Food Research International 183 (2024) 114214

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Unveiling ochratoxin A and ochratoxigenic fungi in Brazilian artisanal Cheeses: Insights from production to consumption

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ARTICLE INFO

Keywords:
Aspergillus section *Circumdati*
Ochratoxin A
Artisanal cheese

ABSTRACT

Ochratoxin A (OTA) is a toxin produced by several *Aspergillus* species, mainly those belonging to section *Circumdati* and section *Nigri*. The presence of OTA in cheese has been reported recently in cave cheese in Italy. As artisanal cheese production in Brazil has increased, the aim of this study was to investigate the presence of ochratoxin A and related fungi in artisanal cheese consumed in Brazil.

A total of 130 samples of artisanal cheeses with natural moldy rind at different periods of maturation were collected. Of this total, 79 samples were collected from 6 producers from Canastra region in the state of Minas Gerais, since this is the largest artisanal cheese producer region; 13 samples from one producer in the Amparo region in the state of São Paulo and 36 samples from markets located in these 2 states. *Aspergillus* section *Circumdati* occurred in samples of three producers and some samples from the markets. *A.* section *Circumdati* colony counts varied from 10^2 to 10^6 CFU/g. Molecular analysis revealed *Aspergillus westerdijkiae* (67 %) as the most frequent species, followed by *Aspergillus ostianus* (22 %), and *Aspergillus steynii* (11 %). All of these isolates of *A.* section *Circumdati* were able to produce OTA in Yeast Extract Sucrose Agar (YESA) at 25 °C/7 days. OTA was found in 22 % of the artisanal cheese samples, ranging from 1.0 to above 1000 μg/kg, but only five samples had OTA higher than 1000 μg/kg. These findings emphasize the significance of ongoing monitoring and quality control in the artisanal cheese production process to minimize potential health risks linked to OTA contamination.

1. Introduction

The production of artisanal cheese in Brazil is of great economic, cultural, social and food importance, with the state of Minas Gerais being one of the largest producers of this type of cheese in the country (Pinto et al., 2009; Pineda et al., 2021). Brazilian artisanal cheeses are typically marketed with short ripening times, ranging between 14 and 22 days, but they can also be cured for periods that exceed 90 days of maturation in ripening rooms or in caves. These environments contain their own microbiota, influenced by the region, climate, and natural conditions, promoting the growth of different types of fungi in cheese, including toxigenic ones. Therefore, artisanal cheese is a product susceptible to fungal growth that can produce toxins, such as ochratoxin A (OTA). Caves are underground rooms, naturally humid environments with their own microbiota. The characteristics of the cave are conducive to the growth of various types of fungi starting on the cheese rind and permeating into the inner layers. In recent years, several artisanal cheese produces have allowed the growth of fungi with white spores, giving rise to the so-called flowery rind cheese. In addition there is another type of cheese with colored spores, referred to as natural moldy rind cheese, which contributes distinctive in sensory characteristics to the products.

Ochratoxin A (OTA) is a mycotoxin produced mainly by *Aspergillus ochraceus*, *Aspergillus westerdijkiae, Aspergillus steynii, Aspergillus carbonarius, Aspergillus niger, Penicillium verrucosum* and *Penicillium nordicum* (Pitt & Hocking, 2022; Pattono et al., 2013; Anelli et al., 2019). This mycotoxin is considered nephrotoxic, teratogenic, immunotoxic and hepatotoxic to domestic and laboratory animals (O' Brien & Dietrich, 2005). Based on ample evidence of carcinogenicity in animal studies, the

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https://doi.org/10.1016/j.foodres.2024.114214
Received 30 November 2023; Received in revised form 19 February 2024; Accepted 10 March 2024
Available online 14 March 2024

International Agency for Research on Cancer (IARC) categorized OTA as a possible human carcinogen (category 2B) (IARC, 1993). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has confirmed a Provisional Tolerable Weekly Intake (PTWI) of 100 ng/kg human body weight for this mycotoxin, which corresponds to 14 ng/kg human body weight per day (JECFA, 1995).

Recently, species of *A. westerdijkiae* and *A. steynii* producing OTA were found in 45 % of cave cheese samples in Italy. In that study, OTA was detected in 36 % of samples (Anelli et al., 2019). Pattono et al. (2013) also detected the presence of OTA in semi-hard cheeses in Italy. This fact suggests that OTA may also be present in cheese manufactured in Brazil, since these *Aspergillus* species are well adapted to warmer climates and have already been isolated from foods produced in Brazil such as coffee, cocoa, black peppers, among others (Taniwaki et al. al., 2003; Copetti et al., 2010; Silva et al., 2021).

So far, there is no Brazilian regulation that determines maximum level of OTA in cheese in general. Few studies have been carried out on the presence of fungi that are potential sources of OTA in cheese produced and marketed in Brazil. Therefore, the aim of this research was to investigate the presence of ochratoxigenic *Aspergillus* species, as well as OTA and investigate the source of this toxin in artisanal cheese from producers to consumers.

2. Materials and methods

2.1. Samples

A total of 130 samples of artisanal cheese with natural moldy rind of approximately 1 kg, at different periods of maturation were collected as shown in Table 1. Of this total, 79 samples were collected from 6 producers from the Canastra region in the state of Minas Gerais, since this is the largest artisanal cheese producer region in the country; 13 samples from one producer in the Amparo region in the state of São Paulo and 36 samples from markets of these 2 states (MG and SP). Some of the cheese have undergone a "*toilette*" process that is generally made with water sandpaper, others were collected from the ripening caves or cellars, with a visible fungal growth. In addition, scrapings were made of the benches (wooden made) on which the cheeses are placed during maturation and storage. The air was aspirated for 5 min with an air sampler (Andersen - Bionergetica, Rio de Janeiro). All these samples were plated directly onto Dicloran 18 % Glycerol agar (DG18). Information about features of cheese such as pingo quality, which consists of the natural starter culture rich in native microorganisms (indigenous lactic acid bacteria), maturation time, besides temperature or humidity control, hygiene aspects, cleaning and others was collected from the cheesemakers.

2.2. Water activity determination

Water activity in the cheese samples was measured in triplicate at 25 °C (±1 °C) using AquaLab – Meter model 4TE New (WA, USA) equipment.

2.3. Fungal counting

Twenty-five grams of cheese sample were taken randomly from the rind and inner parts with a sterilized metal spoon and homogenized with 225 mL of 0.1 % peptone water for 1 min. Serial dilutions were performed and aliquots of 0.1 mL of each dilution plated onto the media Dicloran 18 % Glycerol agar (DG18) for cheese with water activity below 0.95 and Dichloran Rose Bengal Chloramphenicol agar (DRBC) for samples above 0.95. Plates were incubated at 25 °C for 5–7 days (Pitt & Hocking, 2022). The number of *A.* section *Circumdati* colony forming units (CFU), was counted based on morphological characteristics (Klich, 2002; Visagie et al., 2014) and some representative colonies were isolated for further analyses.

2.4. Isolation and characterization of A. section Circumdati

A total of 63 representatives colonies of *A.* section *Circumdati* isolated from 26 cheese samples (42 isolates), 4 air samples (7 isolates) and 10 bench samples (14 isolates) were purified and inoculated at three equidistant points onto Czapek Yeast Autolyzed (CYA) and Malt Extract Agar (MEA), incubated at 25 °C for 7 days. These isolates were divided and grouped morphologically according to their macroscopic and microscopic characteristics following the taxonomic keys of Klich (2002) and Visagie et al. (2014) such as: the colony diameter, exudates production, colony texture, conidia and colony reverse colors, as well as conidial heads, vesicles shape and seriation, metula covering, conidia size, shape and roughness. Each strain was kept at 15 °C in CYA culture medium for prompt work and in silica gel for longer periods of preservation.

2.5. Molecular identification

Out of 63 isolates of *A.* section *Circumdati,* 27 were chosen for molecular identification. The isolates were grown in 7 mL of Yeast Extract Sucrose medium at 25 °C for 24 h. The mycelia were collected, frozen in liquid nitrogen and ground to a fine powder. The macerated material was used to obtain genomic DNA through the DNA purification kit (Mebep Bioscience, Shenzhen, China) according to the protocol recommended by the manufacturer. The DNA was quantified by

Table 1
Sampling of artisanal cheeses at different maturation times and collecting regions. Samples were collected from farms in the state of Minas Gerais and São Paulo and from markets located in both states.

1st. Collection period	Quantity of samples	Maturation (days)	2nd. Collection period	Quantity of samples	Maturation (days)
	São Paulo (SP)			São Paulo (SP)	
	3	< 30		1	< 30
	-	31–90		1	31–90
	4	> 90		4	> 90
	Minas Gerais (MG)			Minas Gerais (MG)	
	21	< 30		27	< 30
	12	31–90		16	31–90
	1	> 90		4	> 90
	Market in SP			Market in SP	
	-	< 30		Not collected	
	6	31–90			
	1	> 90			
	Market in MG			Market in MG	
	2	< 30		-	< 30
	17	NI		3	31–90
	3	NI		4	> 90
Total of samples	**70**		**Total of samples**	**60**	

NI: not informed.

spectrophotometry (NanoDrop®, Thermo Scientific).

Part of the calmodulin gene (*CaM*) sequence was amplified using CMD5 and CMD6 primer pairs (Hong et al., 2006) or CF1 and CF4 (Peterson et al., 2005). Conditions for amplification were as described by Silva et al. (2020). PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific, UK) according to the manufacturer's protocol. Amplicons were sequenced in both directions (forward and reverse) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in a SeqStudio Genetic Analyzer (Applied Biosystems, USA). The sequences obtained were aligned with sequences from all *A.* section *Circumdati* type strains available in the NCBI database (http://www.ncbi.nlm.nih.gov/) using Clustal W (Thompson, Higgins & Gibson, 1994). A maximum likelihood tree was inferred using the Tamura-Nei model (Tamura & Nei, 1993) with gamma distribution (+G). To determine the support for each clade, a bootstrap analysis was performed with 1,000 replicates using MEGA 11 software (Tamura et al., 2021). The contig was obtained through the BIOEDIT v.7.2.5 program.

2.6. Evaluation of *A.* section *Circumdati* for ochratoxin A production

Out of 63 representatives of *A.* section *Circumdati*, 42 isolates were tested for OTA production according to Accensi et al (2004). They were inoculated onto Yeast Extract Sucrose Agar (YESA) and incubated at 25 °C for 7 days. Three plugs were taken and then 2 mL of methanol was applied to extract the metabolites for 2 min. The extracts were filtrated and dried with nitrogen and resuspended in the mobile phase acetonitrile: water: acetic acid (57:41:2) for analysis. A 20 μL of extract was injected in a HPLC system and OTA was detected using an HPLC system (Agilent 1260 infinity), equipped with a fluorescence detector set at excitation = 333 nm and emission = 447 nm, a C18 Shimadzu column (5.0 μ, 250 mm, 4.6 mm), mobile phase composed of acetonitrile (ACN): ultrapure water (H2O): acetic acid (CH_3COOH) (51:47:2), with a flow rate of 0.8 mL/min, in an isocratic regime at 30 °C.

2.7. Extraction and quantification of ochratoxin A in cheese samples

2.7.1. Sample preparation

For the extraction of OTA from artisanal cheese samples, the methodology of Pietri et al. (2022) was followed with some modifications. It was determined that samples with low mold and yeast counts would be extracted in a single part (homogeneous) and the samples with fungal growth on the outside and high mold and yeast counts would be separated into 2 parts, the outer part (rind) and the inner part. Thus, 10 g of the cheese sample were weighed, ground in a blender, then 100 mL of a solution containing acetonitrile (ACN) and 0.13 M sodium bicarbonate solution ($NaHCO_3$) (1:1) was added and stirred in a mixer for 40 min at 4,000 rpm. The sample was centrifuged at 6,000 RPM at 4 °C for 10 min, vacuum filtered using a double filter (Whatman no. 4) following filtration in a microfiber filter (Vicam, Milford, MA, USA). An aliquot of 5 mL of the filtered liquid was taken and diluted with 50 mL of phosphate buffered saline (PBS) pH = 7. The solution was passed through an OTA immunoaffinity column (Ochratest, Vicam, Milford, MA, USA) at a flow rate of around 1 drop/s, to promote the extract clean up. OTA was eluted with 4 mL of HPLC-grade methanol (MeOH) and collected in a glass vial. The extract was dried in nitrogen, suspended in 0.4 mL of mobile phase, an aliquot of 20 μL was injected into a HPLC system and quantified.

2.7.2. Quantification of ochratoxin A by HPLC

An HPLC system was used (Agilent 1260 infinity, Santa Clara, CA), equipped with a fluorescence detector set at excitation = 333 nm and emission = 447 nm, a C18 Shimadzu column (5.0 μ, 250 mm, 4.6 mm), mobile phase composed of acetonitrile (ACN): ultrapure water (H2O): acetic acid (CH_3COOH) (51:47:2) in an isocratic regime at 30 °C. The working flow rate was 0.8 mL/min and the sample injection volume was 0.2 mL. For calibration curve, the OTA standard (Sigma-Aldrich, São Paulo, Brazil) was diluted with ACN at six concentrations (0.009154——0.9154 ng) obtaining an R^2 = 0.9999. OTA levels were measured comparing peak areas with calibration curve obtained with OTA standard solution in duplicate.

Limits of detection (LOD) and quantification (LOQ) were calculated according to Eurachem Guides (Magnusson et al., 2015). The limit of detection (LOD) and limit of quantification (LOQ) obtained were LOD = 0.3 μg/Kg and LQ = 0.95 μg/Kg. To determine the recovery of OTA through this methodology, contamination was carried out at three concentration levels (2.30, 4.55 and 6.85 μg/kg), obtaining recovery of 96, 98 and 92 % respectively. All analyses were performed in duplicate.

3. Results and discussion

3.1. Water activity and *A.* section *Circumdati* from artisanal cheese samples

Table 2 shows the average and range of water activities of the cheese artisanal samples at different maturation days, collected from farms and markets of Minas Gerais (MG), São Paulo (SP). These data show the decrease on a_W along maturation time. Vipotnik et al. (2017) studied the growth and OTA production of *A. westerdijkiae* in a dry-cured ham-based medium, showing that *A. westerdijkiae* were able to grow over a wider range of conditions, with optimum conditions at 0.97–0.93 a_W (3–6 % salt) at all tested temperatures, with maximum levels produced at 20–24 °C. This fact, may give a competitive advantage to *A. westerdijkiae* over the other species in salt-rich and protein rich animal derived products such as dry-cured ham and cheese. In our study, the cheese samples had a_W suitable for growth and OTA production by the isolated species of *A.* section *Circumdati*.

Total counts of *A.* section *Circumdati* from artisanal cheese samples in different maturation days in these three collection sites are shown in Table 3. Species of *A.* section *Circumdati* were found in samples of 2 cheesemakers from Minas Gerais. Producer # 4 had 11 samples out of 21 contaminated with *A. westerdjikiae*, *A. ostianus* and *A. steynii* in the 2 dates of collection. Producer # 6 showed 3 samples with *A. westerdjikiae* out of 12 from the second collection. Producer #7 from São Paulo had *A. westerdijkiae* and *A. steynii* in 7 out of 13 samples. *A.* section *Circumdati* population increased with maturation time. *A. westerdjikiae* was found in the air and bench samples from these 3 producers, *A ostianus* was detected only in the air and bench samples of producer #4, and *A. steynii* was found in the bench sample of producer # 4 and 7. Five samples from Minas and São Paulo markets had *A. westerdijkiae* out of 36 tested. *A.* section *Circumdati* counts varied from 100 to 10^6 CFU/g, showing that some samples had high levels of this group (Table 3). Species of *Aspergillus* section *Circumdati* have been considered important contaminants in products such as cereals and cereal products, grapes and coffee. For a long time, OTA presence in meat products was associated to *P. nordicum* and *P. verrucosum,* as they are traditionally more prevalent in European countries, which have a temperate climate and where the most of studies were carried out (Battilani et al., 2010;

Table 2
Averages and ranges of cheese water activities (a_W) at different maturation times and collection regions. Samples obtained from farms located in the state of Minas Gerais (MG) and São Paulo (SP) as well from markets in both states.

Maturation days	Minas Gerais	São Paulo	Markets
NI	–	–	0.954 (0.826–0.975)
< 30	0.921 (0.855–0.949)	0.972 (0.948–0.986)	0.962 (0.950–0.975)
31 – 90	0.902 (0.871–0.969)	0.901 (0.869–0.934)	0.945 (0.891–0.969)
> 90	0.869 (0.827–0.941)	0.877 (0.815–0.941)	0.915 (0.829–0.957)

NI: Not informed.

Table 3
Aspergillus section *Circumdati* counts (CFU/g) in cheese samples at different maturation times and in two collecting states: Minas Gerais (6 producers), São Paulo (1 producer), and markets from MG and SP.

				Maturation time (days)						
Cheesemakers	1st collection (positive samples)	2 nd collection (positive samples)	Total positive samples/ Totalsamples	NI	20-50	51-90	>90	Species found in cheese samples	Species found in the air	Species found on the bench
Minas Gerais (MG)										
# 1			0/14		< 100					
# 2			0/7		< 100					
# 3			0/13		< 100					
# 4 (Cave)	4	7	11/21		$100 – 10^3$	100 – 10^5	$100 – 10^4$	*A. westerdijkiae, A. ostianus, A. steynii*	*A. westerdijkiae A. ostianus*	*A. westerdijkiae*
# 5			0/14		< 100					
# 6		3	3/12		$100 – 10^2$		$100 – 10^5$	*A. westerdijkiae*	*A. westerdijkiae*	
São Paulo (SP)										
# 7 (Cave and Barn)	2	5	7/13		$100 – 10^6$	$100–10^5$	$100—10^6$	*A. westerdijkiae, A. steynii*	*A. westerdijkiae*	*A. steynii*
Markets (MG and SP)	5	0	5/36	< 100	$100—10^6$	100 – 10^5		*A. westerdijkiae*		

NI: not informed.

Rodríguez et al., 2014). However, increasing evidence has associated *A. westerdijkiae/A. ochraceus* to contamination of OTA in several salt-rich and maturated animal products, such as cured ham, sausages (Iacumin et al., 2011), salami from Argentina (Vila et al., 2016), Italian type salami from Brazil (Parussolo et al., 2019) and more recently in cheeses (Aneli et al., 2019, Patono et al., 2013).

3.2. Molecular identification of A. section Circumdati and ochratoxin a production

In the present study, *A. westerdijkiae* was the predominant species among the fungi of the *Circumdati* section. Of the 27 *A.* section *Circumdati* isolates, 18 were identified as *A. westerdijkiae* (67 %), 6 as *Aspergillus ostianus* (22 %), and 3 as *Aspergillus steynii* (11 %), as shown in Fig. 1.

A. section *Circumdati* currently harbors 27 species, of which around 20 are potentially ochratoxigenic (Houbraken et al., 2020; Visagie et al., 2014). Among these, *A. ochraceus* and *A. westerdijkiae* are often reported as the main ochratoxigenic species (Chiotta et al., 2016; Taniwaki et al., 2018; Wang et al., 2022). In artisanal cheese, *Aspergillus fumigatus, Aspergillus puulaauensis, Aspergillus niger, Aspergillus flavus*, and *A. westerdijkiae* have been reported (Anelli et al., 2019; De Souza et al., 2021; Laurenčík et al., 2008).

All 42 isolates of *A.* section *Circumdati* (*A. westerdjikiae*, *A. ostianus* and *A. steynii*) tested positive for OTA production, thus confirming the source of OTA formation in cheeses collected in this study. Anelli et al. (2019) also found *A. westerdijkiae* and *A. steynii* producers of OTA from cave cheese in Italy. To the best of our knowledge, the present study is the first report of the presence of *A. ostianus* in cheeses. This species has been reported as a poor producer of OTA (Varga et al., 2015; Visagie et al., 2014); therefore, it is likely that the main fungi responsible for OTA contamination of cheese is, in fact, *A. westerdijkiae*. Our results are in accordance with those from Vipotinik et al (2017) in which *A. westerdijkiae* showed wide distribution and adaptability to different environments as well as the ability to produce high amounts of OTA.

Recently, Martin et al. (2023) analyzed samples of Brazilian artisanal cheese from the Canastra region, based on a metataxonomic approach (ITS region). The main yeasts found were *Debaryomyces, Kluyveromyces, Torulaspora* and *Trichosporon* besides filamentous fungi such as *Fusarium, Acremonium* and *Aspergillus.* Among *Aspergillus* spp. they identified some species of: *A. caesiellus, A. falconensis, A. heterocaryoticus*, and *A. sydowii*, which were described by the authors as uncommon species or previously unreported in artisanal cheese. However, Martin et al. (2023) did not report the occurrence of *A.* section *Circumdati*, probably because their samples were not ripened for a longer period of time. Similarly, De Souza et al. (2021) also did not find any species of *A.* section *Circumdati* in samples of Minas artisanal cheese collected in the Serra do Espinhaço region (MG). Although they analyzed cheese samples ripened for 45 days, their results were based only on six cheese samples. In addition, this difference in the composition of the mycobiota can be explained by the different approaches used (metataxonomics, Sanger sequencing, and MALDI-TOF: Matrix-Assisted Laser Desorption Ionization Time-of-Light, identification) or even by the nature of the samples themselves. For example, Martin et al. (2023) reported the association of differences in the mycobiota in relation to seasonality, municipality of collection, and mainly, the origin of sample production (collection farm).

3.3. Occurrence of ochratoxin a in artisanal cheese

Out of 130 cheese samples, OTA was found in 28 samples, representing 22 % of then total (Table 4). Five samples had OTA levels above 1000 μg/kg. Dall'asta et al. (2008) in their study reported having found OTA in Gorgonzola and Roquefort cheese in a range of 0.25 to 3.0 μg/kg, Pattono et al. (2013) found OTA in semi-hard cheese at levels ranging from 18.4 to 146 μg/kg in the inner part of the contaminated cheese and levels from 1.0 to 262.2 μg/kg in the rind region. In the study reported by Anelli et al. (2019) samples of Italian cave cheese the detected ranges varied from 0.2 to 317 μg/kg of OTA.

Little is known about the relationship between ripening time and the occurrence of OTA in cheese; however, it is a point that must be considered, since out of 28 positive OTA samples, only 4 were not matured for more than 30 days. As the ripening time increases, the water activity of the cheese decreases favoring xerophilic fungal growth such as *A.* section *Circumdati*.

Another point to be considered regarding OTA contamination is the cheese maturing environment. Cheese aged in caves generally promote ideal temperature and humidity conditions for fungal growth, favoring their colonization on product. In Anelli et al. (2019), 65.5 % of the samples in which OTA levels were detected were samples whose maturation rooms were in an environment classified as "Cave", which consists of a place with little or no incidence of light, located underground, with temperatures lower than room temperature and with visible presence of fungi on the counters and walls where the cheese was located.

Vipotnik et al. (2017) reported high amounts of OTA production by *A. westerdijkiae* in dry-cured ham-based medium, reaching up to 1934

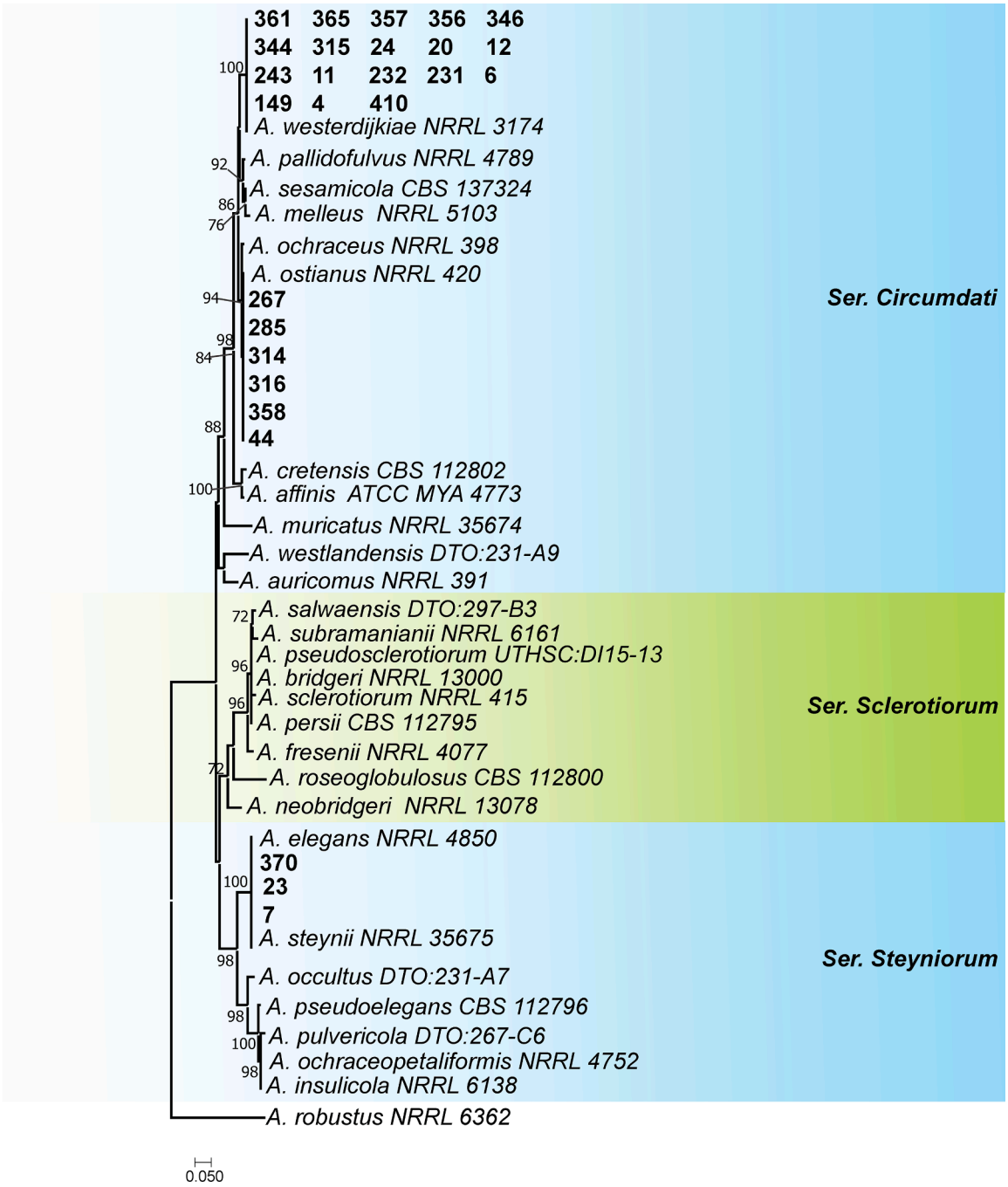

Fig. 1. Maximum likelihood tree (T93 + G) based on *CaM* sequences showing the relationships between *Aspergillus* section *Circumdati* and isolates from artisanal cheese samples. Bootstrap values (BS) higher than 60 % are shown. Isolates from this study are in bold. *Aspergillus robustus* is the outgroup.

μg/kg. This fact needs to be considered as a potential hazard in salt-rich and protein rich animal derived products such as dry-cured ham and artisanal maturated cheese. In our work, *A. westerdijkiae* was the most prevalent species isolated from cheese; all the isolates were able to produce OTA. As far as we know, this is the first report of the natural occurrence of OTA associated to *A. westerdijkiae* in artisanal cheese produced in Brazil.

Based on the information on specific characteristics of the collected cheese samples from the cheesemakers, and the relationship between the presence of OTA in cheese and maturation room conditions of the 7 farms, the following observations were taken:

Producer # 1: This producer is located in South West of Minas Gerais with most of the cheese in the maturation room showing white rind in consequence of *Geotrichum candidum* growth. One part of the maturation room had a space where cheese with more than 30 days ripening was kept with signs of mites and diverse color of fungal growth (reddish, yellow, pink). This cheese (defective cheese) was maturing together with sound cheese. OTA was found in two samples of defective cheese, one sample showed 5.7 μg/kg in the rind and not detected in the internal part; another sample had more than1000 μg/kg in the rind and 4.7 μg/kg in the inner part. In other 5 samples with white fungal growth, with less than 30 days of ripening, OTA was not detected. This producer was advised to clean up the place and avoid a section of the maturation room to not allow mite infection and toxigenic fungal growth. In the next visit, the maturation room was cleaned and all 7 samples did not contain OTA. The maximum period of maturation was 20 days.

Table 4
Occurrence of ochratoxin A (OTA) in artisanal cheese samples (rind and inner part).

Samples	Maturation (days)	Concentration of OTA (μg/kg)	
		Rind	Inner part
1	30	5.7	ND
2	30	> 1000	4.7
3	30	88.5	ND
4	30	12.8	1.2
5	60	126.0	ND
6	60	> 1000	> 1000
7	90	6.7	ND
8	90	22.9	1.0
9	90	> 1000	ND
10	90	22.9	2.7
11	90	2.1	4.5
12	60	5.3	ND
13	19	9.4*	
14	NI	4.8	ND
15	NI	ND	9.9
16	30	31.8	ND
17	90	5.9	ND
18	30	70.8	ND
19	60	76.3	1.8
20	70	70.3	ND
21	90	> 1000	91.7
22	90	> 1000	ND
23	30	9.6*	
24	120	0.4	ND
25	200	10.0	0.8
26	300	0.9	ND
27	120	2.9	ND
28	21	4.9	0.5

ND: Not detected (Limit of Detection = 0.3 μg/Kg).
NI: not informed.
*Samples were analyzed as one.

Producer # 2: This producer is located in South West of Minas Gerais and the maturation room is new. They do not produce cheese with more than 15 days of maturation. All the samples had only white fungi (*Geotrichum candidum)*. The cheese factory was built with a specific layout for production, with tiles on all walls, making it easier to clean, which is performed properly. Around the cheese factory, the land is clean and there are no sources of contamination such as chicken coops and corrals, as observed in other cheese producers. OTA was not detected in any of the samples.

Producer # 3: This producer is located in South West of Minas Gerais. The maturation room had cheese from 8 to 90 days of maturation. In the first visit, the maturation room had a precarious aspect. Two samples of 90 days had OTA at the levels of 23 and 2.1 μg/kg in the rind and 2.7 and 4.5 μg/kg in the rind and inner part, respectively. In the second visit, the maturation room showed a better aspect and although there was still cheese over 90 days, this time OTA was not detected in any sample.

Producer # 4: This producer is located in South West of Minas Gerais. It has one maturation room with cheese from 12 to 20 days; samples from this room did not have OTA presence. In this producer there is a *Cave* under a house with controlled light, with precarious temperature and relative humidity controls, which is used for maturing cheese from 30 to 90 days. The maturation room walls were made with porous stones, which make it difficult to clean providing a favorable place for fungal growth. All the 14 cheese samples from the first and second collection had OTA at a high amount. The concentration of OTA varied from 6.7 to > 1000 μg/kg and ND to > 1000 μg/kg in the rind and inner part of the cheese, respectively. All cheese and shelf samples showed visible growth of *A*. section *Circumdati,* suggesting systemic conditions that may favor growth of this group.

Producer # 5: This producer is located in Midwest of Minas Gerais. All cheese is matured in a special chamber with control of temperature (around 25 °C) and relative humidity (>90 %). Time of maturation reaches more than 100 days, but the majority of cheese is from 30 to 90 days, showing visible mold with *Penicillium* being the major one. No cheese samples (14) had OTA. Perhaps due to high humidity inside the room, along all the maturation period, the most hydrophilic species may take over not allowing the more xerophlic species such as *A*. section *Circumdati* to grow.

Producer #6: This producer is located in Midwest of Minas Gerais. All production follows an organic system. Cheese from the maturation room was from 2 to 200 days. Most samples showed visible fungal growth, with whitish in the first 20 days, changing to yellow, ochre and brown. In the first collection OTA was not found in any of the 4 cheese samples as the room was new, but after 10 months, in the second collection the shelves were more contaminated with colored molds and OTA was found in 3 samples ranging from 0.4 to 10 μg/kg.

Producer # 7: This producer is located in the East of São Paulo who has different types of cheese maturing in different rooms. There is also a *Cave* and attic with very poor conditions, where the cheese is ripened. In the first collection OTA was present in two samples at concentration of 5.3 and 9.4 μg/kg. In the second collection three samples from *Cave* had OTA from 0.9 to 4.9 and ND to 0.5 μg/kg in the rind and inner part, respectively. Although this producer inoculated cheese with starter cultures (*Geotrichum candidum, Penicillium* and yeasts), these starters were not able to inhibit ochratoxigenic fungi because, in this case, the cheese water activity decreased with the maturation period, allowing *A*. section *Circumdati* to grow, which can be observed in Table 3.

Although less than a quarter of the analyzed samples had detected levels of OTA, the present data raises concerns. To date, cheese with natural moldy rind types do not have specific regulations for their commercialization, due to the lack of scientific information. In addition to establishing maximum permitted OTA levels in cheese, more studies are needed on the occurrence and identification of toxigenic fungi, as well as the critical points and factors that favor toxin formation. For comparison, in Italy, since 1999 the Ministry of Health has established a guideline OTA value of 1 μg/kg in pork meat and derived products (Ministero della Sanità1999). Recently, the European Food Safety Authority (EFSA), published a report on "Risk assessment of ochratoxin A in food" evaluating the risk of OTA in foods to human health. In this report, cheese was considered one of the most important contributors to the chronic dietary exposure to OTA, together with preserved meat, and grains and grain-based products (Schrenk et al., 2020). The panel concluded that "ripened cheese might have higher OTA contamination levels, predominantly in the crust as they get contaminated with OTA during manufacturing or storing. Consequently, cheeses with edible rind and the grated ones where the outer part is also included in the final product are assumed to be the main contributors of exposure originated from cheese and more occurrence data on OTA in cheese paste in comparison to cheese rinds are needed.".

This research led us to start a work with the artisanal cheesemakers together with the authorities with the aim of clarifying the existence of potentially toxigenic fungi in the product and environment. So far, several measures have been taken, recommending the use of good practices in the management of artisanal cheese production to ensure food safety from producer to consumer. This research will contribute not only nationally but also internationally to clarify the OTA exposure from cheese to help the authorities and organizations in establishing a code of practice to prevent and reduce OTA contamination in cheese and recommending maximum level limits.

CRediT authorship contribution statement

Caio V.P. Marcelão: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Mariana C. de Souza:** Writing – original draft, Methodology, Formal analysis. **Josué José da Silva:** Writing – review & editing, Methodology, Formal analysis. **Fabiana Aparecida Couto:** Investigation, Conceptualization. **Gustavo Augusto**

Lacorte: Investigation, Conceptualization. **Uelinton M. Pinto:** review and editing. **Juliana T. Maffei:** . **Patrícia B. Zacarchenco:** Methodology and Review. **Beatriz T. Iamanaka:** Methodology and Review. **Marta H. Taniwaki:** Writing original draft, review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

The authors wish to thank São Paulo Research Foundation (FAPESP) Processes 2022/10378-0, 2017/50349-0; 2021/07937-4; 2022/06905-4 for funding this research; CAPES (Financial code 001) and CNPq Process 304036/2022-6 for the grants to C.V.P.M. and M.H.T.

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