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Microbial diversity of Syrah grapes cultivated for winter wine production in Minas Gerais, Brazil

Diversidade microbiana de uvas Syrah cultivadas para a produção de vinhos de inverno em Minas Gerais, Brasil

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ABSTRACT

Microorganisms play a key role in determining the terroir of any region where vineyards are cultivated, influencing the characteristics of the wine produced in that region. This study aimed to determine the mycobiota terroir of Syrah grapes from subtropical vineyards of southern Minas Gerais, Brazil, using independent cultivation methods. Total DNA was extracted from the grapes and internal transcribed spacer/rRNA regions were sequenced. Taxonomic profiles were obtained at the kingdom, phylum, class, order, family, genus, and species levels. The results showed a predominance of the phylum Ascomycota, followed by Basidiomycota. A phylogenetic tree was also constructed with the top 20 species of fungi present in the grapes, mainly represented by the genera *Cercospora*, *Uwebraunia*, *Aureobasidium*, *Leptospora*, *Pseudopithomyces*, *Periconia*, *Acrocalymma*, *Alternaria*, *Aspergillus*, *Penicillium*, *Hansfordia*, *Meyerozyma*, *Candida*, *Wickerhamomyces*, *Acremonium*, *Sarocladium*, *Gibberella*, and *Colletotrichum.* The fungal diversity of the grapes was successfully characterized, but many individuals were not classified, indicating that future studies should be performed to better understand the profile of the species found, as well as their functions in this system.

Index terms: *Terroir*; wine grapes; metagenomics.

RESUMO

Os microrganismos desempenham um papel fundamental na determinação do terroir da região onde os vinhedos são cultivados, influenciando as características do vinho produzido nessa região. Nesse sentido, este estudo teve como o objetivo determinar a micobiota *terroir* de uvas variedade Syrah de vinhedos sub-tropicais localizados no sul de Minas Gerais. O material genético foi extraído para a obtenção do DNA total e seu sequenciamento foi realizado pelo programa Illumina, usando a região ITS rRNA. Perfis taxonômicos foram obtidos em Domínio, Filo, Classe, Ordem, Família, Gênero e Espécie, observando-se que houve uma maior predominância do filo Ascomycota, seguido de Basidiomycota. Também foi construída uma árvore filogenética com as 20 principais espécies de fungos presente nas uvas, respresentadas pelos gêneros *Cercospora*, *Uwebraunia*, *Aureobasidium*, *Leptospora*, *Pseudopithomyces*, *Periconia*, *Acrocalyma*, *Alternaria*, *Aspergillus*, *Pecinicillium*, *Hansfordia*, *Meyerozyma*, *Candida*, *Wickerhamomyces*, *Acremonium*, *Sarocladium*, *Giberella* e *Colletotrichum.* A caracterização da diversidade microbiana das uvas foi obtida com sucesso, no entanto, pôde-se observar um grande número de indivíduos que não foram classificados, levando a crer que estudos futuros nessa área precisam ter continuidade para que, cada vez mais, possamos ter conhecimento dessas espécies e entender como funcionam esses sistemas.

Termos para indexação: *Terroir*; uvas viníferas; metagenômica.

INTRODUCTION

The characteristics of the soil and climate of a particular region, associated with the grape variety and the viticulture and oenological techniques used, contribute to determining the characteristics of the wine, establishing the concept of *terroir* Mas and Portillo (2022) Tsiakis et al. (2022), Castelló (2021) Belda et al. (2017), Bokulich et al. (2016). Microorganisms naturally present in the grape growing environment also influence the final wine characteristics, and the contribution of microorganisms on *terroir* has come to be recognized Bokulich et al. (2016), Gilbert, Van Der Lelie and Zarraonaindia (2014) and Knight et al. (2015).

Knowledge on the microbiota associated with wine grapes allowed the discovery of some yeast species of the *Saccharomyces* genus with oenological properties used as a starter culture in the winemaking process. Non-*Saccharomyces* genera, such as *Kloeckera*, *Cryptococcus*, *Torulaspora*, *Hanseniaspora*, *Candida*, *Pichia*, *Hansenula*, *Debaromyces*, and *Rhodotorula*, are also present in the initial fermentation process and responsible for the sensory properties of wines Çelebi et al. (2020), Contreras et al. (2014).

The southeastern region of Brazil has been consolidating itself as a producing region of Syrah winter wines characterized by different cultivation characteristics when compared to other producing regions. Grapes are usually harvested in the first quarter of the year in Europe, corresponding to the rainy summer with high temperatures in Brazil that besides diluting sugar, color, and aroma components are favorable to diseases. The use of the double pruning technique allows grapes to be harvested in favorable climatic conditions Favero et al. (2011), Palliotti et al. (2017). Grapes are induced to change their stages of growth, ripening, and harvest by carrying out two prunings (in August and January), which allows the formation of productive branches and partial elimination of bunches. With this, plants change their cycle, starting to sprout in February, flowering in March, and starting to form clusters in April. The harvest can be carried out in winter, an indispensable condition for the production of high quality wines Favero et al. (2011).

According to Mota et al. (2020), the composition of Syrah winter wines produced in southeastern Brazil resembles that of Syrah wines from traditional regions such as Australia, Italy, and South Africa. In addition, environmental conditions (soil, climate, topographic relief, and geology) are also considered favorable for the composition of Syrah grapes and the production of high quality wines Gonçalves et al. (2022).

The diversity of microorganisms present in wine grapes grown in this region of Brazil is largely unknown, and therefore, the knowledge of this microbiota through the use of metagenomics tools (Zhang et al., 2021; Stefanini; Cavalieri, 2018; Morgan; Du Toit; Setati, 2017) can accelerate processes to obtain high quality products. Here, we aimed to identify the species of filamentous fungi and yeasts in the microbiota of Syrah grapes from wineries located in the south of Minas Gerais, southeastern Brazil, for assessing their contribution to the microbial *terroir*.

MATERIAL AND METHODS

Study area and sampling

Grape samples were collected from two wineries located in the municipalities of Boa Esperança (21°12′45″ S, 45°34′58″ W, 883 m.a.s.l.) and Três Corações (21°36′49″ S, 45°07′42″ W, 985 m.a.s.l.) in the southern region of Minas Gerais, Brazil, with production areas of 6 and 10 hectares, respectively. Samples of red grapes of the Syrah variety were collected in the final stage of maturation (June/July) during the harvest seasons, 2015 and 2016. A diagonal transect was drawn along the vineyard, in which three grape bunches were collected from three equidistant vines (P1, P2, and P3). The samples were packed in sterile bags and transported to the Mycology and Mycotoxins Laboratory, Food Science Department, Federal University of Lavras for DNA extraction.

Metagenomic analysis

DNA extraction and PCR

Total DNA was extracted from pooled samples (approximately 2 g) from three samples collected at equidistant points in each vineyard using a Wizard Genomic DNA Purification Kit (Promega). The concentration of extracted DNA was measured in a Nanodrop spectrophotometer. Different 18S rRNA/internal transcribed spacer (ITS) regions (18S V4/18S V9, ITS1/ITS2, Arc V4) were amplified using specific primers (18S V4: 528F and 706R; 18S V9: 1380F and 1510R). All PCRs were performed with Phusion® High Fidelity PCR Master Mix (New England Biolabs).

The same volume of 1× loading buffer (containing SYBR Green) was mixed with the PCR products and analyzed by 2% agarose gel electrophoresis. Samples of 400-450 bp were excised for additional tests. PCR products were mixed in equidense ratios and purified with a Qiagen Gel Extraction Kit (Qiagen, Germany).

Library preparation and sequencing

The sequencing libraries were generated using the TruSeq® DNA PCRFree Sample Preparation (Illumina, USA) following the manufacturer's recommendations. The quality of the library was evaluated in a Qubit @ 2.0 Fluorimeter (Thermo Scientific) and Bioanalyzer 2100 (Agilent) system. Finally, the library was sequenced on the Illumina HiSeq 2500 platform (HiSeq 2500 system) and 250-

bp paired-end reads were generated. This step was performed by the GenOne Biotechnologies Laboratory (Rio de Janeiro, RJ).

Bioinformatics

Paired-end reads were assigned to samples based on their unique barcode. Reads were extended using the FLASH software (V1.2.7), a fast and precise tool that can extend the reads when there is some overlap. The consensus sequences (called here raw tags) were filtered to obtain high-quality clean tags with the QIIME (V1.7.0) quality control process. The tags were compared against the reference database (Unite database) using the UCHIME algorithm to detect chimera sequences that were removed. Finally, effective tags were obtained.

Operational taxonomic unit cluster and species annotation

Sequence analyses were performed using Uparse software (V.7.0.1001). Sequences with ≥97% similarity were considered from the same operational taxonomic unit (OTU). The representative sequence for each OTU was annotated with taxonomic information using the Unite database and the BLAST algorithm in QIIME software (version 1.7.0).

To study the phylogenetic relationship of different OTUs and the dominant species between different samples (groups), sequence alignment was performed with MUSCLE software (version 3.8.31). The OTU abundance information was normalized using a sequential number pattern corresponding to the sample with the same sequence.

RESULTS AND DISCUSSION

The mycobiota of the Syrah grape variety from two wineries located in southern Minas Gerais, which employs an inverted pruning system, was investigated based on the partial sequences of rRNA/ITS regions. Two rRNA/ITS libraries from one pooled grape sample of each winery (Winery 1 - Boa Esperança and Winery 2 - Três Corações) were analyzed.

Winery 1 generated a total of 58,351 sequences that were grouped, 55,905 belonging to the phylum Ascomycota and 2,446 belonging to the phylum Basidiomycota. Winery 2 yielded 58,351 sequences, 57,857 from Ascomycota and 494 from Basidiomycota (Figure 1).

The proportion of unclassified sequences was 13 % (7,326) and 45% (26,359) for Wineries 1 and 2, respectively. Figure 2 shows the taxtree with the distribution of the kingdom, phylum (Ascomycota) class, order, family, genus, and species that were verified in both wineries.

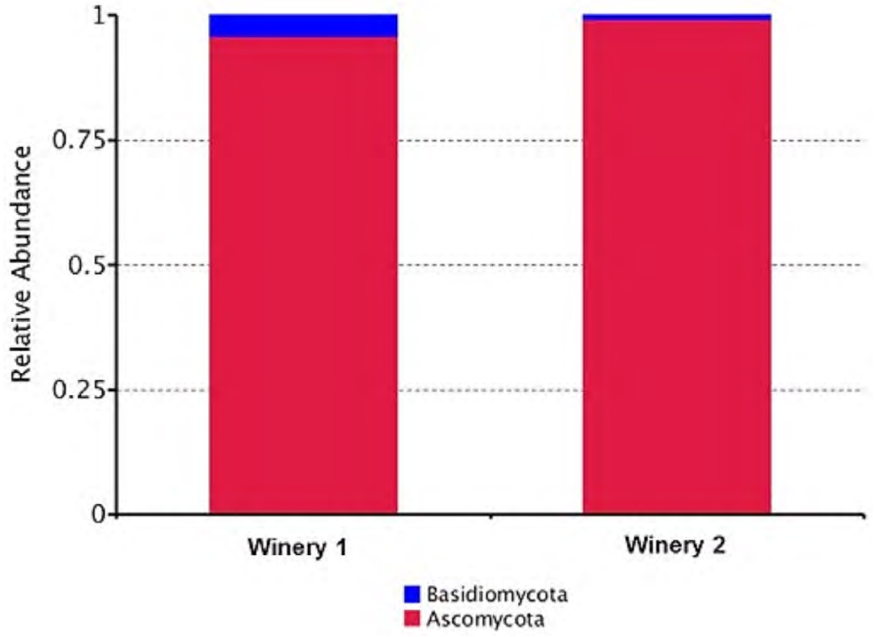

Figure 1: Relative abundance of the identified fungi at the phylum level in Winery 1 (Boa Esperança) and Winery 2 (Três Corações).

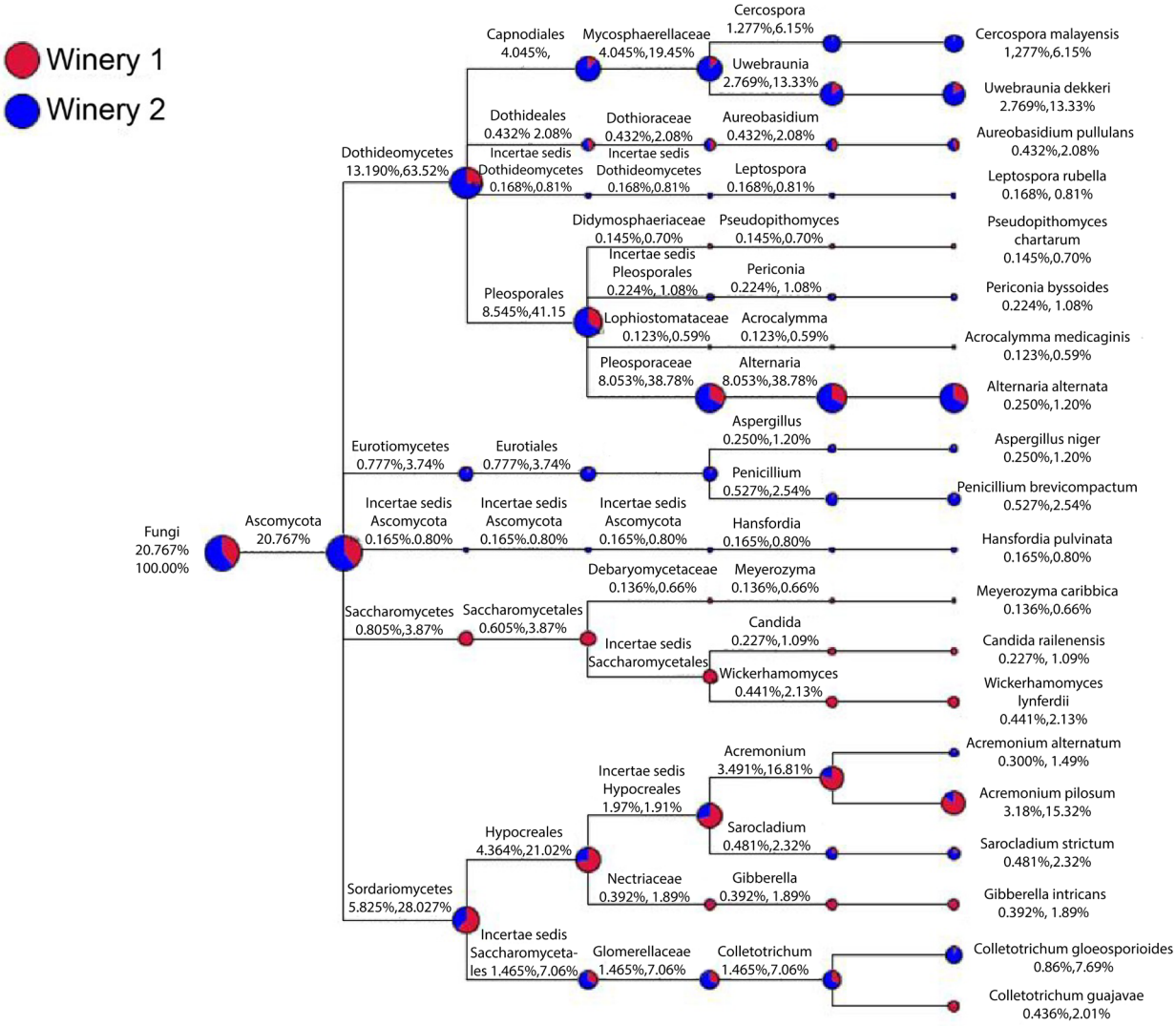

Figure 2: Classification tree with taxonomic affiliations for Winery 1 and Winery 2 generated with MetaGenome Analyzer. The first value represents the percentage of the sequenced and classified microbial community, and the second value represents the proportion in relation to the first.

Regarding the class-level fungal classification, class Dothideomycetes represented almost 50% of DNA samples identified in the two wineries. Winery 1 had a significant count of fungi belonging to the Saccharomycetes class (25%), followed by Sordariomycetes (approximately 10%). Winery 2 had a 5% incidence of Sordariomycetes, followed by approximately 1% of Eurotiomycetes. Winery 1 had 42% of fungi of the order Capnodiales, and 25% of Saccharomycetales. Winery 2 had an incidence of 30% Capnodiales and15% Pleosporales. Winery 1 was most enriched in the family Saccharomycodaceae (25%), whereas Winery 2 had an incidence of 10% Pleosporales, 8% Mycosphaerellaceae, and 3% Trichocomaceae.

Regarding the genus level of the filamentous fungi identified in the Syrah grapes of the wineries studied, *Alternaria* and *Acremonium* had the highest incidence (approximately 5% each) in Winery 1. Other genera showed low abundances, approximately 2%. *Candida* and *Wickerhamomyces* yeasts were also observed. In Winery 2, the genus *Alternaria* had an approximately 10% incidence, followed by *Uwebraunia* (5%). Other genera present were *Cercospora, Aspergillus*, and *Penicilium* approximately 3% each). No yeasts were found at Winery 2.

The 20 main species of filamentous fungi and yeasts shared by Winery 1 and Winery 2, belonging to the phylum Ascomycota, were discriminated: *Cercospora malayensis* (0.49% and 9.81% in Winery 1 and Winery 2, respectively) and *Uwebraunia dekkeri* (5.50% and 18.40%) (order Capnodiales); *Aureobasidium pullulans* (2.38% and 1.88%) (order *Dothideales)*; *Leptospora rubella* (0.13% and 1.25%) (order *Incertae sedis Dothideomycetes)*; *Pseudopithomyces chartarum* (1.48% and 0.19%), *Periconia byssoides* (0.66% and 1.35%), *Acrocalymma medicaginis* (1.50% and 0.01%), and *Alternaria alternata* (32.62% and 42.77%) (order *Pleosporales*); *Aspergillus niger* (0.29% and 1.79%) and *Penicillium brevicompactum* (0.48% and 3.87%) (order *Eurotiales*); *Hansfordia pulvinata* (0.15% 1.22%) (*Incertae sedis Ascomycota*); *Acremonium alternatum* (0.51% and 2.12%), *Acremonium pilosum* (32.88% and 3.96%), *Sarocladium strictum* (0.97% and 3.19%), and *Gibberella intricans* (4.42% and 0.25%) (order *Hypocreales*); and *Colletotrichum gloeosporioides* (0.86% and 7.69%) and *Colletotrichum guajavae* (5.0% and 5.0%) (order *Incertae sedis Sordariomycetes*). Among the yeasts belonging to the Saccharomycetales class, *Meyerozyma caribbica* (1.64% and 0.02%), *Candida railenensis* (2.72% and 0.04%), and *Wickerhamomyces lynferdii* (5.31% and 0.06%) were observed.

Winery 1 and Winery 2 presented very divergent Ascomycete fungal diversity. Winery 1 had some genera that were not observed in Winery 2, such as *Eucasphaeria*, *Cytospora*, *Fusarium*, *Phoma*, *Zygophiala*, *Microdiplodia*, *Metschnikowia*, and *Pichia*. In turn, Winery 2 showed greater diversity and many of its genera were not reported in Winery 1, such as *Gliomastix*, *Gliomastix*, *Engyodontium*, *Lophiostoma*, *Ophiosphaerella*, *Pithomyces*, *Pseudopithomyces*, *Phloeospora*, *Zasmidium*, *Ramichloridium*, *Passalora*, *Toxicocladosporium*, *Septoriella*, *Aspen, Penicillus*, *Aspergillus Talaromyces*, *Neophaeomoniella*, *Rhinocladiella*, *Lambertella*, *Botrytis*, *Hansfordia*, *Dimelaena*, and *Tilletiopsis.*

OTU phylogenetic relationships of the 10 genera with the highest incidence in the two wineries were analyzed together with the relative abundances of OTUs and the information of each species (Figure 3).

Two graphs were integrated: the inner layer is the phylogenetic tree using the OTU representative sequence, where colors correspond to the scientific names and each color represents an individual genus; the outer layer is the relative abundances of OTUs according to the column size. As seen in Figure 3, the most abundant filamentous fungi in the two wineries were *Penicillium brevicompactum*, *Sarocladium strictum*, *Alternaria alternata*, *Acremonium pilosum*, *Uwebraunia dekkeri*, *Aureobasidium pullulans*, *Cercospora malayensis*, *Colletotrichum globospora*, and *Colletotrichum guajavae.* The yeast *Wickerhamomyces lynferdii* was observed.

Regarding the diversity of basidiomycete fungi identified in Winery 1, Incertae sedis Tremellales group had the highest occurrence, with *Hannaella*, *Bulleromyces*, *Cryptococcus*, *Filobasidium*, and *Mrakiella* as its representatives. The Sporidiobolales group had a higher incidence of *Rhodotorula*, *Sporidiobolus*, and *Sporobolomyces*, which are basidiomycete yeasts with an anamorphic stage, in addition to *Exobasidium*, a fungus that shows no yeast-like structure belonging to the order Exobasidiaceae.

In Winery 2, *Incertae sedis Tremellales* also had the highest occurrence, with representatives *Bulleromyces*, *Hannaella*, *Cryptococcus*, and *Filobasidium*. The Sporidiobolales order had a higher incidence of *Sporobolomyces*, *Sporidiobolus*, and *Rhodotorula*, which are basidiomycete yeasts with an anamorph stage. *Exobasidium*, belonging to the order Exobasidiaceae, and Tilletiopsis, belonging to the order *Incertae sedis Exobasidiomycetes* (which show no yeast-like structure) were also present.

Gene sequences from the rRNA/ITS regions were used for sequencing using Uparse software. Sequences with similarity $\geq$ 97% were assigned to the same OTU. Next, a rarefaction curve was drawn to depict the number of species found relative to the number of organisms collected. The more concave downward the curve, the better the community was represented (Figure 4).

The rarefaction technique emerged as a solution to evaluate the diversity indices that depend on the size of the sample. The higher curve is the one that has the greatest diversity. The rarefaction curves tend to increase very quickly at the beginning, showing a slope expressing the most common species found. However, much of the diversity still needs to be discovered. As the number of rare species decreases, the curve becomes less flat, but usually the rarer species do appear. This shows that even if a higher number of samples are collected, few species are likely to be added (Hughes et al., 2001; Youssef; Elshahed, 2009).

The curve in Figure 4 has a steep left side, indicating that a large fraction of the fungal species diversity has not yet been discovered, while the concave side shows the number of rare species observed. Winery 1 ("Grape1") had more OTUs than Winery 2 ("Grape2"), i.e., greater diversity.

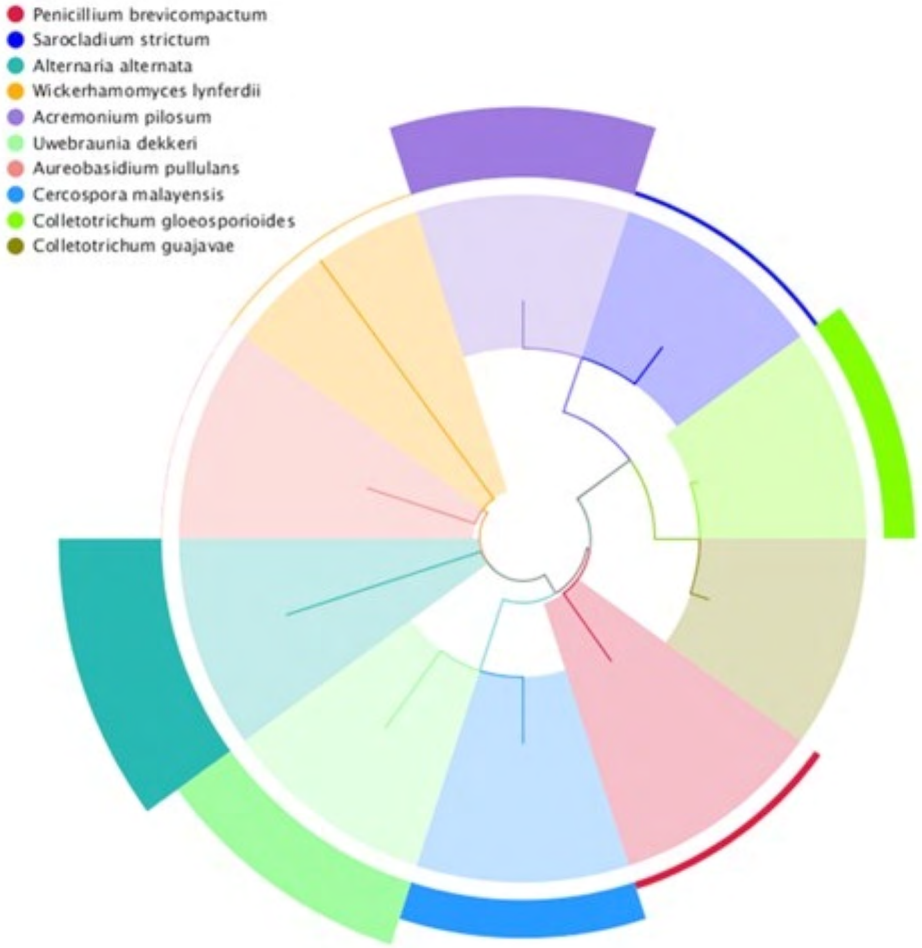

Figure 3: Overall relative abundance of fungi of Winery 1 and Winery 2 represented as an Operational Taxonomic Unit (OTU).

Winery 1 and Winery 2 had very different Ascomycete fungal diversity, and Winery 2 had more unique genera than Winery 1. Some genera found in this study are common in all grapes. In a study of California grape must, there was a predominance of *Cladosporium* spp. (2%), *Botryotinia fuckeliana* (*Botrytis cinerea*) (15.2%), *Penicillium* spp. (9.5%), *Davidiella tassiana* (9.2%), and *Aureobasidium pullulans* (7.3%), in addition to the yeasts *Saccharomyces cerevisiae* (4.0%), *Hanseniaspora uvarum* (5.0%), and *Candida zemplinina* (1.3%) Bokulich et al. (2013).

Yeasts play a key role in the fermentation process of wine grapes and contribute to the characteristics that wines will acquire. The non-Saccharomyces yeasts that include the detected in this study (genera *Kloeckera*, *Cryptococcus*, *Torulaspora*, *Hanseniaspora*, *Candida*, *Pichia*, *Hansenula*, *Zygosaccharomyces*, *Metschinikowia*, *Debaryomyces*, *Issatchenkia*, and *Rhodotorula*) are present in the initial phase of spontaneous fermentation of wines because they predominate on grape surfaces. Groups of oxidative basidiomycete yeasts, such as *Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp., and *Filobasidium* spp. observed in this study are present in whole berries, from the end of germination to the full grape maturation. These yeasts belong to the vineyard environment and are typically associated with the phyllosphere of grapes and soil. Furtheremore, these groups are also reported in vineyards in the Polkadraai region, in Stellenbosch, South Africa, and in Cabernet Sauvignon grapes.

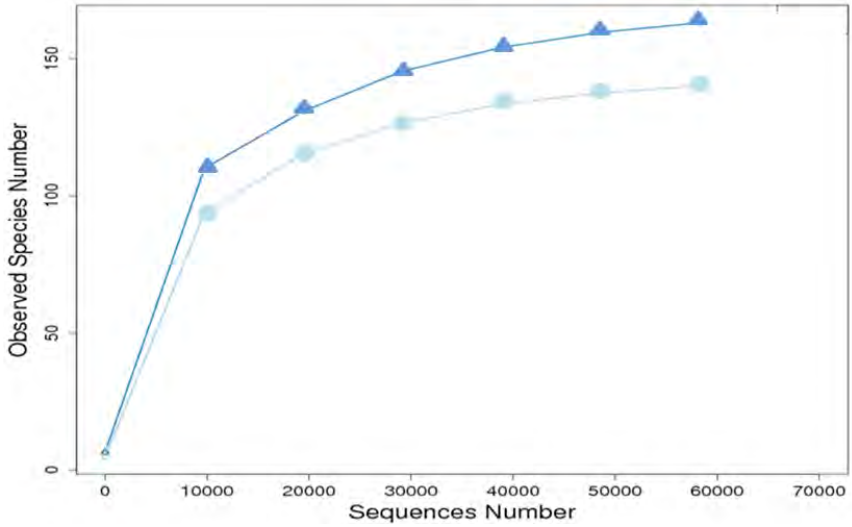

Figure 4: Rarefaction curve with the number of species represented from Winery 1 (circles) and Winery 2 (triangles).

Some yeasts predominant in many grapes had low incidences in the present study because whole, damage-free grapes were evaluated. These grapes exhibit lower incidences of oxidative ascomycete yeasts *Candida* spp., *Pichia* spp., and *Metschnikowia* spp., as well as the fermentative ascomycete yeasts *Hanseniaspora*, *Kloeckera* spp. Nisiotou and Nichas (2007), and *Saccharomyces cerevisiae* Fleet (2003). The genera *Kloeckera* and *Saccharomyces*, despite having low incidences in each winery, are important because they produce valuable metabolites for wine production. In a study conducted by Mamede and Pastore (2004), *Kloeckera apiculata* produced high concentrations of ethyl acetate and isoamyl acetate. In contrast, *Saccharomyces cerevisiae* had a low production of these compounds but a high production of ethanol, a positive factor for wine production. Ethyl acetate is the predominant ester in wine and is produced in small quantities by yeasts during fermentation. Jolly, Augustyn, and Pretorius (2006) determined that *Pichia anomala*, *Kloeckera apiculata*, and *Candida pulcherrima* have high ester production.

In ripe grapes that have been injured, in which nutrients and sugar have higher concentrations, yeasts such as *Hanseniaspora*, *Metschinikowia*, *Candida*, and *Pichia* spp predominate. The latter was found only in Winery 1, while the others were common to both wineries.

Some genera found in grapes, such as *Rhodotorula glutinis* and *Sporobolomyces roseus,* may inhibit the development of species *Penicillium expansum* and *Botrytis cinérea*, considered undesirable because they produce mycotoxins and compounds that reduce wine quality. Thus, those genera exhibit potential as a biocontrol agent, such as *Tilletiopsis albescens*, which has been shown to inhibit the growth of *Penicillium italicum.*

Some cited genera and their respective species detected in this study had not been previously reported in grapes and are characteristic of the studied region; some of these species can be found in soils.

Studies of microbial diversity in wine grapes have enabled the discovery of species with positive oenological properties, providing knowledge of a growing number of fungi and bacteria that actively participate in fermentation processes and contribute to the sensory qualities of wine.

CONCLUSIONS

Metagenomics allows to know the uncultivated microbiota of a given sample. With this technique, we taxonomically characterized fungi and yeasts present in wine grapes grown in the southern of Minas Gerais. Knowledge of this diversity is of paramount importance, as it helps to understand how this mycobiota can influence the final quality of the wine produced.

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AUTHORS CONTRIBUTIONS

Conceptual idea: Batista, L. R., Methodology design: Batista, L. R., Lira, N. A., Data collection: Lira, N. A., Passamani, F. R. F., Aguilar, M. A., Data analysis and interpretation: Lira, N. A., Passamani, F. R. F., Evangelista, S. R. and Writing and editing: Lira, N. A., Passamani, F. R. F., Evangelista, S. R., Goulart, N. M. V., Aguilar, M. A., Batista, L. R.

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