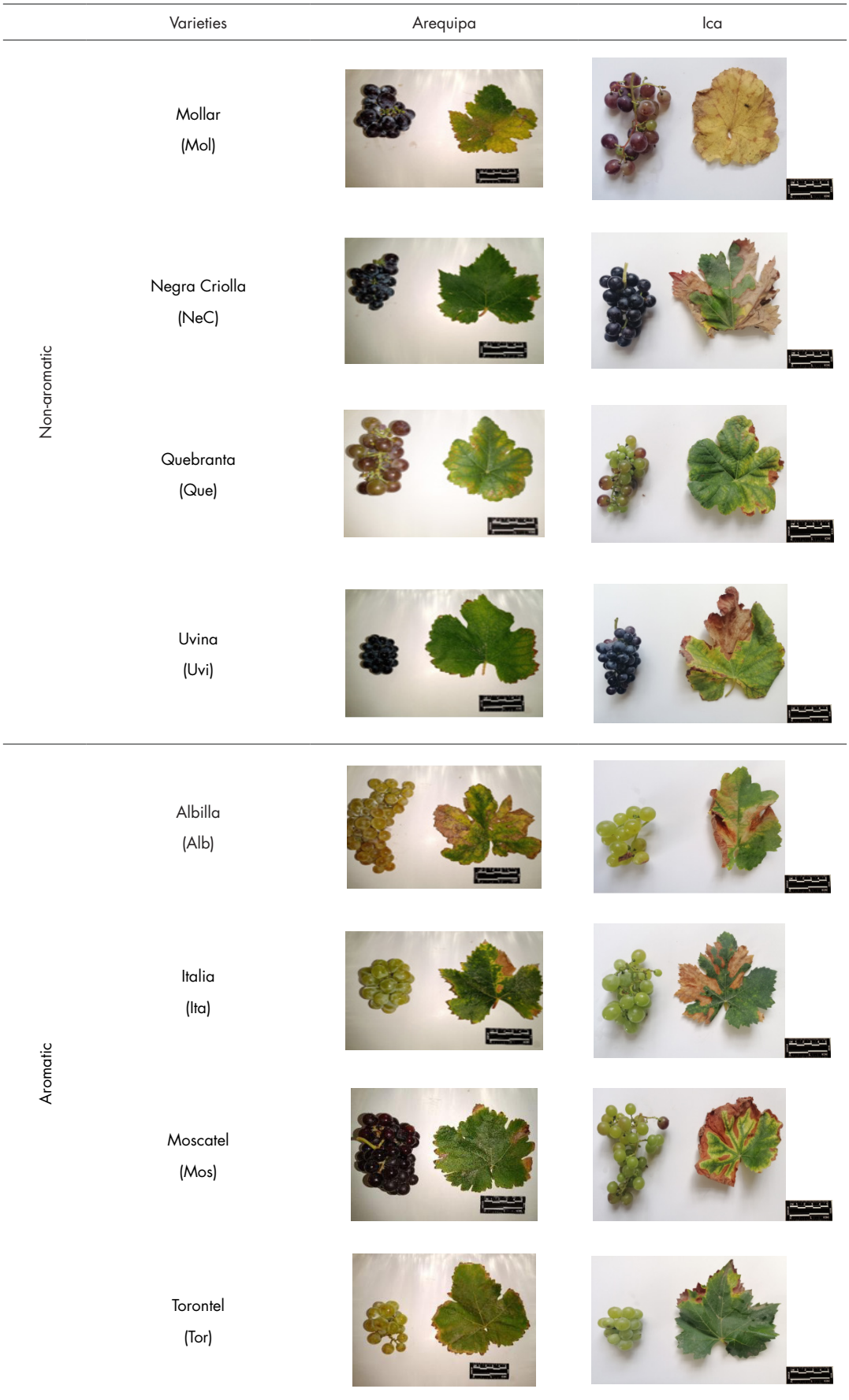



**FIGURE 3.** Berries and leaves of the eight varieties of Pisco grapes from Arequipa and Ica used in the present study. Non-aromatic: Mollar, Negra Criolla, Quebranta and Uvina. Aromatic: Albilla, Italia, Moscatel and Torontel.

## 3. DNA isolation

Berries were washed with distilled water, and then homogenised using a frozen mortar and pestle. Samples were transferred to 2 mL microvials for centrifugation at 4000 x g. The supernatant from individual samples was collected in 50 mL conical tubes then cold centrifuged. The resulting solutions were mixed for a final centrifugation and subsequent storage at –20 °C until DNA extraction, according to Oyuela (2019).

DNA was extracted using the CTAB protocol (Li *et al*., 2013). The extracted DNA was qualified by means of 1.2 % agarose gel electrophoresis, run in TAE buffer and stained with GelRed, taking note of the degree of degradation of each sample. A dilution test was also performed to detect DNA supersaturation. Quantification was performed using a Qubit® 2.0 155 fluorometer (Thermo Fisher Scientific™) and NanoDrop spectrophotometer. To assess purity, the spectrophotometric absorbance ratios 260/280 nm and 260/230 nm were measured.

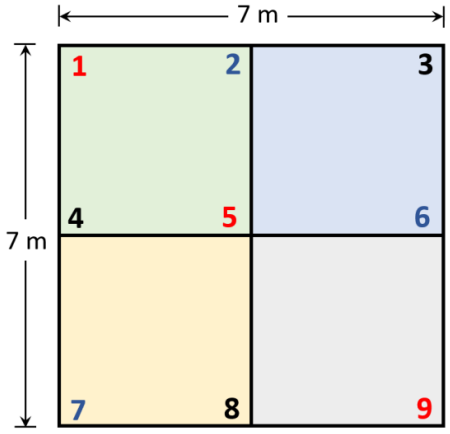



**FIGURE 4.** Design of berry sampling and grouping in composites (Adapted from Oyuela, 2019). Each point is a part of a composite sample that is homogenised and mixed to generate three biological replicates. Biological replicate A (red): generated by mixing points 1, 5 and 9; Biological replicate B (blue): generated by mixing points 2, 6 and 7; Biological replicate C (black): generated by mixing points 3, 4 and 8.

## 4. Sequencing of amplicons of bacteria (16S rRNA) and fungi (ITS region)

Massive sequencing was performed applying NGS of the V3-V4 variable region of the 16S rRNA gene as a marker of the prokaryotic community, and ITS1 (Internal Transcribed Spacer), located between the ribosomal genes 18S rRNA and 5.8S rRNA, as a marker of the fungal community (Oyuela, 2019; Aleynova *et al*., 2023). Sixteen samples were processed (8 varieties of Pisco grapes from two geographic regions). The sequencing was developed in the MiSeq system by Gen Lab del Perú S.A.C.

## 5. Bioinformatic processing

Readings were demultiplexed with the bcl2fastq v.2.17.1.14 software (Illumina). Data processing and preliminary analyses were carried out *via* the MiSeq System using the MiSeq Reports (MSR) software (Illumina). The classification of organisms is based on the Greengenes database (https://greengenes.lbl.gov/). In the preliminary data, a cleaning and decontamination of the general data was carried out for both 16S and ITS in order to select the results of interest for the investigation.

## 6. Statistical Analyses

The alpha and beta diversity metrics were calculated using the R package phyloseq (McMurdie and Holmes, 2013). For both bacterial and fungal samples, three Alpha diversity metrics [Chao 1, Shannon and Simpson] (Tong-Lu *et al*, 2023) were evaluated.

To examine the multivariate relationships between the communities of fungi and bacteria associated with berries, the method of Multidimensional Non-Metric Scaling (NMDS) was used, choosing the Bray-Curtis distance (Bakker, 2024) to evaluate similarities and differences between microbial communities, according to the aromatic and non-aromatic Pisco grape varieties. The statistical analyses were performed using the free R software (R Core Team, 2022).

# RESULTS

## 1. Microbial diversity in Pisco grape berries

The microbial diversity of 16 samples of Pisco grape berries from the two study areas (regions) Arequipa and Ica was analysed.

Relative abundance analyses (> 1) of bacterial and fungal genera identified in each Pisco variety were performed, grouped by study area (Figure 1). The similarities and differences with respect to abundance per variety were clearly visible, with the identification of genera of bacteria, such as *Ralstonia, Gluconobacter, Pantoea, Tatumella, Bradyrhizobium* and *Komagataeibacter,* and some unclassified species from the Orbaceae family (Table 1 and Figure 5*)*.

Regarding the relative abundance of the fungal microbiota at the genus level, *Cladosporium, Starmerella, Pichia, Hanseniaspora, Wallemia, Claviceps* and *Alternaria,* as well as some unclassified species from the Saccharomycetales order and others within the Ascomycota division, were identified as those that stood out in terms of relative abundance (Table 3 and Figure 6). Among the genera, many others were reported as having percentages of less than 1 %.

## 2. Diversity and composition of the microbial communities of Pisco grape berries

To determine the relationship of the similarities and differences among microbial communities with the Pisco grape varieties, a Non-Metric Multidimensional Scaling (NMDS) analysis was performed (Figure 7) using the Bray-Curtis distance matrix. A relationship was there by observed between the structure of the community and aromatic/non-aromatic classification of the grape varieties: communities (graph points) of the aromatic ones are close to each other, regardless of whether they are from Arequipa or Ica.

**TABLE 1.** Percentages of predominant genera of bacteria in Pisco grape samples by study area.

| Pisco grapes | Arequipa | Percentage (%) | Ica | Percentage (%) |
|---|---|---|---|---|
| Alb | *Tatumella* | 40 | Orbaceae_unclassified | 37 |
| | *Gluconobacter* | 18 | *Gluconobacter* | 37 |
| Ita | *Gluconobacter* | 78 | *Gluconobacter* | 68 |
| | Orbaceae_unclassified | 6 | Orbaceae_unclassified | 17 |
| Mol | *Ralstonia* | 65 | *Gluconobacter* | 62 |
| | *Gluconobacter* | 6 | Orbaceae_unclassified | 26 |
| Mos | *Gluconobacter* | 60 | *Ralstonia* | 46 |
| | *Tatumella* | 27 | *Bradyrhizobium* | 13 |
| NeC | *Gluconobacter* | 43 | *Ralstonia* | 38 |
| | *Tatumella* | 22 | *Bradyrhizobium* | 15 |
| Que | *Ralstonia* | 44 | *Ralstonia* | 44 |
| | *Pantoea* | 30 | *Bradyrhizobium* | 12 |
| Tor | *Gluconobacter* | 71 | *Ralstonia* | 45 |
| | *Ralstonia* | 13 | *Bradyrhizobium* | 17 |
| Uvi | *Gluconobacter* | 27 | *Ralstonia* | 41 |
| | *Komagataeibacter* | 20 | *Bradyrhizobium* | 9 |

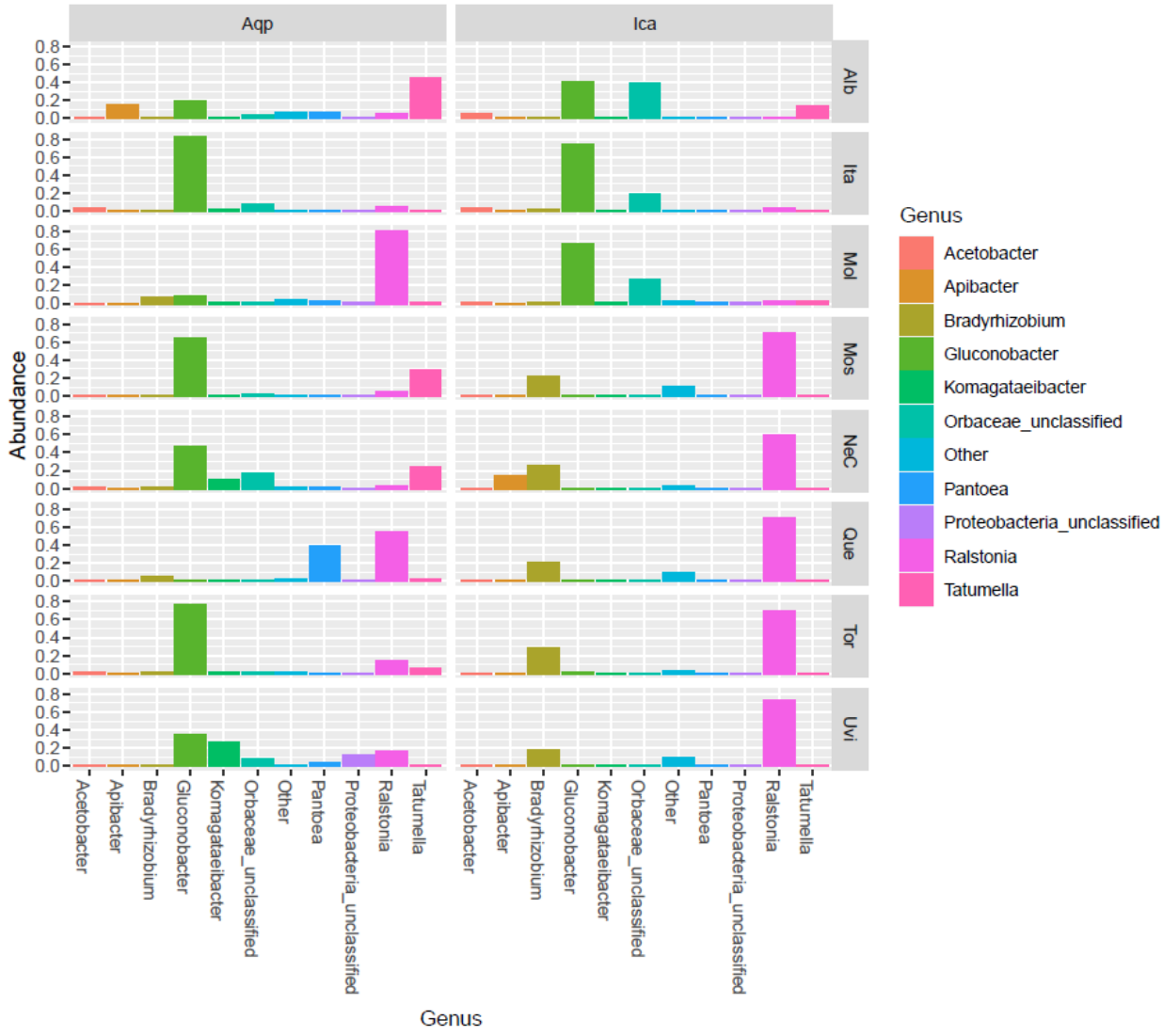



**FIGURE 5.** Distribution of the bacterial microbiota of berries at the genus level in the regions of Arequipa and Ica.

**TABLE 2.** Percentages of predominant genera of fungi in Pisco grape samples by study area.

| Pisco grapes | Arequipa | Percentage (%) | Ica | Percentage (%) |
|---|---|---|---|---|
| Alb | *Starmerella* | 24 | *Starmerella* | 50 |
| | Ascomycota_unclassified | 23 | *Hanseniaspora* | 18 |
| Ita | *Starmerella* | 60 | *Pichia* | 50 |
| | *Hanseniaspora* | 15 | *Hanseniaspora* | 24 |
| Mol | *Cladosporium* | 82 | *Cladosporium* | 68 |
| | *Alternaria* | 4 | *Hanseniaspora* | 9 |
| Mos | *Cladosporium* | 44 | *Cladosporium* | 83 |
| | Ascomycota_unclassified | 24 | *Wallemia* | 10 |
| NeC | *Starmerella* | 48 | *Cladosporium* | 88 |
| | *Hanseniaspora* | 22 | *Claviceps* | 4 |
| Que | *Cladosporium* | 92 | *Cladosporium* | 68 |
| | *Alternaria* | 1.3 | *Wallemia* | 19 |
| Tor | *Cladosporium* | 35 | *Cladosporium* | 75 |
| | *Hanseniaspora* | 24 | Saccharomycetales_ unclassified | 11 |
| Uvi | *Starmerella* | 42 | *Cladosporium* | 85 |
| | *Cladosporium* | 35 | *Alternaria* | 2 |

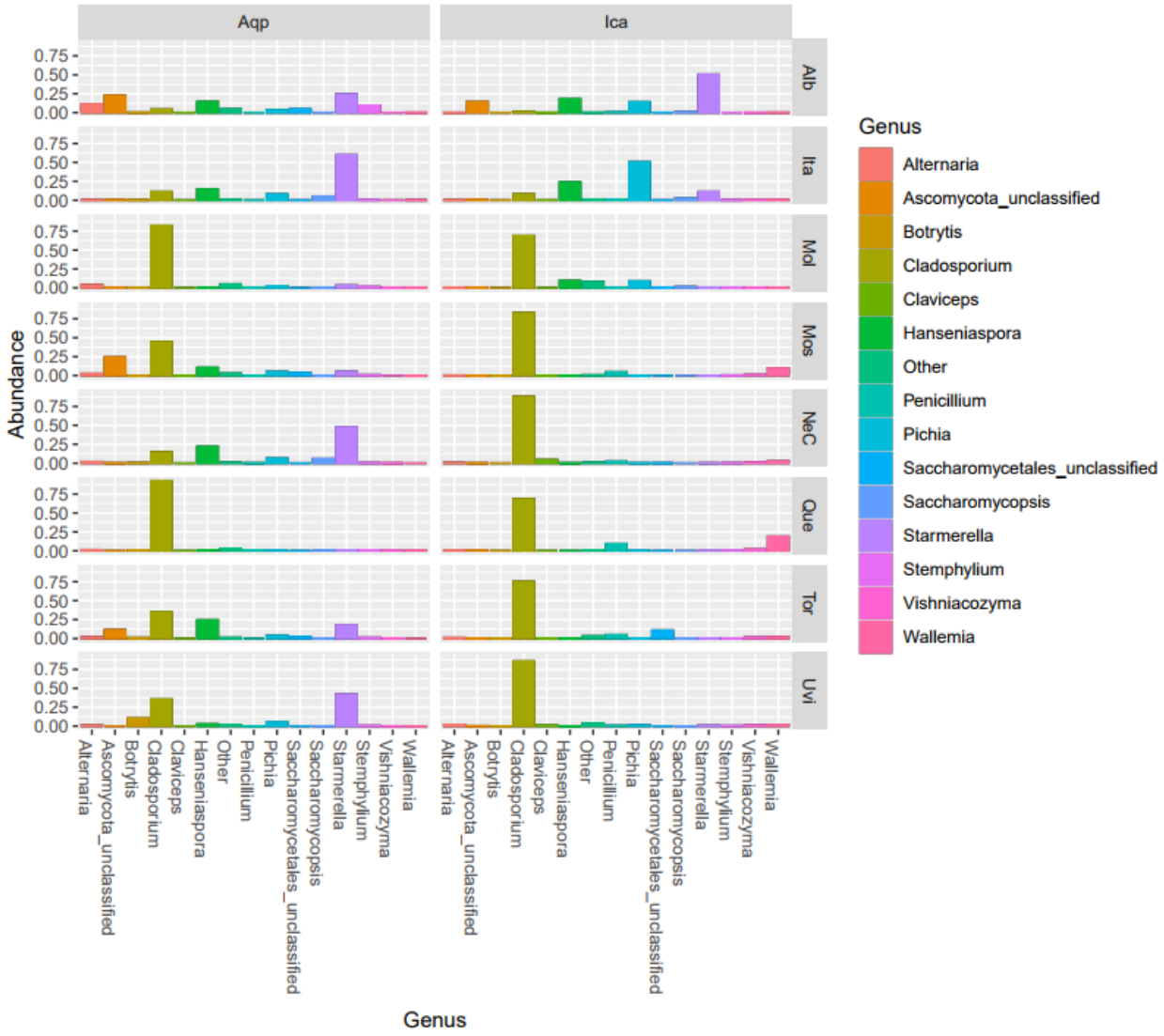



**FIGURE 6.** Distribution of the fungal microbiota of berries at the genus level in the regions of Arequipa and Ica.

The pattern is repeated in both bacteria (Figure 7A) and fungi (Figure 7B). Likewise, we observed that the communities of the non-aromatic Pisco grapes are more dispersed.

## 3. Richness and diversity of the microbiota of Pisco grape berries

To estimate the expected species richness of the tested samples, the Alpha Chao 1 index was calculated. In terms of bacteria (Figure 8), an important finding was that there were no significant differences between the Italia, Negra Criolla, Quebranta and Uvina varieties; *i.e.*, they contained similar microorganisms, which was not the case of the other four varieties. In terms of fungi (Figure 9), the Pisco grapes that did not show any significant differences were Albilla, Moscatel, Quebranta and Torontel, unlike the Italia, Mollar, Negra Criolla and Uvina varieties, which showed more heterogeneity.

Another index that measures diversity, richness and similarity is the Shannon index. For 16S bacteria (Figure 8), the index varied between 1.32 and 4. Results highlight that Pisco varieties from the Ica region are less diverse, whether aromatic or non-aromatic, and only some taxa are dominant. By contrast, in the Arequipa region, the distribution is more uniform and diverse. The Shannon index analysis of fungi – ITS (Figure 9) in grape berry samples varied between 0.70 and 2.02. Here, unlike the analysis in bacteria (from both Arequipa and Ica), the species richness and abundance had similar dispersions.

In addition, we applied the Simpson alpha diversity index. According to this index, the species diversity within the bacterial communities of the Pisco grape varieties in the Arequipa region were Torontel, Italia and Moscatel, while in the Ica region, the most representative varieties were Italia, Mollar and Albilla.

Likewise, at the fungal community level in the Arequipa region, the varieties with the greatest diversity according to the Simpson index were represented by Italia, Quebranta and Mollar, while in the Ica region, they were Negra Criolla, Uvina and Albilla.

## 4. Edaphoclimatic, geographical and cultivation practices that influence the identity of Pisco

We analysed the edaphoclimatic factors, biodiversity elements, cultivation practices and surrounding crops, among others (Table 3) that may play a role in defining the composition and diversity of the microbiome of Pisco grapes and the unique characteristics of Pisco from each region.

The microbiota identified in each region was not found to be dominated by any particular genus. However, there were interesting findings, such as the presence of *Chryseobacterium* (in the Arequipa vineyard), a bacteria typical of volcanic ash and *Wallemia* (in the coastal areas), a fungus with an affinity for saline soils (Table S1 and Table S2).

Regarding cultivation practices, fungicides are applied on white varieties, such as Albilla, Italia and Torontel, in the Majes Tradición Vineyard (Arequipa) but not in CITEagroindustrial Ica or the Álvarez Vineyard in Ica. In terms of surrounding vegetation, the vineyard in Arequipa is very close to slopes with wild vegetation characterised by desert plants, some of which are used as living fences; meanwhile, the samples

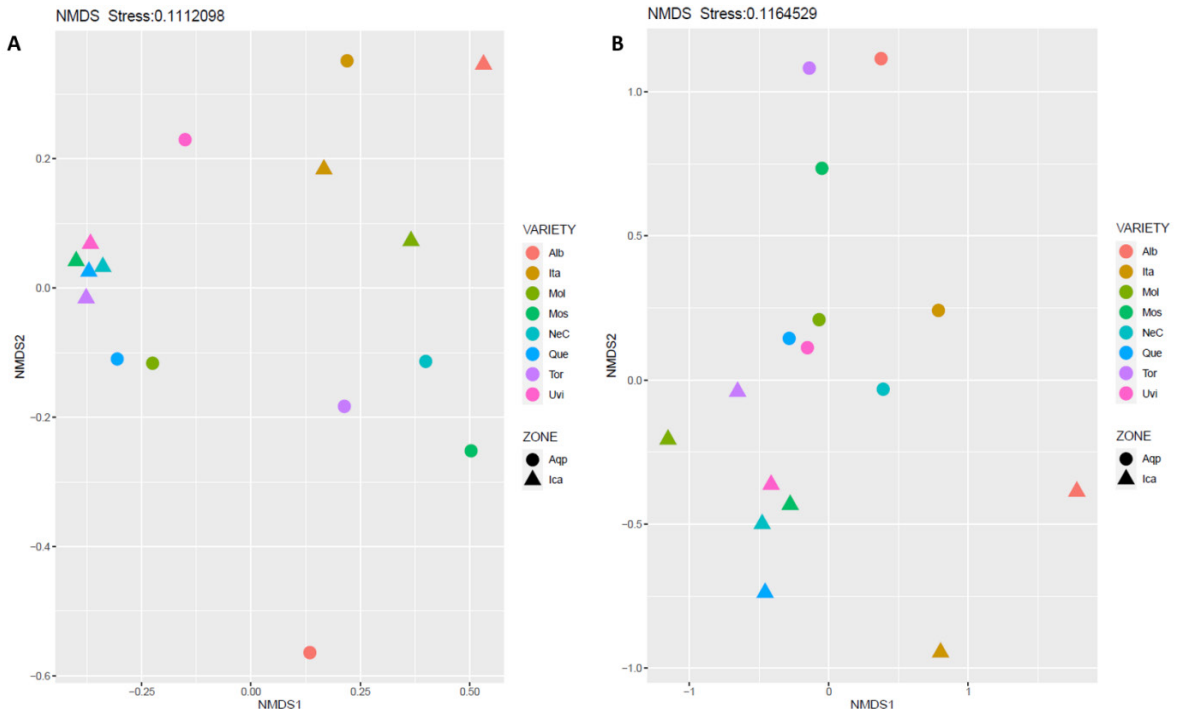



**FIGURE 7.** Non-metric multidimensional scale ordering (NMDS): A) Bacterial microbiota of berries at genera level; and B) fungal microbiota of berries at genera level in the Arequipa and Ica regions (aromatic and non-aromatic Pisco grapes).

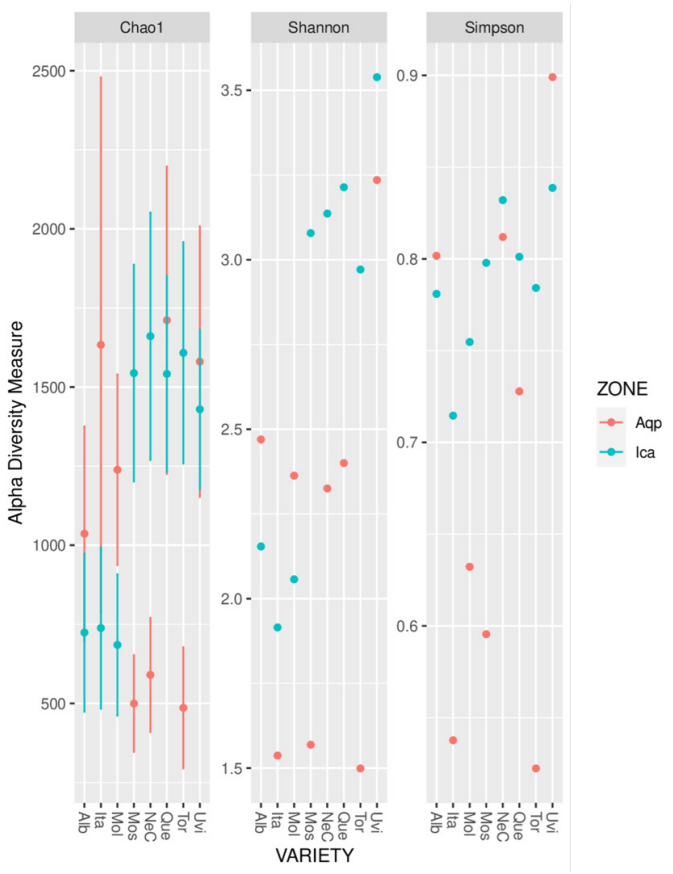



**FIGURE 8.** Richness and alpha diversity measurements of bacteria based on 16S sequences.

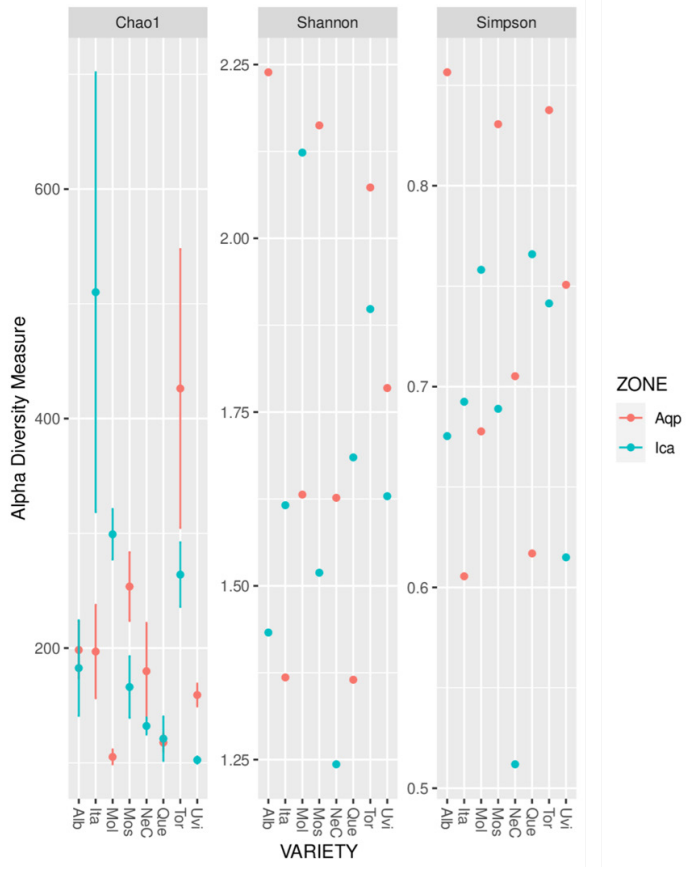



**FIGURE 9.** Richness and alpha diversity measurements of fungi based on ITS sequences.

**TABLE 3.** Edaphoclimatic and biodiversity elements, and cultivation practices characteristic of the study areas (vineyards).

| Variable | Majes Tradición Vineyard–Arequipa | CITEagroindustrial Ica / Vineyard Álvarez–Ica |
|---|---|---|
| | Climate | |
| Temperature | 29 °C | 26 °C |
| Rainfall | 0 mm | 0 mm |
| Relative humidity | 77 % | 61 % |
| Elevation | 618 masl | 407 masl |
| | Soil | |
| pH | 7.2 | 7.1 |
| | Biodiversity | |
| Surrounding vegetation | Sparse vegetation due to the desert climate. Predominantly, families of *Portulacaceae* and *Chenopodiaceae,* grasses, aromo (as a living fence), among others. | *Neltuma pallida* "huarango" plantations, table grapes, mango trees and grasses. |
| Arthropods and birds | Mainly spiders, crickets and passerines (pigeons). | Mainly spiders, bees and *Mimus longicaudatus* "chaucatos". |
| | Vineyard management (cultivation practices) | |
| Phytosanitary applications | Micronised, "flowable sulphur" applications (1 or 2 applications per year). Application (as needed) of fungicides (azoles) only on white varieties. Foliar nitrogen, phosphorus, potassium fertilisers and microelements. | Sanitary application of sulfur to bunches and leaves. Foliar nitrogen, phosphorus and potassium fertilisers and microelements. |
| Conduction system | Parronal in triple T, formation pruning, production pruning. | Spanish pattern, galera iqueña, formation pruning, production pruning. |
| Irrigation system | Optimised drip irrigation. | Drip irrigation, gravity irrigation |

from Ica are not surrounded by native vegetation, but by fruit trees and other crops. Furthermore, in both areas there are particular elements of biodiversity, like arthropods and birds, which also contribute to the microbial terroir.

Thus, all this detailed information regarding edaphoclimatic conditions, surrounding biodiversity and vineyard management allowed us to determine how these factors influence the microbial terroir of Pisco.

## DISCUSSION

In recent years, many studies have shown the importance of better understanding the microbiota associated with grape berries for application in the wine industry, especially in terms of its contribution to the wine terroir (Zhang *et al*., 2020). Unlike most studies that have worked on grapes for winemaking, the present study analysed the microbiota on Pisco grape berries, the main input in the production of Pisco.

This study has some limitations. Sample-collecting was carried out once a year (due to the natural harvest cycle). A sensory analysis was not carried out because once the berries are harvested they are subject to destemming, pressing, fermentation, distillation, resting and bottling, and thus at each stage the equipment, material, surfaces and the distillate itself will contribute to any differences in microbiota composition (Hall *et al*., 2019; García-Izquierdo, 2024). Additionally, samples in the final stage were not taken for organoleptic evaluations due to the SARS-CoV-2 pandemic and associated difficulties, such as access to warehouses being partially restricted and changes to the usual time frame of the winemaking process itself, given the state of emergency at that time. If sensory analyses had been carried out by Pisco varieties, mainly at the distilled Pisco stage, they would have provided greater support for the conclusions of this work, as they would have revealed the influence of regional characteristics on the organoleptic properties of Pisco, thus complementing the determination of the Pisco terroir.

Nevertheless, this work represents a first step in describing the microbiota present in the eight Pisco varieties from the Arequipa and Ica regions, serving as preliminary findings that can be complemented with a sensory analysis to determine the aromatic profile of Pisco.

In the light of the fact that spontaneous (*i.e*., not induced) fermentation is essential in the production of Pisco, specifically that involving non-*Saccharomyces* yeasts, this study reveals

key information on the microorganisms that contribute to the uniqueness of Pisco in the regions examined.

## 1. The distribution of microbial communities provides information on the microbiome of Pisco grapes varieties

The characterisation of microbial diversity using a metagenomic approach has highlighted the importance of microorganisms and their interactions (Fadiji and Babalola, 2020; Tronchoni *et al*., 2022), and hence their relevance to the wine industry to know the microbiome population in soil, grape, grape leaves, grape juice and wine (Wei *et al*., 2018). In this study, the microorganisms that colonise Pisco grape berries were identified, the most predominant bacterial genera being *Ralstonia, Gluconobacter, Pantoea, Tatumella, Bradyrhizobium, Komagataeibacter* and some unclassified species from the Orbaceae family These findings coincide with those of studies by Marsano *et al*. (2016), who characterised the microbiota associated with the winemaking process from the three wine varieties Cabernet, Negramaro and Primitivo: they found *Giliamella*, *Gluconobacter* and *Komagataeibacter* to be the dominant genera of bacteria present in the samples at the beginning of wine fermentation. Berbegal *et al*. (2019), identified, *via* NGS analysis, the genus *Gluconobacter* to be one of the main acetic acid bacteria in malolactic fermentations in wine, and in their research on the endophytic microbiome of *Vitis vinifera*, Campisano *et al*. (2014) detected the presence of the genera *Ralstonia, Burkholderia*, and *Pseudomonas* in all their samples of grapevine branches. In the present study, we also identified *Ralstonia* to be one of the dominant genera.

It is worth highlighting that in this study at the bacterial level the Orbaceae family was identified as dominant under the denomination 'unclassified', indicating that there is not enough preliminary information from other studies carried out on berries. However, this family has been reported in fermented samples of grapes in a study by Shi *et al*. (2022), who found it highly relevant, because it showed a positive correlation with the aromas of these distillates.

Meanwhile, the most abundant fungal microorganisms at the genus level identified in the present study were *Cladosporium, Starmerella, Pichia, Hanseniaspora, Wallemia, Claviceps* and *Alternaria,* which are similar to those identified in a study carried out by Hall *et al*. (2019); in research on the epiphytic microbiota of grapes with acid rot, these authors recorded yeasts such as *Candida, Hanseniaspora, Pichia* and *Saccharomyces* in greater abundance, which shows the etiological role they play in diseases such as acid rot. Additionally, the study by Pinto *et al*. (2015) identified filamentous fungi (moulds) in the initial fermentation of musts, such as *Alternaria, Aspergillus, Botrytis, Cladosporium, Penicillium* and *Rhizopus*, which are undesirable due to their impact on the quality of the wine; of these reported genera, two of them were identified in this work: *Cladosporium* and *Alternaria*–although the latter in a lower percentage, but still of concern at the time of distillate production. In addition to *Cladosporium* and *Hanseniaspora,* Bokulich *et al*. (2014) and Wei *et al*. (2022) have reported that other functionally related genera to be part of the wine microbiome; both these studies used wine grape musts and NGS analysis.

Using samples taken from the fermentation stage, Onetto *et al*. (2024) found *Aureobasidium, Cladosporium, Saccharomyces, Hanseniaspora* and *Metschnikowia* to be the dominant genera at the fungal level, unlike the results of our study on grape berries, which revealed the predominant genera to be different, except for genus *Hanseniaspora* (identified as the fourth predominant genus). Onetto *et al*. (2024) used musts which were at the beginning of fermentation, while our study used recently harvested grapes, thus confirming *Hanseniaspora* to be an oxidative, sensitive and oxygen-habitant yeast, but with the potential to be used in the sensory profile of distillates, influencing the quality of the final product (Stefanini and Cavalieri, 2018; van Wyk *et al*., 2023). In addition, according to a study by Leal *et al*. (2024), the most representative genera of the fungal microbiome were *Phaeomoniella, Devriesia, Fusarium, Diplodia* and *Alternaria*, showing differences with this study; this is mainly due to the fact that the samples analysed by Leal and colleagues were at the level of the bark of the vine trunk and sampled in two seasons over two years, thus confirming how fungal microorganisms vary in their predominance in each part of the vine.

## 2. Contrasting the richness and diversity of the microbiota in the different varieties of Pisco grapes

This being the first study of this kind performed on Pisco, we discuss the richness and diversity of the microbiota in the light of similar analyses that have been performed on wine grapes.

The results of the Alpha Chao 1 bacterial diversity show Pisco grape varieties Mollar, Albilla, Moscatel and Torontel to have the highest richness. The results for the alpha diversity of fungi show the Italia, Mollar, Negra Criolla and Uvina varieties to have the highest richness and diversity. Liu *et al*. (2019) analysed the richness and diversity of nine varieties of wine grape berries: Chardonnay (Cha), Riesling (Rie), Pinot noir (Pin), Gem (Gem), Cabernet-Sauvignon (Cab), Zinfandel (Zin), Longan (Lon), Syrah (Syr) and Riesling (Rie), of which the Chardonay variety showed the highest richness in bacterial communities, and the Longan variety the highest richness in fungal communities.

The Shannon index indicates that the Pisco grape varieties of the Ica region are bacterially less diverse (*i.e*., few taxa cover most individuals) than the Pisco grape varieties in Arequipa, on which the distribution of bacterial communities is more uniform and diverse. Meanwhile, the Shannon index indicates that both regions have similar dispersion in the richness and abundance of fungal species. For comparison, Zhang *et al*. (2020), who studied samples of wine grape leaves, reported the Syrah variety to have the highest diversity of bacterial communities, and the Longan variety the highest diversity of fungal communities. This thus highlights the importance of the Shannon index for measuring microbial richness in these kinds of studies.

Furthermore, according to the Simpson index for the analysis of bacterial communities in the present study, the Pisco grape varieties with the highest diversity of species in the Arequipa

region were Torontel, Italia and Moscatel, and in the Ica region, Italia, Mollar and Albilla. In turn, at the fungi level, the varieties with the highest species diversity recorded were Italia, Quebranta and Mollar for Arequipa and Italia, Mollar and Albilla for Ica. This index shows the tendency to be smaller when the community is more diverse, which is also demonstrated, when using the Simpson index for wine grapes, Wei et al. (2023) found that Fengzao grape was more diverse than the Kyoho grape, and that the fruit had the highest diversity of microbial species in comparison to the stem and leaf.

### 3. Influence of edaphoclimatic, geographical and cultivation practices on the regional identity of the Pisco terroir

The terroir is closely linked to the regional identity of a wine or distillate, and is therefore important in the Pisco and distillate industry. In this context, edaphoclimatic factors, biodiversity elements and cultivation practices in each region condition the microbial populations that will be associated with the production of Pisco. This study took into account data on temperature, precipitation, relative humidity, altitude and soil pH, as well as cultivation practices related to phytosanitary applications, conduction and irrigation systems; all of these factors affect the microbiota identified in the Pisco grape berries, whether from Arequipa or Ica. Zarraonanindia *et al*. (2015) addressed in detail the critical factors determining vine terroir, such as soil pH, soil carbon and the carbon–nitrogen relationship, since most of the microbial taxa associated with the grape came from the soil and showed specificity for the local vineyard analysed. Another study that demonstrates the relevance of environmental conditions is that of Karagöz *et al*. (2012), who consider soil pH to be a determinant of bacterial communities and their interaction with the plant. The interaction of environmental factors, such as topography, climate, soil properties, cultivars and agricultural management, affects soil microbial communities, have also been reported (Burns *et al*., 2015; Mezzasalma *et al*., 2018; Rivas *et al*., 2021; Cruz-Silva *et al*., 2023).

Given that the microbial assembly function is inherently difficult to measure and define due to its highly changing nature (Nannipieri *et al*., 2003; Lazcano et al., 2020), complementary research is required to correlate the identified taxa of oenological importance with different sensory patterns or physicochemical tests in order to better define the Pisco terroir; this would, for instance, contribute to a higher quality wine and thus fulfill consumer expectations (Belda *et al*, 2017; Hervé, 2020). There is also a need for carrying out individual evaluations of microbiotas in order to determine to what extent and with what stability they could preserve the regionality of Pisco profiles over time.

## CONCLUSION

This study characterised for the first time the microbial communities present in the eight varieties of Pisco grapes from two PDO regions of Peru: Ica and Arequipa. The predominant genera in the bacterial communities were *Ralstonia*, *Gluconobacter*, *Pantoea*, *Tatumella*, *Bradyrhizobium*, *Komagataeibacter,* and some unclassified species in the Orbaceae family. In the fungal communities, the predominant genera were *Cladosporium*, *Starmerella*, *Pichia*, *Hanseniaspora*, *Wallemia*, *Claviceps*, *Alternaria* and some unclassified species in the order Saccharomycetales and within the division of Ascomycota. Edaphoclimatic factors and cultivation practices seem to have a major effect on microbial populations in the vineyards, even more than varietal or aromatic/non-aromatic classifications. These results are an important starting point, since all eight varieties of authorised Pisco grapes have been evaluated, providing fundamental information for both grape growers and Pisco or wine makers.

Future research will focus on A) examining correlations between edaphoclimatic factors and cultivation practices and the sensory components of Pisco for a complete characterisation of the Pisco microbial terroir, B) the microbial characterisation of Pisco grapes from the three other PDO regions (Lima, Moquegua, Tacna), and C) classifying the significant number of unidentified microorganisms that were observed here; this will soon be made possible as a result of the constant updating of reference molecular databases.

Peru has five PDO regions for Pisco, and that strictly follow procedures set out in the Pisco PDO Regulations, which are administered by the Pisco Regulatory Council; these include the Peruvian Technical Standard NTP 211.001: 2006 on the use of Pisco varieties and their processing from harvest to storage (NTP 212.033, 2007). In this study, we have highlighted the significance of the regional identity of Pisco for the Pisco industry, and sought to show how the identified microbial communities are key players in the development of the Pisco microbial terroir. As a consequence, various complementary lines of research are warranted, such as a study of the first stages of fermentation, during which microbiota, especially the non-*Saccharomyces*, play a very important role in conferring a fermentative aromatic profile, which together with the contribution of varietal aromas, can more consistently reinforce the concept of the Pisco "aromatic footprint".

Applying metagenomics can help understand the phytopathogenic and oenological importance of microbiota in Pisco grape berries to improve the quality of the Pisco distillate and increase recognition of its terroir (unique footprint) in each region.

## ACKNOWLEDGEMENTS

We thank the directors and staff of CITEagroindustrial Ica, Vineyard Álvarez (Ica), and Majes Tradición Vineyeard (Arequipa), for access to the sampling sites and providing essential biological material and logistic support. We are particularly grateful to Dr. Hanna Cáceres and Eng. Alejandro Ponce from CITEagroindustrial Ica, Mrs. Mirtha Álvarez from Álvarez Vineyard, and Eng. Marcos Zúñiga from Majes Tradición Vineyard for sharing their knowledge on Pisco and Pisco grapes.

Funding was provided by Gen Lab del Perú S.A.C. through their Gen Lab Incentive Programme 2019.

The authors also thank Oksana Huerta for her assistance during sample collection, Y. Pamela Aroni, Elmer Ramos and Fiorella Chávez for their help in the laboratory, Guillermo Trujillo for the support on sequencing and data analyses and Dr. Kenneth Young for his writing review of the article.

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