Fungal Community and Physicochemical Profiles of Ripened Cheeses

Michele Aragão

Federal University of Lavras: Universidade Federal de Lavras

Suzana Evangelista

Federal University of Lavras: Universidade Federal de Lavras   https://orcid.org/0000-0002-7680-0149

Fabiana Passamani

Federal University of Lavras: Universidade Federal de Lavras

João Pedro Guimarães

Federal University of Lavras: Universidade Federal de Lavras

Luiz Abreu

Federal University of Lavras: Universidade Federal de Lavras

Luis Batista (✉ luisrb@ufla.br)

Federal University of Lavras: Universidade Federal de Lavras

Abstract

Ripened cheeses are traditionally produced and consumed worldwide. Canastra's Minas artisanal cheese (QMA) is a protected geographical indication (PGI) traditional ripened cheeses. The influence of fungi on the cheese ripening process is of great importance. This study aimed to apply culture-dependent and -independent methods to determine the mycobiota of QMA produced in the Canastra region, as well as to determine its physicochemical characteristics. Samples from different producers were collected in the cities of São Roque de Minas and Piumhi (MG). Illumina-based amplicon sequencing, and Matrix Assisted Laser Desorption Ionization Time-of-Flight - (MALDI-TOF) Mass Spectrometry (MS) methods were used. The physicochemical analysis showed that the QMA had a moisture content between 18.24% and 21%, fat content between 20.5% and 40%, sodium chloride percentage around 0.9%, and pH of 5.5 to 5.3. The population of fungi ranged between 6.3 and 8 log CFU/g. *Fusarium* sp., *Geotrichum candidum*, *Paecilomyces* sp., *Trichosporon coremiiforme*, *Candida catenulata*, *Aspergillus* sp., *Trichosporon japonicum*, *A. oryzae*, *Kluyveromyces*, *Torulaspora,* and *Debaryomyces* were the most prevalent fungi. *A. ochraceus* potentially mycotoxin-producing was detected. Promising species that can contribute to the product quality were identified as *Geotrichum candidum* and *Candida catenulate*. However, future studies should evaluate the ability of *A. ochraceus* to produce mycotoxin in cheese, as well as control the growth of certain species of fungi during ripening, resulting in a safe and high quality product.

Introduction

Ripened cheeses are widely consumed and are appreciated for their characteristic flavor. Canastra's Minas artisanal cheese (QMA) is a protected geographical indication (PGI) traditional food, handmade in this region from raw cow's milk in Brazil. A natural starter known as "pingo" is used during QMA's production. It consists of the part of the whey collected after pressing the cheeses from the previous day. This endogenous inoculum, rich in native microorganisms, contributes to the fermentation and ripening of the product (Nóbrega, 2008). After being produced, the cheese undergoes a ripening process, which is important for improving its sensory characteristics by modifying texture, aroma, and flavor of the cheese (McSweeney, 2004).

Bacteria and yeasts are the main microorganisms present during fermentation, while filamentous fungi are also observed during ripening. Wolfe et al. (2014) observed that the presence of several fungal genera can have a positive or negative impact on bacterial growth. Filamentous fungi and yeasts play an important role in increasing the pH during cheese rind formation. Such medium deacidification can happen due to the positive responses of bacterial growth to the presence of certain fungal genera. The action of fungal enzymes leads to the metabolization of milk constituents, such as proteins and lipids, contributes to improving quality of texture, flavor, and aroma of the final product (Cardoso et al., 2015; Hymery, 2014; Zheng et al., 2018).

The microorganisms present during the production process may come from the raw material, such as milk (Kamimura et al., 2020), as well as from the production environment (Bokulich and Mills, 2013).

Some genera of microorganisms are commonly found in cheese production regardless of the producing region; however, the species may vary depending on the type of cheese produced (e.g., washed rind) (Kamimura et al., 2019a). The main microbiota variation during cheese ripening is related to the environmental conditions, with moisture being one of the main drivers of the microbiota (Wolfe et al., 2014).

The high-throughput sequencing approach is an efficient culture-independent method used in food studies, and is known to produce fast results at an affordable cost. This type of analysis allows to observe important microorganisms contributing to the product quality, as well as the contaminants involved in fermentation and offers insights into the metabolic potential of a microorganism. With this technique, it is now possible to evaluate important microbiota not seen before with the use of culture-dependent techniques only (Doyle et al., 2017; Filippis et al., 2018; Walsh et al., 2017). Studies using metaprofiling (target genes) methods have been carried out on cheeses and have contributed to identification of bacterial microbiota (Escobar-Zepeda et al., 2016; Filippis et al., 2017; Gonçalves et al., 2018; Kamimura et al., 2019 b).

With regards to fungi, some studies have already been carried out on cheeses matured in different countries and regions. The main fungi in French "Tomme d'Orchies" cheeses were *Yarrowia lipolytica*, *Galactomyces geotrichum*, *Kluyveromyces* sp., and *Debaryomyces* sp. (Ceugniez et al., 2017). In evaluating 12 types of French cheese, Dugat-Bony et al. (2016) observed that the most common species were *G. candidum*, *D. hansenii*, *Yarrowia lipolytica*, *Clavispora lusitania*, *Cyberlindnera jadinii*, *S. cerevisiae*, and *Torulaspora delbrueckii*, and the genera were *Kluyveromyces* and *Candida*. *Galactomyces* and *Debaryomyces* were the main yeast genera present in Italy's Fontina cheeses (Guzzon et al., 2017). In artisanal cheeses evaluated at different maturation periods in China, the genera with the highest levels of abundance were *Kluyveromyces* and *Torulaspora* (Zheng et al., 2018). Bokulich and Mills (2013) evaluated cheese making facilities and observed that *Debaryomyces* sp. and *P. commune* were dominant in the production environment, demonstrating that the microbiota present on the processing surfaces will influence the final product, both during fermentation and maturation. Wolfe et al. (2014) developed cheese rinds as microbial community models, characterizing in situ diversity standards and developing an in vitro system for community reconstruction. Roughly 25% of the observed fungi were not start cultures, therefore originating from environmental sources. The following genera were found: *Brachybacterium, Debaryomyces, Galactomyces, Candida, Scopulariopsis, Fusarium, Acremonium, Penicillium, Aspergillus, Sporendonema*, and *Chrysosporium*.

Currently, there are no studies characterizing the fungal microbiota of ripened cheeses from Brazil. Such information would allow verification of the microorganisms responsible for the sensory characteristics of the product, as well as the existence of mycotoxigenic fungi. Characterization of this mycobiota would make it possible to identify measures regulating growth of certain fungi species during ripening, resulting in a safe, high quality product as well as contribute to the characterization of the *terroir*.

Therefore, the objectives of this study were to analyze the mycobiota present in the ripened Minas artisanal cheese produced in the micro region of Canastra using both culture-dependent and -independent methods, and to determine physicochemical characteristics of the cheese responsible for its geographical indication.

1. Material And Methods

1.1 Sampling

Samples were collected at certified producers located in the cities of São Roque de Minas and Piumhi, Canastra region in the state of Minas Gerais, Brazil. The samplings of three cheeses from each of the four different farms were collected and coded as A1 (Capão Grande Farm, altitude of 854 m), A2 (São Bento Farm, altitude of 860 m), A3 (Bela Vista Farm, altitude of 766 m), and A4 (Alvorada Farm, altitude of 769 m). By March 2017, the cheeses had already been ripened for 50 days. The cheeses were packaged in sterile bags and transported to the Mycology and Mycotoxins Laboratory of the Food Science Department at the Federal University of Lavras for further analysis.

1.2 Physicochemical analysis

The QMA samples were subjected to physicochemical analyses in triplicates. All analyses were performed at the Milk and Dairy Products Laboratory of the Food Science Department of the Federal University of Lavras according to the procedures of the Instituto Adolfo Lutz (2008). The moisture content was determined by the gravimetric method. The sodium chloride content was measured using the Mohr argentometric method. The pH values were determined using a pH Meter (Quimis). The fat content was measured by the butyrometric method (Van Gulik method), using a butyrometer for cheese.

1.3 Evaluation of the mycobiota using a culture-dependent method

Direct replication from the cheese rind and serial dilution were used to evaluate fungi from the QMA samples. For direct replication, 20 points of visible fungal colonies from the cheese rind were collected using a sterile toothpick and inoculated in the Malt Extract Agar (MA) (Merck, Darmstadt, Germany). For decimal serial dilution, 25 g of the cheese rind obtained from six equidistant points were coarsely ground and added to 225 mL of 0.1% saline peptone solution and homogenized using a Stomacher (Mayo Homogenius HG 400, São Paulo, Brazil) subjected to 490 strokes/min for 2 minutes. Aliquots of 0.1 mL of the appropriate serial dilutions were spread on the surface of the culture media and incubated at 25°C for 5 to 7 days. The following culture media were used in these experiments: DRBC - Dichloran Rose Bengal Chloramphenicol medium (Merck, Darmstadt, Germany) and Dichloran Glycerol Medium Base (DG18) (Merck, Darmstadt, Germany). After the incubation period, colony forming units (CFU) were counted and the square root technique was used to determine the total number of fungi to be isolated. The isolates were then transferred to MA purification medium for monosporic isolation and incubated in BOD at 25°C (filamentous fungi) and 28°C (yeasts) for 7 days. After this period, slides from the pure

colonies were prepared to determine genera based on microscopic characteristics. The isolates were preserved and later reactivated for identification.

The *Aspergillus* and *Penicillium* isolates were grown in CYA - Czapek Dox Agar culture media (Sigma-Aldrich, St. Louis, MO), incubated at 25ºC and 37ºC, and in MEA - Malt Extract Agar (Sigma-Aldrich, St. Louis, MO) at 25ºC, for a period of 7 days. Incubation in MEA culture medium at 25ºC for 7 days was used to perform the morphological characterization of the other genera. Macro- and microscopic characteristics of fungi colonies were observed and described according to Klich (2002), Pitt (2000), Samson et al. (2002), Samson et al. (2014), and Varga et al. (2011). The isolated yeasts were inoculated on Yeast Extract Peptone Glucose Agar (YEPG) plates (1% yeast extract (Merck), 2% bacteriological peptone (HiMedia), 2% glucose (Merck), 1.5% agar (Merck) at pH 5) and incubated at 28ºC for 18 hours. Yeast colonies were characterized for morphology as described by Kurtzman et al. (2011).

The fungi were then identified using Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry. This technique has been used to identify filamentous fungi mainly in the medical field, but these days it is also applied in experiments with food (Quero et al., 2019; Lima and Santos, 2017). It has good results for microbiota evaluation in cheeses, allowing for a reliable identification of a large number of cultivable species (Andrade et al., 2017; Nacef et al., 2017), as well as in genotypic methods such as PCR (Dušková et al., 2012). Each strain was prepared in triplicate and analyzed on a microflex LT MALDI-TOF spectrometer (Bruker Daltonics, Bremen, Germany), using MALDI Biotyper 3.0 automatic system as described in Resende et al. (2018).

After being identified, the isolates were deposited at the Culture Collection of Microorganisms of the Food Science Department (CCDCA) at the Federal University of Lavras.

1.4 Chemotaxonomic analyses of potentially toxigenic filamentous fungi

The isolated filamentous fungi identified as potentially producing ochratoxin A (OTA) and aflatoxins B1, B2, G1, and G2 (AFB1, AFB2, AFG1, and AFG2) as well as the potential producers of citrinin (the species of the *Penicillium* genus of the Citrinin section), were cultured in Coconut Agar culture medium (400 mL coconut extract, 15 g agar, 400 mL distilled water) to evaluate mycotoxins production. After 7–10 days incubation at 25ºC, mycotoxin production was verified with UV light at λ = 366 nm in Chromatovisor CAMAG (UF-BETRACHTER) as described by Mohamed et al. (2013). The isolates characterized as mycotoxin-producers were evaluated using the thin layer chromatography to confirm the production and to identify the mycotoxins according to Freire et al. (2017).

1.5 Mycobiota evaluation using a culture-independent method

The three cheese samples collected in each property were mixed to obtain a composite sample for each location. The samples were then subjected to metaprofiling analysis performed by the GenOne

Biotechnologies Laboratory (Rio de Janeiro, RJ).

1.5.1 Metagenomic DNA extraction and amplification of PCR products

Briefly, approximately 500 mg from each sample were used for genome DNA extraction. Total sample DNA was extracted using the CTAB-SDS method as described in Stefanova et al. (2013). DNA concentration and purity were monitored using 1% agarose gels. Final DNA concentration was adjusted to 1 ng/µL using sterile water.

Amplification of the Internal Transcribed Spacer (ITS) region of the ribosomal DNA was performed using specific primers and barcodes to identify samples. ITS is the most used region for fungi and the ITS2 region was selected for this type of analysis. The primers used in this step were ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). All PCR reactions were performed using Phusion High-Fidelity PCR Master Mix (New England Biolabs).

Equal volumes of 1× loading buffer (containing SYBER green) and PCR products were mixed and then separated on 2% agarose gels. Samples with fragment sizes between 300 and 450 bp were chosen for further analysis. Those selected PCR products were then purified using Qiagen Gel Extraction Kit (Qiagen, Germany).

1.5.2 Library preparation and sequencing analysis

Libraries were generated using NEBNext Ultra DNA Library Pre Kit (Illumina, USA) and index codes were added following manufacturer's instructions. The library quality was assessed by the Qubit 2.0 Fluorometer (Thermo Scientific, USA) and Agilent Bioanalyzer 2100 system (Santa Clara, USA). The library was then sequenced on an Illumina platform and 250 bp paired-end reads were generated.

Paired-end reads were attributed to samples according to their barcodes which were later cut off with the primer sequence. These were then assembled using the FLASH 1.2.7 software (Magoč and Salzberg, 2011) that merged the pairs to identify the overlaps generated from the opposite end of the same DNA fragment in order to reconstruct the ITS region. Paired fragments were filtered by the QIIME 1.7.0 software (Caporaso et al., 2010) to obtain better quality fragments. Subsequently, the fragments were compared with the reference database (Gold Database) using the UCHIME algorithm (Edgar et al., 2011) to detect chimera sequences which were further removed.

Sequences with an average Phred quality score < 25, containing at least one ambiguous base or exhibiting at least one error in primer sequences, were discarded. Operational taxonomic units (OTUs) were clustered at 97% identity with QIIME's algorithm. Any OTU comprising < 200 reads (almost 0.005% of total sequences for each run) was removed, as recommended by Bokulich et al. (2013), as previously described by Dugat-Bony et al. (2016).

Sequences were analyzed using the Uparse 7.0.1001 software (Edgar, 2013) and those with 97% similarity were assigned to the same Operational Taxonomic Units (OTUs), and representative sequences were then selected for annotation. For each representative sequence, the GreenGene Database (DeSantis et al., 2006) was used based on the RDP classifier (Version 2.2) (Wang et al., 2007) algorithm to annotate taxonomic information, as previously described by Bi et al. (2019).

To determine the phylogenetic relationship between the OTUs, as well as the difference between the dominant species in each sample, the sequence alignments were performed using MUSCLE 3.8.31 software (Edgar, 2004). OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences, as previously described by Zhu et al. (2020).

1.6 Statistical analysis

Analysis of variance (ANOVA) with subsequent Tukey test using the Sisvar 5.6 software was carried out to assess the statistical differences between the samples (Ferreira, 2014).

2. Results

2.1 Physicochemical analyses and counting of filamentous fungi and yeasts

Cheese samples collected from four different producers were analyzed for chemical composition and fungi population. The results of the analyses are summarized in Table 1. Moisture percentage ranged from 18.2% (sample A2) to 28.2% (sample A1); samples A3 and A4 showed similar values around 21%. With regards to the fat content, all samples were statistically different from each other ($p < 0.05$), ranging from 20.5% (sample A3) to 40% (sample A4). The sodium chloride (NaCl) content was around 0.9% and there was no significant difference between the samples ($p > 0.05$). The pH values were also similar ($p > 0.05$), with the pH in samples A1 and A3, and samples A2 and A4, being around 5.5 and 5.3, respectively.

Table 1
Physicochemical compositions of samples A1, A2, A3, and A4 of Minas artisanal cheese from the Canastra micro region and populations in culture media (DRBC and DG18)

Sample	Physicochemical characteristics				Total average populations Log CFU/g	
	% Moisture	% Fats	% NaCl	pH	DRBC medium	DG18 medium
A1	28211 ± 024^a	32 ± 0^a	093 ± 008^a	552 ± 004^a	753 ± 4^{aA}	805 ± 15^{dB}
A2	1824 ± 067^b	29 ± 0^b	098 ± 008^a	529 ± 0005^b	630 ± 2^{bC}	758 ± 4^{eD}
A3	21136 ± 024^c	205 ± 0^c	093 ± 003^a	553 ± 001^a	678 ± 2^{cE}	664 ± 2^{fF}
A4	22749 ± 323^c	40 ± 0^d	087 ± 013^a	533 ± 002^b	632 ± 1^{bG}	723 ± 1^{gH}

$^{a-g}$ Mean values in the same column with different superscripts differ statistically ($P < 0.05$). $^{A-H}$Mean values in the same line with different superscripts differ statistically (P < 0.05).[1]DRBC = dichloran rose bengal chloramphenicol; [2]DG18 = dichloran glycerol medium base

The fungal population present in the cheese samples ranged from 6.4−8 log CFU/g. Sample A1 had the highest total fungi population (7.5 log CFU/g in DRBC and 8.05 log CFU/g in DG18).

2.2 Fungal diversity determined by culture-dependent method and mycotoxin production evaluation

Out of 292 purified isolates obtained from the plating experiments, 16 different species were identified (Fig. 1). Our results demonstrate that the region's main dominant fungi were *Geotrichum candidum*, *Paecilomyces sp.*, *Trichosporon coremiiforme*, *Candida catenulata*, *Trichosporon japonicum*, *A. oryzae*, *A. ochraceus*, and *Fusarium sp*.

The main fungi identified in sample A1 were *Fusarium* sp., *Geotrichum candidum*, *Paecilomyces* sp., *Trichosporon coremiiforme*, *Candida catenulata*, and *Aspergillus* sp. There was a predominance of yeasts in sample A2, with the most abundant species being *Trichosporon coremiiforme*, followed by *Trichosporon japonicum*. At the same time, sample A3 showed the highest diversity of fungi, comprising predominantly of *Aspergillus oryzae*, *Fusarium* sp., *A. ochraceus*, and *Penicillium* sp. In sample A4, the main identified fungi were *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp., and *Geotrichum candidum*.

Among the 16 different fungi observed, 75% of the identified genera were common among the samples. *Byssochlamys* sp. was found only in sample A4, while *Trichothecium roseum*, *Cladosporium cladosporioides*, and *A. ochraceus* were identified in sample A3. With regards to the other isolates, the fungi *Fusarium* sp. and *Geotrichum candidum* were common to samples A1, A3, and A4, and *Trichosporon coremiiforme* and *Candida catenulata* were common to samples A1, A2, and A4. The

genera *Paecilomyces* sp. and *Aspergillus* sp. were present in samples A1 and A4, *A. oryzae* and *Alternaria* sp. in samples A1 and A3, *Trichosporon japonicum* in samples A2 and A3, and *Penicillium* sp. in samples A3 and A4.

Sample A3 was the only one to have isolates of the species *A. ochraceus* testing positive for OTA production. The other filamentous fungi were not identified as Ochratoxin A, Aflatoxins B1, B2, G1, and G2, nor citrinin producers.

2.3 Fungal diversity determined by ITS amplicon sequencing

The fungal microbial community composition was characterized for each sample using metaprofiling analysis. A total of 63,857, 48,294, 65,315, and 54,691 high-quality sequences were obtained for samples A1, A2, A3, and A4, respectively (projectID PRJNA682028 and submission SUB8669502, GenBank - NCBI). Sequences with 97% similarity or higher were assigned to the same OTUs, which were later grouped into 154, 139, 189, and 103 OTUs, respectively. Representative sequence for each OTU was screened for further annotation. The Venn diagram (Fig. 2) was generated from the results of all OTUs to show not only the number of OTUs that were shared among samples, but also that differed among them. The taxonomy at the species level was not possible for all OTUs. Sample A3 showed the greatest OTU 'richness' (41 OTUs). At the same time, samples A1, A2, and A4 presented 7, 16, and 9 exclusive OTUs, respectively. Most of the OTUs, representing 70% of the total OTUs obtained, were common between the analyzed samples, with 64, 52, and 50 OTUs shared between 4, 3, and 2 samples, respectively.

The region's most abundant OTUs assigned at the genera level were *Fusarium, Microascus, Debaryomyces, Acremonium, Torulaspora, Trichosporon, Kluyveromyces, Kodamaea, Candida*, and *Aspergillus* (Fig. 3). Next, we evaluated the OTUs for each sample at the genera level. For sample A1, the most abundant OTUs were *Fusarium, Microascus, Debaryomyces, Acremonium*, and *Torulaspora*. Sample A2 had the lowest diversity, whereas sample A3 had the greatest one (confirming the results obtained using the culture-dependent method). The most prevalent genera in sample A2 were *Trichosporon, Fusarium, Kluyveromyces*, and *Torulaspora*. For sample A3, the most common genera were *Kodamaea, Candida, Fusarium, Acremonium, Torulaspora, Aspergillus, Kluyveromyces*, and *Debaryomyces*. For sample A4, the predominant genera of filamentous fungi were *Fusarium, Acremonium, Debaryomyces*, and *Candida*.

Among the main genera of OTUs, the following species were present in the QMA samples: *Kodamaea ohmeri, Acremonium citrinum*, and *Candida catenulata* at 42%, 23%, and 24%, respectively (Fig. 4). The other species identified in these samples were *Gibellulopsis nigrescens, Microascus brevicaulis, Nectria bactridioides, Neosetophoma samarorum, Exophiala salmonis, Aspergillus tamarii, A. westerdijkiae, A. charticola, A. rutilum, Sarocladium strictum, Fusarium redolens, Cryptococcus aerius, Operculomyces laminatus, Mortierella enlongata, Torulaspora delbrueckii*, and *Kluyveromyces lactis*, totaling 10.97%. Several minor contributing species were also identified: *Fusarium oxysporum, Fusarium solani, Candida parapsilosis, Candida etchelsii, Acremonium persicinum, Cryptococcus terricola, Mortierella exigua,*

Trichoderma piluliferum, Minimedusa polyspora, Microdochium bolleyi, Gibberella intricans, Dendryphion nanum, Veronaea musae, Thelebolus ellipsoideus, Rhizophlyctis rosea, Metarhizium carneum, Cladorrhinum foecundissimun, Tetragoniomyces uliginosus, Scutellinia crucipila, Mucor hiemalis, Pseudogymnoascus pannorum, Tetracladium furcatum, Phaeonectriella lignicola, Monographella nivalis, Trichosporon dulcitum, Atractiella solani, Septoriella oudemansii, and *Saccharomyces cerevisiae*.

3. Discussion

Currently, there is an increase in consumption of artisanal ripened cheeses such as QMA. These products are an important source of income for small producers who add value to the product through the ripening process. These cheeses are colonized by fungi during maturation and the knowledge of this mycobiota is important both for assessing the influence on sensory quality and the presence of mycotoxigenic fungi. This study was designed to evaluate the physicochemical characteristics and the mycobiota present in Canastra's Minas artisanal cheese.

Since this is an artisanal product, changes in milk composition (mainly in fat content) and raw materials could provide explanation for some variations detected by the physicochemical analyses. The pH is influenced by fermentation during the production process, and the pH values presented here were within the expected range for the ripened cheeses (Kamimura et al., 2019a).

The presence of fungi in ripened cheeses is of great importance due to their role in the final product characteristics, such as texture and flavor. Important volatile compounds are formed during cheese ripening as a result of the enzymatic activity of fungi (Zheng et al., 2018). The enzymes (proteases and lipases), present during the ripening process, can also come from milk (Ozturkoglu-Budak, 2018).

Both culture-dependent and -independent methods of cultivation demonstrated that sample A3 showed a greater diversity compared to the other three samples. At the same time, most of the genera (~ 70%) were shared among the analyzed samples, suggesting that they are responsible for the unique characteristics of cheese from this region.

In the culture-dependent method, the use of direct replication made it possible to observe some fungi not detected by the serial dilution method. *A. oryzae, Paecilomyces sp., Aspergillus* sp., *Penicillium* sp., and *Byssochlamy*s sp. were present in the cheese rind, although in smaller quantities.

Species that are common in cheeses, such as *Geotrichum candidum* and *Candida catenulata* (Borelli, 2006; Cardoso, et al. 2015), were observed using the culture-dependent method. These yeasts can produce enzymes (proteases, lipases, and β-galactosidase) affecting metabolism of the cheese constituents, such as casein, lipids, lactate, and amino acids (Dugat-Bony et al., 2015). They also contribute, in a beneficial way, to the production of aromatic compounds, such as ethanol, ethyl acetate, 3-methylbutanol, and acetic acid, leading to the development of sensory characteristics of cheese ripening (Zheng et al., 2017). *Geotrichum candidum* is one of the most well-known species that contributes to the sensory characteristics of cheese as a result of the enzymatic action of proteases and

lipases (Boutrou and Guéguen, 2005). The detected genera, such as *Penicillium, Aspergillus*, and *Cladosporium*, are common in cheeses and can be found throughout the ripening process (Yuvaşen et al., 2018). These fungi also influence the cheeses' texture as a result of the enzymatic action; however, the amount of mycelium visible on the cheeses' surface should not be used as a parameter to evaluate texture, since it varies depending on the temperature of the ripening environment and species involved in the process (Jurado and Ruiz-Navarro, 2018). *Aspergillus oryzae* is normally isolated from soil and plants. Therefore, it can be naturally present in the air and in the environment where the cheeses are ripened, since cheese ripening rooms are usually located next to crops. *Aspergillus oryzae* produces the Beta-Galactosidase enzyme (Viana, et. al, 2018), which is used to break down lactose. It also produces proteolytic enzymes (Kumura et. al, 2011) so is capable of growing on the cheese's surface using lactose and proteins as substrate. It has already been detected in cheese (Ayana, et. al, 2014) and is commonly used in the production of proteases from whey.

Despite having some limitations as in the DNA extraction and target genes, the culture-independent methods allow us to verify the presence of microorganisms not detected through plating. This could happen if some of the microorganisms cannot be cultured, or if their population is smaller than required in order to be detected by plating. Culture-independent methods are fast, and, furthermore, they enable us to evaluate the entire microbial community present in cheeses (Jany and Barbier, 2008; Pangallo et al., 2014, Perin et al., 2017). It was possible to classify 292 isolates into 16 species using the culture-dependent method, whereas 47 species were classified by the culture-independent method (ITS region). Three species were common to both methods. The use of culture-dependent and independent methods made it possible to observe a greater number of species, demonstrating the importance of using the methods in characterizing the mycobiota profile.

Debaryomyces and *Kluyveromyces* are usually detected in cheeses and play an important role in their quality; however, in this study they were only identified through the culture-independent method (Banjara, 2015; Laurenčík, 2008; Santos et al., 2017). The population present in the ripened cheese could have been lower than the detectable threshold when the analysis was being performed. This could explain the fact that these genera were not found in the culture-dependent method. Their detection through fold dilution may not have been possible because this method requires a population greater than 2 log CFU/g

These genera positively contribute to the sensory characteristics of cheeses as a result of *Debaryomyces*' lipolytic and proteolytic activities, production of ammonia, and the increase of cheese surface pH, also a desirable event (Cardoso, 2015; Gori, 2007). *Kluyveromyces* can have lipolytic and β-galactosidase activities (Cardoso, 2015). This further demonstrates the important contribution of these microorganisms to development of the sensory characteristics of these cheeses during ripening (Santos, et al., 2017). *Trichosporon*, *Candida*, and *Fusarium* were the prevalent genera detected by both methods. *Fusarium* has been known for many years as a contaminant on the surface of ripened cheeses (Bockelman, et al., 1997); however, certain species like *Fusarium domesticum*, can contribute and participate in the production of cheese (Metin, 2018). *Trichosporon*, a genus of fungus identified by a culture-independent

method in traditional ripened cheese from the Wielkepolska region in Poland (Rychlik, et al., 2017), is also found in raw milk (Delavenne, et al., 2011).

Filamentous fungi and yeasts detected in cheese can come from the milk used during the production (Buehler, et al., 2017; Lavoie et al., 2012), since it does not undergo pasteurization, as well as from the processing environment (Bokulich and Mills, 2013; Wolfe et al., 2014). This mycobiota is influenced by geographical factors, such as longitude, latitude, and altitude, which determine climate, air temperature, and atmospheric humidity. Thus, the mycobiota will be specific to each region (Kamimura et al., 2019b; Zheng, et al., 2018).

Many fungi contribute to the quality of the product; however, there is a concern related to food safety due to the production of mycotoxins by some species. There is a possibility of mycotoxigenic fungi development on the surface of the cheeses during ripening. In the present study, only *A. ochraceus* had tested positive for OTA production.

However, even if the development of mycotoxigenic fungi occured, it would not necessarily result in mycotoxin production. Certain conditions are required: some fungi need a high water activity (0.95) for the production of mycotoxins (Casquete et al., 2018). As water activity decreases during ripening, the risk of mycotoxin production is consequently reduced. Biotic factors are also responsible for modulating potentially mycotoxigenic fungi proliferation and mycotoxin production. Some genera of bacteria are more effective for this biological control, either by competition for nutrients or by the production of organic acids and inhibitory compounds (Hymery et al., 2014). However, further studies are needed to verify whether these species would be able to produce mycotoxins in cheese.

To decrease the presence of this fungus and to ensure safety, one possibility is to increase the population of beneficial fungi found in this study during ripening, which, in addition to development of the sensory characteristics of the product, inhibit the growth of the mycotoxigenic fungus. The cultures used in the cheese ripening should be selected according to their safety, to be both non-mycotoxin-producing and contributing to the flavor and aroma of the cheese (Leistner, 1990). The use of more than one starter culture would improve the product's safety as well as its sensory characteristics (Irlinger and Mounier, 2009).

5. Conclusion

This was the first study evaluating the fungi population present in ripened cheeses in Brazil, contributed to the characterization of geographical indication of Canastra's Minas artisanal cheese. The main observed fungi were *Fusarium* sp., *Geotrichum candidum*, *Paecilomyces* sp., *Trichosporon coremiiforme*, *Candida catenulata*, *Aspergillus* sp., *Trichosporon coremiiforme*, *Trichosporon japonicum*, *A. oryzae*, *Kluyveromyces*, *Torulaspora*, and *Debaryomyces*. *A. ochraceus* potentially mycotoxin-producing was detected but its production capacity when present in cheese must be evaluated. The use of culture dependent and independent methods made it possible to observe a greater number of species. Promising species for future studies of the influence on product quality were identified as *Geotrichum candidum* and

Candida catenulata. Further studies should be carried out to characterize the isolates as a way of improving the quality and safety of Minas artisanal cheese.

Declarations

Conflicts of Interest

The authors declare that no conflict of interest exists in the submission of this manuscript.

Authors' Contributions

Michele de Oliveira Paiva Aragão: carried out experiments, interpreted the results and drafted the manuscript. Suzana Reis Evangelista: data analysis, drafted the manuscript and editing. Fabiana Reinis Franca Passamani: data analysis and drafted the manuscript,

João Pedro M. Guimarães: data analysis and drafted the manuscript, Luiz Ronaldo de Abreu: designed the study and reviewed this manuscript. Luís Roberto Batista: designed the study, conception, methodology, funding, project administration, reviewed this manuscript. All authors read and approved the final manuscript.

Availability of data and material, Code availability, Ethics approval, Consent to participate, Consent for publication: 'Not applicable'

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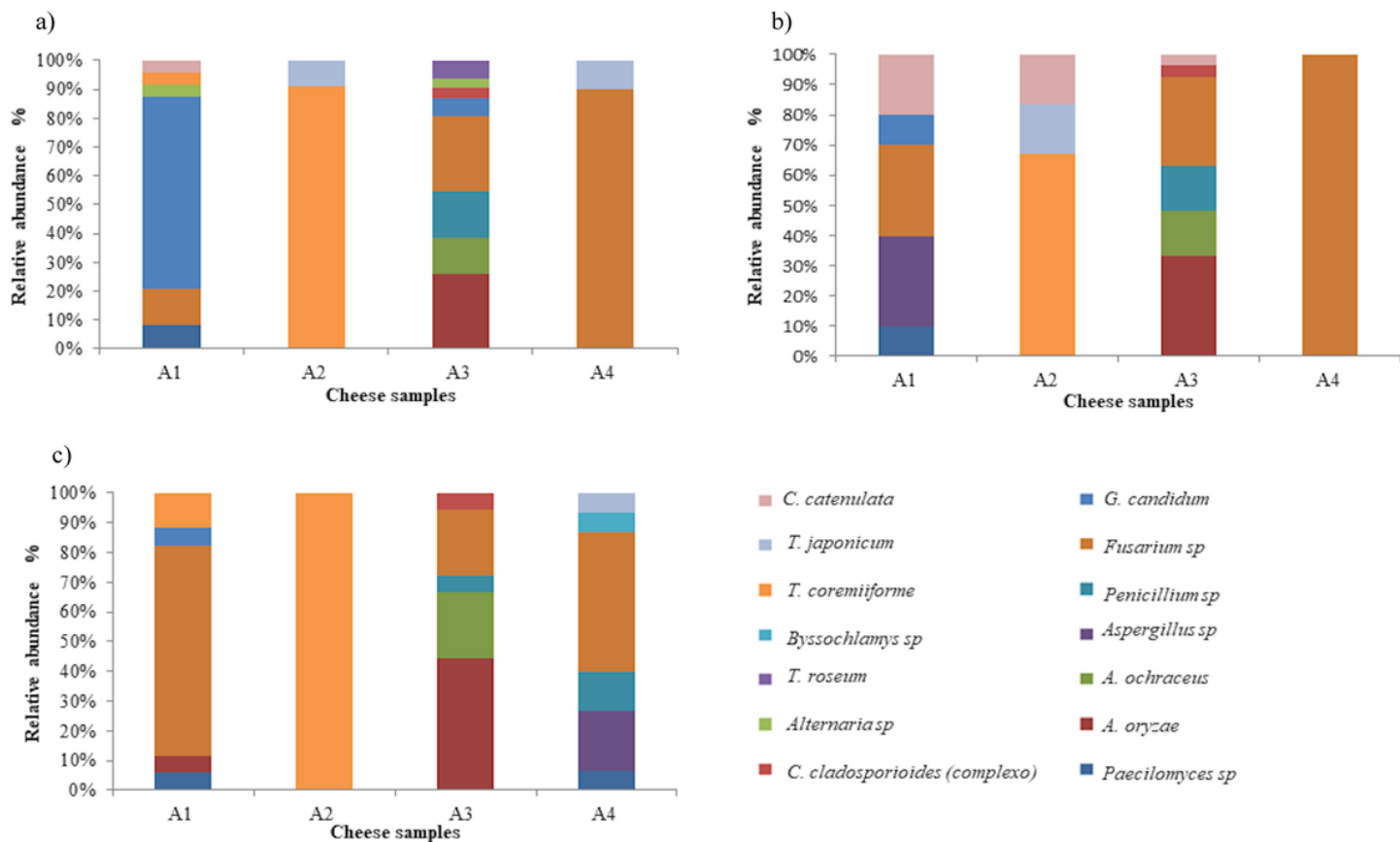
Figures

Figure 1

Relative abundance of fungi population present in the cheese samples cultured using the following media: (a) dichloran rose bengal chloramphenicol (DRBC), (b) dichloran glycerol medium base (DG18) and (c) malt extract agar (MA)
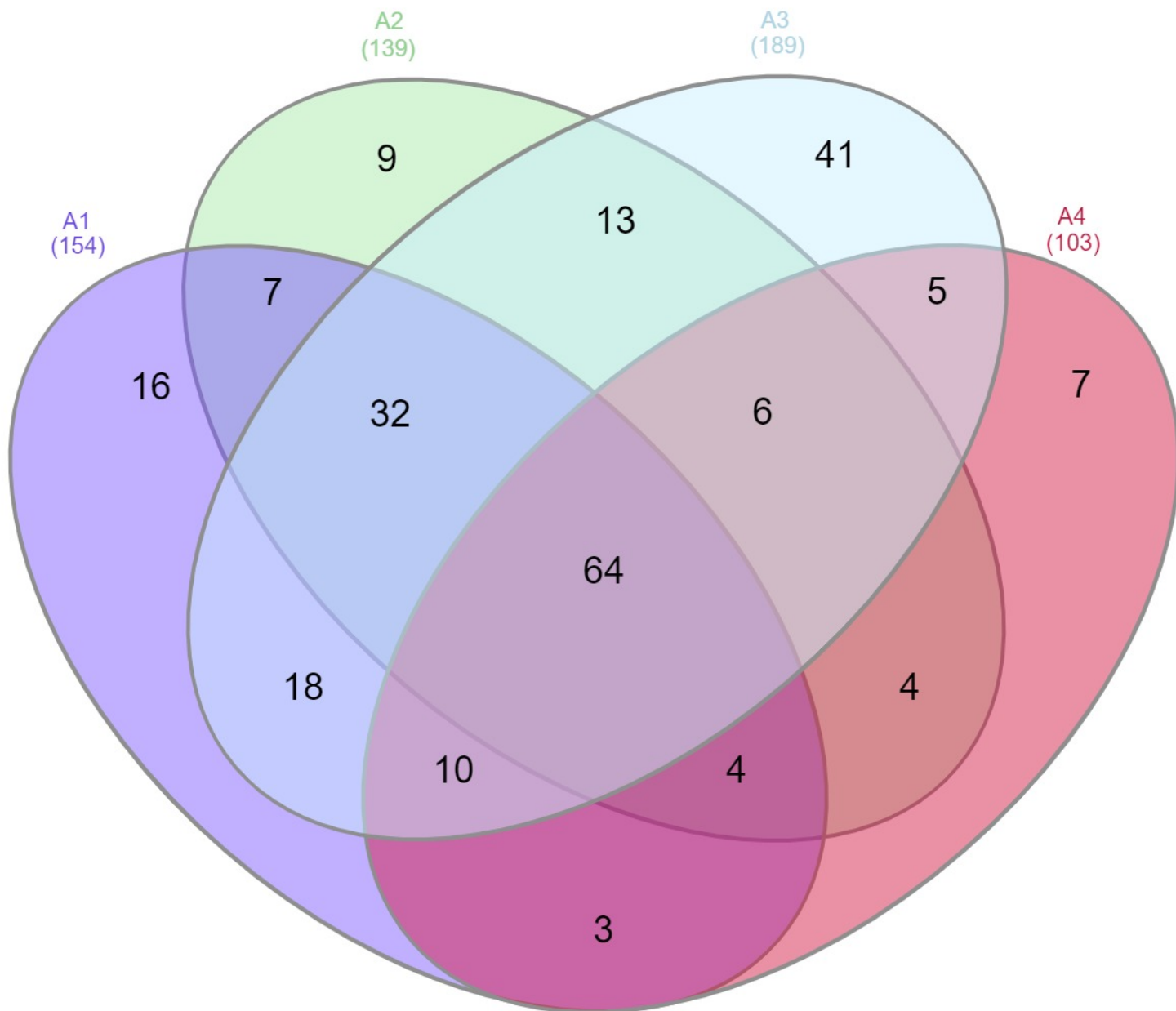
Figure 2

Venn diagram based on all obtained OTUS showing the number of shared and exclusive OTUs among the samples
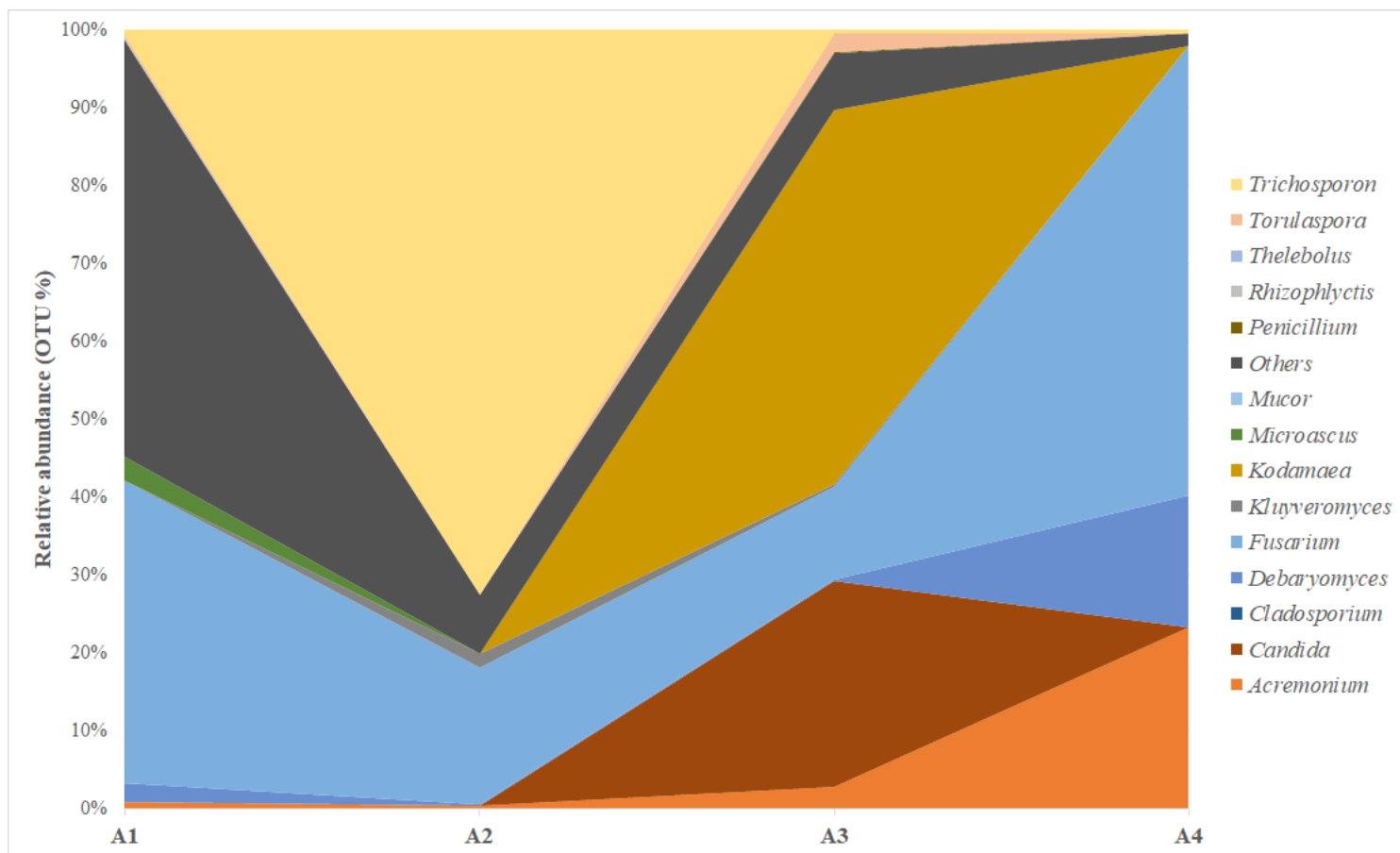
Figure 3

Percentage of operational taxonomic units (OTU) related to the fungi genera observed in each cheese sample analyzed. Any OUT that could not be identified are represented by "Other"
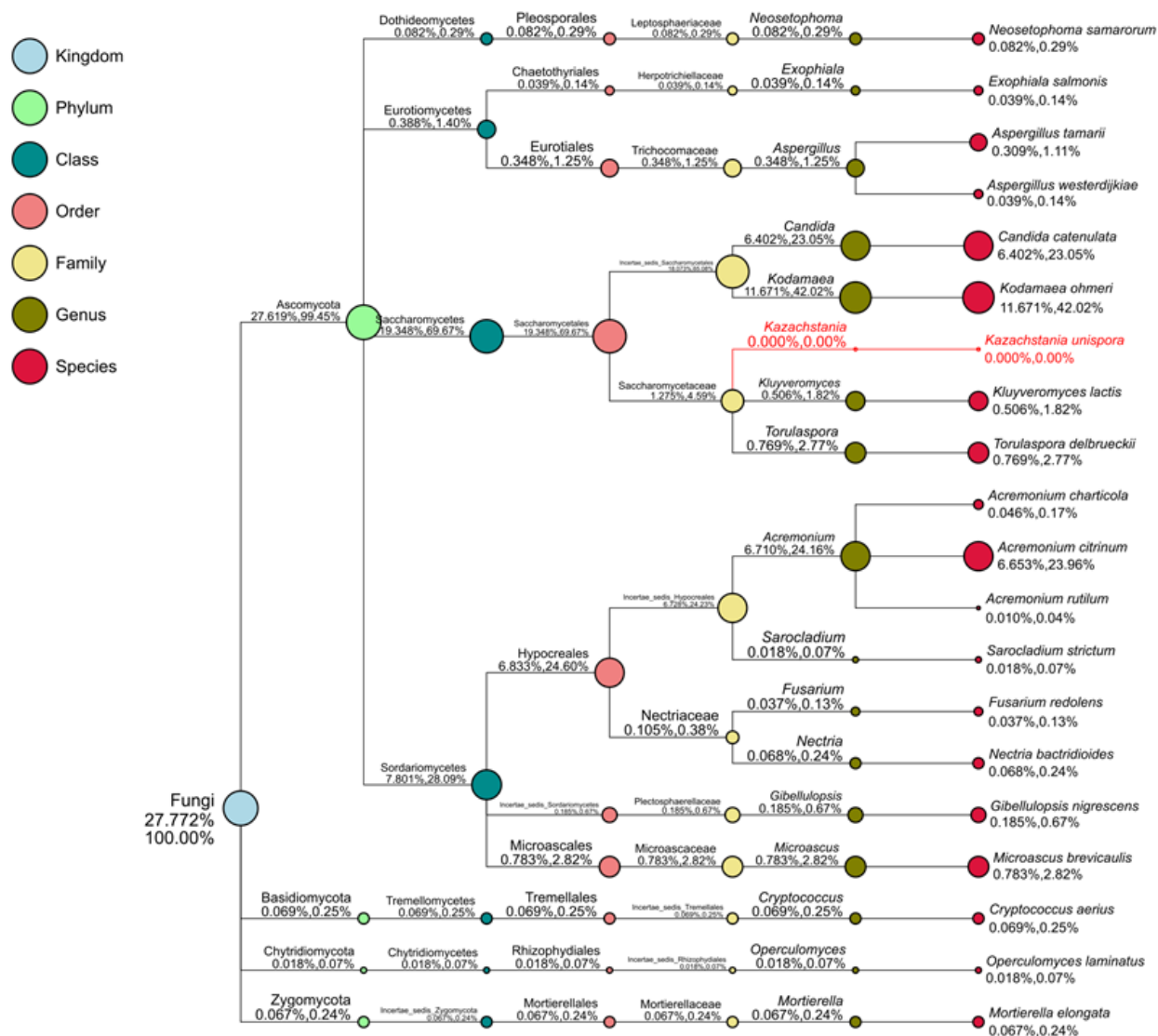
Figure 4

MEtaGenome Analyzer classification tree with taxonomic affiliations for ripened Canastra cheese. 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