Occurrence of Toxigenic Molds in Brazilian Cheese

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ABSTRACT

Several samples of cheese obtained from regular retailers in Campinas, Brazil, were examined for molds and mycotoxins. Strains of Aspergillus and Penicillium were isolated and evaluated for their potential for producing toxins. Two of the isolated Penicillium species produced citrinin, while another produced patulin. However, no mycotoxins were detected in any of the cheese samples tested. Experiments with different types of cheese and a semisynthetic cheese were carried out in order to verify production of citrinin and patulin. It was observed that citrinin may be produced in cheese with high water activity and in semisynthetic cheese. However, patulin does not appear to be stable in cheese, even in semisynthetic cheese with high water activity and high carbohydrate contents.

The cheese industry has greatly expanded in Brazil over the past decade. Successful development of such industrial activity depends on two basic principles: constant increase of productivity rates and reducing to the minimum losses that occur along the production and marketing chains. Considerable losses are caused by product spoilage through undesirable mold growth.

Cheese is an excellent substrate for mold growth. It can become moldy during the ripening process as well as during refrigerated storage. Certain molds produce toxic metabolic substances known as mycotoxins. Most molds commonly found in a wide variety of cheese belong to the same genus Penicillium (28). Certain species of this genus are able to proliferate and produce toxins at low storage temperatures (7). Mycotoxins that have been detected in cheese contaminated by Penicillium species include: ochratoxin A, citrinin, patulin, penicillic acid, mycophenolic acid, and penitrem A (28). Aspergillus species in cheese are usually found in much lower proportions than Penicillium species. Among the mycotoxins most commonly produced by these species and detected in cheese, special reference should be made to sterigmatocystin and aflatoxins (20,28). Other important fungi growing on cheese include Cladosporium, Geotrichum, Mucor, and Trichoderma (15,28).

In studies carried out on Cheddar and Swiss cheese, Bullerman and Olivigni (8) and Bullerman (5), respectivley, isolated species of Penicillium and Aspergillus that produced the following mycotoxins: patulin, penicillic acid, ochratoxin, and aflatoxin. Toxicological analysis of these mold extracts showed higher toxicity than presented by known mycotoxins.

Aran and Eke (2) observed that 90-93% of molds isolated from kasar cheese consisted of Penicillium species. The mycotoxins produced by these molds were identified as citrinin, cyclopiazonic acid, patulin, and penicillic acid.

Natural presence of mycotoxins in cheese has been reported. Bullerman (5) detected penicillic acid in 4 of 33 samples of moldy cheese, stored at 5°C for 6 weeks. The amount estimated was 0.5 μg of penicillic acid per g of cheese. Norholt et al. (20) reported the presence of sterigmatocystin in hard cheese. The toxin was found in 9 of 39 samples, in concentrations ranging from 5 to 600 μg/kg.

Experiments involving the inoculation of toxigenic molds in cheese samples have demonstrated that toxins such as patulin, penicillic acid (18,22), and PR toxin (30) are not stable in cheese because they react chemically with amino acids and compounds containing sulfhydryl groups (7,18). On the other hand, aflatoxin (7,18,23,24,35) and sterigmatocystin (20) have been shown to be stable in cheese. These mycotoxins are usually produced only at high temperatures, i.e., above 10°C (7,28).

The only information available on the occurrence of mycotoxins in cheese in Brazil was reported by Fonseca et al. (11). They investigated the presence of aflatoxin, ochratoxin A, and zearalenone in samples of cured cheese obtained from several dairy shops in Sao Paulo State. The presence of the above-mentioned toxins was not detected in all of the samples. There are no data available about the presence of mycotoxins produced by Penicillium species such as citrinin, patulin, or penicillic acid nor have studies been conducted on toxigenic molds in Brazilian cheese. For that reason, the present research was conducted in order to determine the occurrence of toxigenic molds in cheese, as well as the presence of mycotoxins such as citrinin, patulin, penicillic acid, ochratoxin A, and aflatoxin in commercial samples of Brazilian cheese, and the production of mycotoxins in cheese by the toxigenic molds isolated in this experiment.

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MATERIALS AND METHODS

Sampling of cheese

A total of 36 cheese samples was obtained from different dairy shops in the city of Campinas, Brazil. These 36 samples were divided into four groups: 9 samples of grated Parmesan cheese, 9 samples of processed cheese, 9 samples of Prato cheese, and 9 samples of hard Parmesan cheese. Each group of 9 samples corresponded to three different brands in triplicate.

Mycological analysis

Samples of 25 g of cheese were ground and dissolved in 225 ml of 2% sodium citrate. Serial dilutions of this solution were plated on potato dextrose agar (PDA) (Difco, Detroit, MI) along with the antibiotic chlorotetracycline hydrochloride - chloramphenicol (Sigma, St. Louis, MO) in order to avoid bacterial growth. The plates were then incubated at 25°C and observed after 3-7 d (19), when the counts were counted, isolated and transferred to PDA tubes, according to their macro- and microscopic characteristics for further identification.

Isolation and identification of molds

The isolated molds were divided into groups according to their genus, i.e., Penicillium, Aspergillus, Cladosporium, Absidia, and others. Appropriate counts were carried out in order to obtain the total count for each of the above-mentioned mold groups. The isolated molds were classified to the genus level using the keys of Raper and Fennell (27) and Barnett (4).

Toxin production of isolated molds

Each mold pertaining to the genera Penicillium and Aspergillus was examined for toxin production by inoculation in 100 ml yeast-extract sucrose (YES) broth at 25°C for 10 d. The culture was filtered and the filtrate extracted with two volumes of chloroform (9). The extracts were concentrated in a steam bath and analyzed on thin-layer chromatographic plates (TLC) (Merck art. 5721) to detect the presence of the mycotoxins, together with the following standards: patulin, citrinin, penicillic acid, ochratoxin A, sterigmatocystin, and aflatoxins (B1, B2, G1, and G2) (Sigma). The TLC plates were developed in a solvent solution of toluene/ethylacetate/90% formic acid (proportion: 50/40/10 v/v/v) (31). After all the solvent had evaporated, the plates were examined under ultraviolet light at 365 nm wavelength (Ultra Violet Products, Inc., San Gabriel, CA). Presence of patulin and penicillic acid was determined after spraying the plates with an aqueous solution of 0.5% 3-methyl-2-benzothiazolinone HC1, (Roth, Karlsruhe) heated at 130°C for 15 min, after which the plates were observed under ultraviolet light at the same wavelength as indicated above.

Determination of mycotoxins in cheese

All the cheese samples were examined, after removal of the packaging material for the presence of the following mycotoxins: aflatoxins B1, B2, G1, G2, and M1, ochratoxin A, patulin, penicillic acid, and citrinin. Official methodology for multi-toxin analysis is not available. For this reason, several test methods were used to determine the presence of the above-mentioned toxins in the different cheese samples which were spiked with each toxin. Samples of Prato, hard Parmesan, and grated Parmesan cheese were analyzed using a combination of 200 ml ethyl acetate and chloroform (1:1) as an extraction solvent after the samples were defatted with petrolatum-ether. Extracts of grated cheese were passed through a clean-up column, as described by Siriwardana and Lafont (33). In this case benzene was replaced for toluene, because of its lower toxicity. Processed cheese was analyzed using the method described by Pons et al. (26), but penicillic acid was very difficult to visualize by this method.

After a 20-d incubation at 25°C, samples of moldy Prato cheese were analyzed in order to determine the presence of the above-mentioned mycotoxins, using the same method as previously described for Prato cheese.

Inoculation of toxigenic molds in cheese samples

Samples of Prato, hard Parmesan, and grated Parmesan of different brands, along with one sample of homemade imitation cheese (semisynthetic medium), were inoculated with toxigenic strains isolated from cheese samples used in this experiment in order to evaluate toxin production and toxin stability in cheese.

The imitation cheese with high carbohydrate content was prepared with the following ingredients: 1 l of milk, 200 g of margarine, 200 g of grated Parmesan cheese, and 200 g of starch. The ingredients were homogenized in a food mixer into a paste which was subsequently heated until it was of a uniform consistency. The heated mixture was poured into appropriate containers for cooling and hardening.

A 50-g sample of each cheese was inoculated separately with spores of: a) a patulin producing Penicillium sp strain 7, previously isolated from Prato cheese and b) a citrinin producing Penicillium sp strain 19, previously isolated from grated Parmesan cheese. Inoculation of the Penicillium strains in cheese samples was performed as described by Youssef and Marth (34). Cheese samples were incubated at a temperature of 25°C until mold growth became visible. The time necessary for visible mold formation on the different cheese samples varied from 10 to 30 d.

Extraction of citrinin and patulin was performed using the same technique as described above for each cheese. In addition, patulin was extracted using the clean-up procedure described by Scott and Kennedy (29). Quantitative determination of citrinin in cheese samples was performed comparing the fluorescence produced by the sample extract with the fluorescence of the citrinin standard.

Determination of physical-chemical properties of imitation (semisynthetic) cheese

A potentiometer [pH Meter, E-512 (Metrohm-Herisau)] was used to determine the pH of the sample. The total dry matter content of the cheese was determined by drying the samples mixed with sand to constant weight (7 h) in a drying oven at 102°C (14). Protein content was determined with the official Kjeldahl method (13). The fat content of the cheese sample was determined with the butyric acid method, developed by Gerber-Van Gulik (12). Determination of lactose content was performed on 2.0-g and 0.5-g cheese samples following the procedure described by Acton (1).

Fat content in the dry matter (FDM) was calculated with the formula: FDM = % total fat x 100%/TDM. The salt content of the cheese sample was determined by titrating the excess of added silver nitrate with ammonium thiocyanate (32). The ash content of the cheese sample was determined in a muffle furnace set to 550°C ±5°C (3). The acidity of the cheese was determined by titration with 0.1 N NaOH and expressed as a percentage of lactic acid (25).

Determination of water activity in cheese

Water activity of the cheese samples was determined in triplicate using a Novasina box (Model E.E.J.A. 3/AM) equipped with three sensors at a temperature of 25°C in a thermostatic chamber.

RESULTS AND DISCUSSION

Mycological analysis of cheese

Mold counts performed on samples of Prato cheese, hard Parmesan, grated Parmesan, and processed cheese are shown in Table 1. The occurrence of Penicillium species
were predominant in Prato (99.9% of all molds isolated) and hard Parmesan cheese (84.2-98.9%). Grated Parmesan cheese showed the presence of higher proportions of mold species other than Penicillia. It was found that processed cheese was completely free from any kind of mold growth. This might be due to the heat treatment that this type of cheese is submitted to (90-100°C), as well as to the addition of inhibitory substances added during the manufacturing process (sorbic acid and salts).

Mycotoxins produced by molds isolated from cheese samples

The capability of the molds detected in the cheese samples to produce the following mycotoxins: aflatoxin B₁, B₂, G₁, G₂, ochratoxin A, sterigmatocystin, patulin, penicillic acid, and citrinin revealed that 1 of 9 strains of Penicillium isolated from Prato cheese was capable of producing patulin. One of 8 strains of Penicillium isolated from hard Parmesan cheese proved to be able to produce citrinin. One of 4 strains of Penicillium isolated from grated Parmesan cheese also produced citrinin. According to Leistner and Pitt (16), about 80% of the species of the Penicillium genus are considered to be toxigenic. Two species of Aspergillus isolated from grated Parmesan cheese did not produce toxins. In this experiment, the ability of these molds to produce known mycotoxins was found to be relatively low. However, it should be noted, that only 6 mycotoxins were tested; therefore, it has not been possible to evaluate the real toxigenic potential of the molds found on samples of four different kinds of cheese. These results match with the data obtained by Bullerman (6) who observed that mycotoxigenic molds are present in relatively low proportions compared to the total mold count of the cheese samples, considering exclusively the production of known mycotoxins.

Determination of the presence of mycotoxins in cheese samples

Aflatoxin (B₁, B₂, G₁, G₂, M₂, M₃), ochratoxin A, patulin, penicillic acid, and citrinin were not detected in the samples of Prato, hard Parmesan, grated Parmesan, and processed cheese analyzed immediately after removing the commercial packaging materials. None of the mycotoxins listed above were detected in samples of Prato cheese after 20 d incubation at a temperature of 25°C. All Prato cheese samples showed considerable mold growth after incubation. In most cases, the samples were contaminated exclusively by Penicillium species which probably were not able to produce the mycotoxins tested, but if produced, these toxins apparently were unstable in the cheese.

Water activity in cheese

Water activity in Prato, hard Parmesan, grated Parmesan, and processed cheese is shown in Table 2. All cheese varieties, except for grated Parmesan cheese, showed high water activity rates. High water activity favors the growth of most molds. Mold growth expands rapidly after removal

<table>
<thead>
<tr>
<th>TABLE 1. Occurrence (OCUR) and population (POP) of different genera of molds in cheese samples: Prato, hard Parmesan, grated Parmesan, and processed cheese.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Types of cheese</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Prato Brands</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>Hard Parmesan</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>Grated Parmesan</td>
</tr>
<tr>
<td>G</td>
</tr>
<tr>
<td>H</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>Processed cheese</td>
</tr>
<tr>
<td>J</td>
</tr>
<tr>
<td>K</td>
</tr>
<tr>
<td>L</td>
</tr>
</tbody>
</table>

* Average of triplicate.

CFU/g = Colony Former Units/g.
- = Not present.
of packaging material since these microorganisms depend on oxygen to develop.

**TABLE 2.** Water activity (A_w) of Prato, hard Parmesan, grated Parmesan, and processed cheese.

<table>
<thead>
<tr>
<th>Brands</th>
<th>Prato</th>
<th>Hard Parmesan</th>
<th>Grated Parmesan</th>
<th>Processed Cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.97</td>
<td>0.92</td>
<td>0.85</td>
<td>J 0.96</td>
</tr>
<tr>
<td>B</td>
<td>0.96</td>
<td>0.88</td>
<td>0.76</td>
<td>K 0.96</td>
</tr>
<tr>
<td>C</td>
<td>0.96</td>
<td>0.93</td>
<td>0.79</td>
<td>L 0.95</td>
</tr>
</tbody>
</table>

* Average of triplicate.

**Toxin production in cheese**

After stimulating the growth of toxigenic *Penicillium* and further toxin production in the cheese, it was observed that the mold developed plainly on all of the Prato cheese samples. In samples of hard Parmesan and grated Parmesan cheese, the inoculated mold did not develop, although in some cases the presence of other molds could be observed. In this case it was analyzed if the toxins were present, but none of the tested toxins were detected. The results are shown in Table 3.

**TABLE 3.** Production of patulin and citrinin by *Penicillium* sp. 7 and *Penicillium* sp. 19, respectively inoculated in Prato, hard Parmesan, grated Parmesan and semisynthetic cheese, and incubated at 25°C.

<table>
<thead>
<tr>
<th>Production (μg/kg)</th>
<th>Patulin</th>
<th>Citrinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>nd</td>
<td>15,535</td>
</tr>
<tr>
<td>B</td>
<td>nd</td>
<td>6,690</td>
</tr>
<tr>
<td>C</td>
<td>nd</td>
<td>8,173</td>
</tr>
<tr>
<td>D</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>E</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>G</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>Semisynthetic</td>
<td>nd</td>
<td>350,625</td>
</tr>
</tbody>
</table>

* Average of triplicate.  
  nd = Toxins not detected.  
  - = Presence of inoculated *Penicillium* not detected.

All of the Prato cheese samples inoculated with *Penicillium* sp. 19 (citrinin-producing mold) showed the presence of citrinin in amounts that varied from 6,690 to 15,535 μg/kg. The quantity of citrinin determined in samples of semisynthetic cheese was 350,625 μg/kg. Studies on the stability of citrinin in cheese are contradictory and the amounts produced also vary greatly (28). Nowotny et al. (21) detected citrinin in amounts as high as 450 μg/g in samples of Gouda cheese and 80 μg/g in samples of Edam cheese contaminated by a strain of *Penicillium citrinum* and incubated at 25°C. On the other hand, Engel (10) observed that a strong toxigenic strain of *P. citrinum* did not produce citrinin in autoclaved Tilsit cheese incubated at 27°C.

The presence of the mycotoxin patulin was not detected in any of the Prato cheese samples, although mold growth was extensive. One of the reasons for the lack of patulin and penicillic acid production in cheese is the insufficient A_w values of cheese and the fact that cheese contains small amounts of carbohydrates and high amounts of protein to be suitable for the production of these mycotoxins (7). Furthermore, it has been observed that patulin in foods with high protein contents such as meat and cheese is unstable because of its high reactivity with amino acids and compounds containing sulhydryl groups (7,18).

We included in our experiment an imitation cheese with high carbohydrate contents (12.5% based on the starch added). Table 4 shows the physical-chemical composition of the imitation (semisynthetic) cheese. This imitation cheese was inoculated parallel to other samples of cheese in order to evaluate the influence of carbohydrate content on the production and/or stability of mycotoxins. Citrinin was detected in a very high amount (350,625 μg/kg) in this imitation cheese, probably because the higher proportion of carbohydrate allows higher citrinin production and/or that citrinin is more stable in the presence of carbohydrates. As for patulin, although the mold proliferated regularly on all inoculated cheese samples, no patulin was detected. It may be that the mold did not produce patulin or that patulin was present in extremely small concentrations which could not be detected. However, it is also possible that patulin is not stable in the types of cheese tested.

**TABLE 4.** Physical-chemical composition of imitation (semisynthetic) cheese.

<table>
<thead>
<tr>
<th>Samples</th>
<th>pH</th>
<th>Acidity (% lactic acid)</th>
<th>Fat content (%)</th>
<th>Total Dry Matter (%)</th>
<th>Ashes (%)</th>
<th>Protein (%)</th>
<th>Lactose (%)</th>
<th>Salt (%)</th>
<th>Fat in the Dry Matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prato</td>
<td>5.70</td>
<td>0.42</td>
<td>15.50</td>
<td>42.50</td>
<td>1.87</td>
<td>7.95</td>
<td>4.00</td>
<td>0.88</td>
<td>36.47</td>
</tr>
<tr>
<td>Hard Parmesan</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Grated Parmesan</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Semisynthetic</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

Reiss (cited in 18) observed that patulin production in bread decreased proportionally when subjected to longer incubation periods. Although bread has a high carbohydrate content, it also contains compounds with sulhydryl groups such as nonfat dry milk and egg protein which react with patulin. Once this reaction has taken place the patulin moiety can no longer be detected with conventional methods. Therefore, in our experiment, although the imitation cheese contained high carbohydrate levels (starch), the other compounds rich in proteins (grated Parmesan, milk, and margarine) probably reacted with patulin.

This experiment suggests that toxigenic *Penicillium* may occur in cheese, and citrinin may be produced in cheese with high water activity. On the other hand, cheese proved not to be a good substrate for patulin production even when high amounts of carbohydrates and high water activity are present.
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