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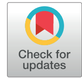
Morphological and molecular characteristics of stored-product mites found on Brazilian ripened cheeses



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ABSTRACT

The production and consumption of cheese that contains mites has become a gastronomical trend in Brazil. However, the making of these cheeses has been a traditional process, and thereby empirical in nature, without the regulatory oversight of the Brazilian authorities. Depending on the species, these mites might present health risks for those consuming them. Therefore, we conducted initial morphological and molecular analyses to characterize the diversity of cheese mites present on these Brazilian ripened cheeses. Our results revealed that the mites were predominantly *Tyrophagus putrescentiae* and to a lesser degree *Sancassania aff. feytaudi*. Future studies need to be performed to indicate whether these species meet the requirements to be characterized as generally recognized as safe (GRAS status) and are capable of conferring unique and desirable sensory characteristics to the cheese.

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1. Introduction

In some situations, during ripening, the presence of a fine powder can be observed close to the cheese or on its rind. This powder, brown in color and similar to “termite powder”, is made up of excreta resulting from the colonization of the cheese by mites, fungal hyphae (Stejskal et al., 2015; Carvalho et al., 2018), live and dead mites and deteriorated cheese (Deong and Roadhouse, 1922; Carvalho et al., 2018).

The presence of mites in Brazilian cheese is related to safety and technological concerns, causing, at a minimum, economic losses, considering that mites feed on the cheese and reduce the weight of pieces during ripening (Robertson, 1952; Carvalho et al., 2018). Several countries legislate maximum tolerance limits of mites in cheese (Carvalho et al., 2018). However, in countries such as France and Germany, the presence of mites during the ripening and sale of Mimolette and Milbenkäse cheese types is essential for the development of specific characteristics that are valued by consumers (Bruckner and Heethoff, 2016).

With the rise of production of artisanal cheese in Brazil and the introduction of different ripening techniques, Brazilian cheesemakers have begun to develop empirical techniques for ripening with the presence of mites in order to differentiate their products sensorially, as is done for the previously mentioned French and German cheeses. However, to the best of our knowledge, there are no studies on the presence of mites in Brazilian cheeses citing either negative results (contamination and economic losses) or positive ones (mites as a technological adjunct).

Mite species are usually identified by morphological and taxonomic analyses (Dawood and Ali, 2015; Melnyk et al., 2010), but considering the complexity of this form of identification and the morphological similarity between cryptic species, the use of molecular markers has become a necessity and a complementary approach (Khaing et al., 2014). However, there are still few records of DNA sequences from mite species stored in databases, even for cytochrome oxidase I (*COI*), a mitochondrial marker widely used in species identification. Therefore, an increase in this type of data in the registry, together with morphological analysis, is fundamental for future comparative studies (Carvalho et al., 2018).

There is a scarcity of information about mites present in Brazilian cheeses, emphasizing the importance of using a polyphasic

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approach for taxonomic identification. This work first establishes a method for sampling mites from cheese, which maintains the integrity of body structures that are taxonomically important. It also aims to identify the mite species found in cheeses from different regions of Brazil using morphological characteristics and the *COI* molecular marker.

2. Materials and methods

2.1. Protocol for sampling mites from cheese

There is no specific method described in the literature for sampling stored-product mites from ripened cheeses for subsequent taxonomic and molecular identification. Thus, we previously tested three distinct protocols in a 5 kg cheese wheel containing a high amount (>10 mites/cm^2) of stored-product mites. This cheese wheel was produced in Santa Catarina State (Brazilian South region). Our protocols (Ps) consisted of a fractionated piece (approximately 150 g) of cheese sampled and maintained in a vacuum sealed plastic bag (P1), a fractionated piece (approximately 150 g) of cheese sampled and maintained in a plastic bag sealed without vacuum (P2) and only the stored-product mites present on the surface (approximately 10 cm^2) of a fractionated piece (approximately 150 g) of cheese (P3). To collect the mites on P3, we scraped the rind area with a spatula and collected 1 g of the fine brown powder. The powder was conserved in a centrifuge tube with a cap containing 5 mL of 70% (v/v) ethanol. Twelve cheese samples from three Brazilian states (Minas Gerais, São Paulo and Santa Catarina) were collected using P3. The samples were maintained in isothermal boxes with ice and delivered to the laboratory for sorting, counting, and taxonomic identification.

2.2. Mite identification

2.2.1. Morphological identification

The mites present in the cheese samples were observed under a stereomicroscope (SMZ800, Nikon, Japan) at 30×. In the cheese samples where different morphological types were observed, an estimation of the proportion of each type was performed. Each sample was mixed with distilled water and then strained through a 360 mesh sieve. The mites present in the cheese were captured and then maintained in 50 mL of 70% (v/v) ethanol. The mites collected were mounted on microscopic slides in Hoyer medium and dried for 10 days in a drying chamber with a temperature of approximately 50 °C. After drying, the slides were sealed with crystal varnish and observed under a differential interference contrast (DIC) microscope with phase contrast (Eclipse Ni, Nikon, Japan).

Specimens were identified based on dichotomous identification keys for families and species. The genus *Acarus* was based on Griffiths (1970); the genus *Sancassania* (=*Caloglyphus*), on Türk and Türk (1957) and Klimov and O'Connor (2003); and the genus *Tyrophagus*, on Johnston and Bruce (1965), Hughes (1976), Fain and Fauvel (1993) and Fan and Zhang (2007).

2.2.2. Molecular identification

After morphological identification, two individuals were used to obtain a biological duplicate of morphologically equal organisms for molecular identification. In the cheese samples that had more than one morphological type, two individuals of each type were used. The samples were maintained in 70% (v/v) ethanol at −20 °C until DNA extraction.

Total genomic DNA extraction was conducted for each organism using a QIAamp® DNA Micro Kit (Qiagen, USA), following the specifications for tissue DNA extraction protocol. The selected individual was added to a 1.5 mL tube containing 180 μL of cell lysis buffer with a brush and the aid of a stereoscopic binocular microscope with a 50× magnification (LED TIM-2B, Opton, Japan). The mite was then macerated with the aid of a plastic macerator for small tubes, and 20 μL of proteinase K was added. From this moment, the extraction protocol followed the steps contained in the manufacturer's manual. The extracted DNA was stored at −20 °C.

For *COI* sequence identification, the gene fragment was amplified using the following primer pairs of oligonucleotides: COI-F 5′ GTT TTG GGA TAT CTC TCA TAC 3′ and COI-R 5′ GAG CAA CAA CAT AAT AAG TAT C 3′ described by Yang et al. (2011). The reaction conditions applied were 1 U of *Taq* DNA polymerase (Thermo Scientific Inc., USA), 1X buffer, 2.5 mM $MgCl_2$, each dNTP at 160 μM and each primer at 0.4 μM for a 50 μL final volume. The amplification conditions used were an initial denaturation stage at 94 °C for 3 min, 35 cycles at 48 °C for 30 s and 72 °C for 1 min, followed by a final extension step of 72 °C for 7 min.

PCR products separated through electrophoresis were visualized on 1% (w/v) agarose gels stained with Gel Red™ (Biotium, USA) under a UV light transilluminator. The amplicon was purified and sequenced in both directions using the same primers of amplification (Macrogen, Korea). The sequence identity obtained was checked using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) available from the GenBank database of the U.S. National Center for Biotechnology Information (http://www.blast.ncbi.nlm.nih.gov/).

3. Results and discussion

3.1. Protocol for sampling mites from cheese

During the screening process in the laboratory, it was verified that the mites resulting from P1 were all degenerated and it was not possible to identify them. P2 preserved only a small number of live mites in good enough condition for morphological identification (only eight individuals). In P3, the mites were dead due to their immersion in ethanol; however, they remained preserved and in good condition for morphological and molecular identification (327 individuals preserved in 5 mL of ethanol).

Mites are aerobic organisms that, during ripening, burrow caves in the rind entering the cheese wheels. They are always found in the external parts and/or in cracks formed on the surface (Marcellino and Benson, 2013). This aerobic requirement may explain why mites packed in vacuum plastic bags are degraded and in no condition for identification. The pressure exerted by the vacuum on the physical structure of these individuals may also have contributed to this situation. The results pointed to the use of P3 as the best option for mite sampling.

One problematic aspect related to ripening cheese with mites is the presence of these organisms in large quantities on the product surface. At points of sale, these mites can migrate from one cheese to other cheeses and/or food products and into the surrounding environment, causing a local infestation. This suggests that to avoid the dissemination of these organisms at the time of commercialization, vacuum packing cheese could be useful, beyond merely using it as a method of controlling the contaminant.

3.2. Mite identification

Although a 378 bp fragment was amplified, the molecular identification was made from 338 bp due to the direct sequencing of the PCR products. The morphological and molecular identification demonstrated that the mites present in the Brazilian sampled cheeses were predominantly *Tyrophagus putrescentiae* (Schrank, 1781) with the exception of the Minas (Serro) type cheese (sample

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