Occurrence of filamentous fungi isolated from matured blue cheese

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Abstract

Matured blue cheese can be made from cow’s milk associated with the inoculated fungus *Penicillium roqueforti*, which guarantees specific sensorial characteristics. Recently, Brazil’s fine cheese production and consumption have increased by more than 200%, highlighting the relevance of microbiological quality control of these products. Fungal contaminations are responsible for significant losses in cheese production and provide a suitable environment for mycotoxins production, constituting a hazard to public health. In this work, we evaluated the mycological contamination profile of matured blue cheeses commercialized in Brazil. Samples of ten different brands were analyzed by serial dilution method, by plating in the Dicloran Rose of Bengal Chloramphenicol (DRBC) culture medium and Dicloran Glycerol Medium Base (DG18). Subsequently, different fungi morphotypes were isolated and morphologically identified. As a result, 461 fungi were isolated and identified, notably *Aspergillus aculeatus*, *Penicillium roqueforti* and *Penicillium solitum*. All samples were contaminated by filamentous fungi, amongst those, many already reported as mycotoxin producers, which underlines the relevance of microbiological monitoring.

Keywords: *Aspergillus*; Dairy; Food microbiology; Mycology; Food safety; Morphological characterization.

Resumo

O queijo azul maturado pode ser fabricado a partir do leite de vaca, associado à inoculação do fungo *Penicillium roqueforti*, o qual confere características sensoriais específicas. Recentemente, a produção e o consumo de queijos finos no Brasil aumentaram mais de 200%, destacando a relevância do controle de qualidade microbiológica desses produtos. Contaminações fúngicas são responsáveis por perdas significativas na produção de queijos e provêm um ambiente propício para a produção de micotoxinas, constituindo-se um risco à saúde pública. Neste trabalho, avaliamos o perfil de contaminação micológica de queijos azuis maturados comercializados no Brasil. A mostras de
dez diferentes marcas foram analisadas pelo método de diluição seriada, através de plaqueamento em meio de cultura Ágar Dicloran Rosa de Bengala Cloranfenicol (DRBC) e Ágar Dicloran Glicerol Médium Base (DG18). Subsequentemente, diferentes morfotipos fúngicos foram isolados e morfologicamente identificados. Como resultados, 461 fungos foram isolados e identificados, notavelmente, Aspergillus aculeatus, Penicillium roqueforti e Penicillium solitum. Todas as amostras analisadas apresentaram contaminação por fungos filamentosos, muitos dos quais já reportados como produtores de micotoxinas, enfatizando a relevância do monitoramento microbiológico.

Palavras-chave: Aspergillus; Laticínios; Microbiologia de alimentos; Micologia; Segurança de alimentos; Caracterização morfológica.

1 Introduction

Blue cheese is characterized by its moldy flavor and blue-green veined appearance, created by maturation process and Penicillium roqueforti growth that produces lytic enzymes such as lipases and decarboxylases (Cantor et al., 2017). These enzymes can convert the fatty acids via β-oxidative pathways, increasing the presence of methyl-ketones, mostly 2-pentanone, 2-heptanone and 2-nonanone, directly involved in blue cheese sensory characteristics (Cao et al., 2014). Gorgonzola is produced from pasteurized cow milk, and is one of the most consumed blue-veined cheeses. Since 1996, Gorgonzola manufacturers awarded a European protected designation of origin (European Union, 1996). In Brazil, it is established that blue cheese needs to be ripened for 35 days (Brasil, 2007), if the maturation is performed at least for 90 days, this matured blue cheese (MBC) can be commercialized as Roquefort type or Gorgonzola type, being the latter produced exclusively from cow milk (Brasil, 1952).

Although pasteurization is an efficient method for milk microbiological control (Van Asselt et al., 2017), undesirable contaminations may occur during manufacturing procedures, namely introduction of starter culture, ripening or brining (Banjara et al., 2015). Brine salting is a crucial step of MBC manufacture, which conveys characteristics NaCl gradient from surface to the core, meanwhile halotolerant microorganisms are also selected for further ripening (Martin & Coton, 2016).

P. roqueforti proteolytic activity increases the pH, creating a suitable environment for fungal growth and development in long ripening cheeses (Bernini et al., 2015; Mucchetti & Neviani, 2006). Among the commonly isolated cheese-associated fungi, the genera Penicillium, Aspergillus, Cladosporium cladosporioides complex, Geotrichum, Mucor and Trichoderma are the most frequently described (Hymery et al., 2014).

However, not all the isolated fungi species exert beneficial effects; some mold growth may produce secondary metabolites, known as mycotoxins, hence constituting a potential risk to public health (Chen et al., 2010). Cheese properties and safety are directly related to the microbial community in the product; therefore, many studies have been conducted to assess commonly associated bacteria, yeast and fungal species (Dugat-Bony et al., 2016; Dobson, 2017; Ramos-Pereira et al., 2019). Nonetheless, in Brazil, one of the world’s largest dairy producer, mycological contamination data regarding milk-based products are still scarce (Lemos et al., 2018). Therefore, this work assesses the mycological profile in ten different brands of MBCs commercialized throughout Brazil.

2 Material and methods

This study was conducted in the Food Mycology and Mycotoxins Laboratory – Department of Food Science, at Federal University of Lavras – UFLA, city of Lavras, Minas Gerais, Brazil. Ten samples, each from a different brand, of MBCs were obtained from stores in Southern Minas Gerais (21° 14’ 43” S, 44° 59’ 59” W). All samples were produced in dairy factories under federal inspection and are allowed to be traded across the country. After purchase, these samples were stored under refrigeration (4 °C).
Samples were analyzed using the serial dilution method, to obtain the first dilution; 25.0 g of each MBCs were diluted in 225 mL of peptone water 0.1%. Next, this solution was macerated and kept under agitation in Stomacher® (Metroterm, Brazil), using a paddle speed of 490/min for 2 minutes. Then, sample aliquots of 0.1 mL (1:10, 1:100, 1:1000 and 1:10000) were spread on petri plates surfaces containing Dichloran Rose-Bengal Chloramphenicol Agar (DRBC) and Dichloran Glycerol Medium Base (DG18) (Merck Millipore, United States) media, according to the spread plate technique. DRBC and DG18 media were prepared according to Hocking & Pitt (1980). Plates were incubated in BOD, at 25 °C, from 5 to 7 days, for posterior filamentous fungi analysis. Each sample of the study was run in triplicate.

After colony growth in plates, colony forming units (CFUs) were counted as described in other study (Pitt & Hocking, 1997). Colonies were individually characterized by size, conidia color, mycelium and reverse color, and then they were grouped into morphotypes. A representative sample of each sample was obtained through the square root method (Sengun et al., 2009) accounting to the total number of counted isolates (n= 461). Subsequently, fungi colonies were selected and transferred to petri plates containing Malt Extract Agar (MEA) medium (Merck Millipore, United States) and incubated in BOD at 25 °C, during seven days.

Pure colonies were transferred to identification media, according to each genus. Isolates of Aspergillus and Penicillium genera were grown in petri plates containing Czapek Yeast Agar (CYA) (Merck Millipore®) medium, at 25 °C and 37 °C, and MEA medium at 25 °C (Klich, 2002), while other genera were grown in MEA only at 25 °C. After seven days of incubation, the macroscopic and microscopic characteristics of the filamentous fungi were observed, to discriminate morphological hallmarks (Samson et al., 2014; Visagie et al., 2014).

Observed macroscopic features were colony color, mycelial characteristics, presence or absence of exudate, reverse color, colony diameter, presence or absence of soluble pigmentation and sclerotia/cleistothecia morphology. The microscopic properties evaluated were branch types, conidiophores length, texture and width, metulae and phialides length and texture, conidia form and texture. These structures were observed through optical microscope (Bioval, Brasil). Fully characterized isolates were stored in microtubes, and integrated into the culture collection of the Food Mycology and Mycotoxins Laboratory – Department of Food Science, Federal University of Lavras – UFLA, Lavras – MG.

3 Results and discussion

We were able to retrieve filamentous fungi from all ten samples, accounting a total of 461 isolates. One sample (2) presented the highest level of retrieved fungi isolates (106) or 23% of total, while samples 7 and 10 presented the fewest fungal presence (14 and 15) isolates or 3.03% and 3.35% respectively (Figure 1).

**Figure 1.** Total amount of fungi isolates found in ten different samples of Brazilian matured blue cheese.
In MBCs, it is expected to found mainly *P. roqueforti* or common cheese-associated fungi (Seratić et al., 2011). In fact, the vast majority of fungi obtained in this work were classified as *P. roqueforti* (n = 282). Other fungi species were also obtained and classified into two main groups, the common cheese-associated and possible contaminants (Non *P. roqueforti*).

Figure 2 shows the distribution of non *P. roqueforti* isolates (n = 179) across samples. Most samples with high amount of fungi isolates (Figure 1), also contributed mainly to the total amount of non-*P. roqueforti* found. However, sample 1 data highlights the relevance of complete morphological characterization, since most of this sample’s amount of isolated fungi (Figure 1) do not belong to any other species rather than *P. roqueforti* (Figure 2).

Cheese-associated fungi isolates are those species already reported as players in cheese ripening microbiota such as: *P. brevicaespactum*, *P. commune*, *P. verrucosum*, *A. versicolor*, and *Geotrichum candidum* (Martín & Coton, 2016). Whilst contaminants fungi are introduced mainly through animal feed or the environment (Hymery et al., 2014). Non-*P. roqueforti* species are a concern in matured cheeses as it can be an indication of mycotoxin producers, such as *Aspergillus* spp. (Ozturkoglu-Budak & De Vries, 2017).

Assuming that the pasteurization process was properly conducted, the majority of milk contaminants should have been previously removed or inactivated (Heiman et al., 2016), indicating that contamination may also occur either from starter strains, environment, processing equipment or even from incorrect manipulation. Although filamentous fungi grow very slowly, their growth can produce hazardous secondary metabolites known as mycotoxins, constituting risk to consumers, especially in long-ripening milk products as MBCs. Thus, we assessed all non-*P. roqueforti* species to address their taxonomic filiation.

We have found several species belonging to both Aspergillus and Penicillium genera. The majority of isolates belong to *A. aculeatus* (30.72%) and *P. solitum* (21.79%) species (Figure 3). Unexpectedly, *Geotrichum candidum* was the third most prevalent species (16.2%).
In addition, *G. candidum* and *Cladosporium* sp. were already characterized as commonly associated to mold-ripened cheeses microbiota. Thus, these isolates should not be considered exogenous contaminants. The genera *Penicillium*, *Aspergillus* and *Cladosporium*, have been documented in other studies that showed strong presence along cheese production chain (Ozturkoglu-Budak & De Vries, 2017).

*Geotrichum candidum* is a common milk-associated dimorphic yeast. It has been shown that this microorganism can contribute positively in taste and aroma of dairy products, further *G. candidum* growth can exert antagonistic effect on undesirable microorganisms such as *Mucor* spp. and *Listeria monocytogenes* (Alper et al., 2011). A recent whole genome sequencing study compared *G. candidum* and other Saccharomycotina yeasts and bring to light the differential gene content of this yeast, which may explain its biochemical properties (Morel et al., 2015). For instance, this dairy yeast has four carboxylesterase/type B lipase genes with no clear homologs in Saccharomycotina, but equivalents in Pezizomycotina, these lipases are secreted extracellular enzymes, and can play a key role in cheese aroma formation through triacylglycerol metabolism.

One of the most prevalent fungi reported here is *Cladosporium* spp., this fungus is an air-carried fungus, and it generally comes from the ventilation system or dust, being commonly found in cheese (Nevalainen et al., 2015). This fungus presence can cause black tight spots on cheese’s surface, deteriorating the aesthetical, nutritional and organoleptic properties of the cheese (Messini et al., 2017).

We also characterized many other species of *Penicillium* genus such as: *P. solitum*, *P. verrucosum*, *P. brevicompactum*, *P. commune* which, amongst others, have already been documented as commonly associated to mold-ripened cheese (Desmasures, 2014) however, their clear role in cheese-ripening is still unclear. Another cheese mycological survey performed in Spain found the same general profile described here, assessing *C. sphaerospermum*, *G. candidum* and *P. solitum* as the most prevalent species in ovine raw milk cheese (Manchego) (Marín et al., 2015). Interestingly, Marín and colleagues further reported several *Fusarium* spp. occurrences, indicating that pasteurization increases food safety of Gorgonzola type cheese.

Furthermore, we characterized *P. camemberti*, which is widely used in fine cheese manufacturing like camembert and brie cheeses (Hymery et al., 2014), and can be encountered mainly due to its introduction during cheese making. Meanwhile, other species (*A. aculeatus*, *A. flavus*, *A. japonicus*, *A. niger*, *A. parasiticus*, *P. aurantiogriseum* and *P. citrinum*, *P. corylophilum*) are unexpected in ripened cheeses, and probably constitute environmental contamination (Dobson, 2017).

Overall, matured blue cheese is a complex food ecosystem with variable gradients of pH and NaCl, but low levels of O₂ and CO₂. This heterogeneity creates different habitats on the surface and in the core of the
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cheese, which select specific micropopulations (Cantor et al., 2017). Disturbances in this regular distribution are mainly associated to contamination during salting stage, or due to air flow.

One of highest concerns regarding fungal contamination are the mycotoxins, these low-molecular weight molecules are secondary metabolites, hence are not directly essential for fungal growth (Hymery et al., 2014). Despite its relevance, no clear function has been established for secondary metabolites yet. Some authors claim that these molecules are related to niche competition (Fox & Howlett, 2008). Mycotoxins can be harmful to humans causing mycotoxicosis, ranging from simple food poisoning to carcinogenic effects, depending on the type of mycotoxins, concentration and exposure rate (Rocha et al., 2014). On top of the list, mycotoxins are known for their industrial processing resistance; since mold can grow on a wide range of foods at any stage of production, food fungi surveillance is a relevant practice to ensure consumers health.

The main mycotoxins reported in cheese are roquefortine C, patulin, cyclopiazonic acid, mycophenolic acid, citrinin, aflatoxin, ochratoxin A amongst others (Dobson, 2017). Here we also report the presence of notorious mycotoxin producing fungi, namely: A. flavus, A. japonicus, A. parasiticus, A. niger, P. verrucosum and P. citrinum. Even though morphological characterization by itself is not suitable to detect mycotoxin production, it still constitutes a reliable tool to perform overall mycological profile and can be extended by enzymatic detection assays. Aflatoxin M1 is the only mycotoxin controlled by the Brazilian Health Regulatory Agency (ANVISA) in cheese, a maximum tolerable limit is 2.5 µg/kg (Brasil, 2011),

Aspergilli species reported in this work belong mainly to Nigri section, known as black aspergilli, A. aculeatus is also a member of Nigri section and was by far the most prevalent species characterized in all analyzed samples (Figure 3). This group is responsible for producing a variety of bioactive natural products, including aculeacins (antibiotics and antifungal agents), CJ-15,183 (antifungal agent) and secalonic acids (toxins) (Yodsing et al., 2018). Although no clear risk of A. aculeatus presence in cheese has been assessed yet, this speciesields an array of molecular weapons against other microorganisms, and could impair proper P. roqueforti development affecting the final quality of blue veined cheese.

Our results show that P. roqueforti and cheese-associated fungi species presented the highest prevalence in all analyzed samples, which corroborates the current data (Martin & Coton, 2016) and other microbiological surveillance data in blue cheese (Banjara et al., 2015). We also reported the presence of other aspergilli species, which may be caused by environmental contamination, with a higher prevalence of A. aculeatus, emphasizing the relevance of mycological monitoring of matured blue cheese for both industry and consumers.

4 Conclusion

1. There was no direct correlation between total amount of isolated fungi and contaminants, thus morphological characterization is necessary to assess contaminated samples;
2. A. aculeatus was the most prevalent exogenous species in the analyzed samples, this fungus massive presence can indicate that P. roqueforti growth was impaired, compromising the quality of the products;
3. Mycotoxin producing species have been characterized, however mycotoxin presence was not assessed.

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