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Toxigenic penicillia spoiling frozen chicken nuggets

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ABSTRACT

Frozen chicken nuggets are classified as pre-prepared frozen meals. These products are convenient to consumers as they are easy to prepare and allow for long storage by freezing. Over the years, spoilage of frozen food products caused by fungi has been a continual problem for the food industry since mold can develop when frozen foods are allowed to attain temperatures of −10 °C, or above. The growth of fungi on the food surface results in economic losses and represents a hazard to public health due to the possibility of mycotoxin production. The aim of this study was to identify the species of filamentous fungi involved in the spoilage of frozen chicken nuggets and determine their ability to produce mycotoxins under laboratorial conditions. A total of 7 samples of frozen chicken nuggets were analyzed by dilution plating in potato dextrose agar (PDA). These products had been returned by customers due to visible mold growth on their surface. The predominant species found were *Penicillium glabrum, Penicillium polonicum, Penicillium manginii*, *Penicillium crustosum, Penicillium commune*, and *Penicillium solitum.* Analysis of the profile of secondary metabolites was carried out in HPLC after growing the isolates in Czapek yeast autolysate agar (CYA) and yeast extract agar and sucrose (YESA) and extracting the extrolites with a solution of ethyl acetate, dichloromethane, methanol, and formic acid. Some isolates of these species showed an ability to synthesize mycotoxins such as cyclopiazonic acid citreoviridin, roquefortine C, penitrem A, and verrucosidin under standard conditions. Considering the occurrence of fungal spoilage in frozen food and the potential hazard involved, more studies on psychrophilic fungi growth in foods stored at low temperatures are necessary.

1. Introduction

According to data from the annual report of the Brazilian Poultry Union (UBABEF), the production of poultry meat reached 12,308 million tons in 2013. Brazil has maintained its position as the world's largest exporter and third largest producer of chicken, behind the USA and China. With regard to marketing, exports of processed chicken products have remained stable at 161,000 tons (UBABEF, 2013). The current tendency of the poultry industry is to focus on product diversification as an alternative means of raising the consumption of poultry meat and adding value to the product, thus increasing financial return (Silva, 2004). Consequently, one segment that has expanded considerably in recent years is that of convenience products, such as frozen chicken nuggets.

The expansion in the trade of convenience foods has been stimulated by the increase in the number of working women and the decline in family size, resulting in a reduction in time spent preparing food. The entry of more women into the workforce has also led to improvements in kitchen appliances and increased the variability of ready-to-eat or frozen foods available on the market (Barbosa-Cánovas, Altunakar, & Mejía-Lorio, 2005; Barbut, 2002).

Commodities preserved by freezing are usually the most perishable ones, which also have the highest price. Therefore, the demand for these commodities is lower in developing areas. In addition, the need for adequate technology for the freezing process is a major drawback for developing countries to compete with industrialized countries. The frozen food industry requires accompanying developments and facilities for storing and transporting products from the processing plant to the consumer (Mallett, 1993). The use of low temperatures maintains the quality and prolongs shelf life by keeping the product temperature at the point where metabolic activity and growth of microorganisms present are minimized (Ashby, 2008).

The microorganisms present in processed and frozen foods may come from the raw material (meat cuts, starches, breading flours, and spices) and survive through the processing steps (especially heat) or they may contaminate the product in post-frying/baking stages of air cooling and packaging (Kuhen & Gunderson, 1962). The stages of distribution and storage both at stores and at consumers' homes are crucial for controlling microbial growth. The ability to thrive at temperatures that are close to, or below, the freezing point of water requires a vast array of adaptations to maintain the metabolic rates and sustained

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growth compatible with life in these severe environmental conditions (Feller & Gerday, 2003). Maintaining the desired or ideal holding temperature is a major factor in protecting perishable foods against quality loss during storage and distribution (Ashby, 2008). If temperature abuse occurs, a portion of these contaminating microorganisms may develop and cause depletion in the nutritional value of the product (Dainty, 1996; Huisin't Veld, 1996), changes in sensory characteristics, and the occurrence of food-borne diseases (Currie et al., 2005).

There is no official estimate of the total financial loss each year due fungal spoilage of frozen chicken nuggets. However, internal data from a nugget factory suggest losses of production ranging between 1.0% and 1.5% in Brazil. Sivasankar (2002) conveniently define food spoilage as having occurred when a consumer refuses it as food. This may vary from society to society, person to person, or even from one type of food to another. Fungal spoilage of food renders the food unacceptable to the consumer, leading to monetary loss (Kotler, 2003). Moreover, it can endanger health by exposing consumers to toxic secondary metabolites produced by mycotoxins. Despite the importance of this subject, very few studies have investigated fungal spoilage of frozen foods (Hegazy & Agami, 2011; Kuhen & Gunderson, 1962, 1963).

This study aimed to assess the main fungal species involved in the spoilage of frozen chicken nuggets as well as to verify the main secondary metabolites and mycotoxins that can be produced by the prevalent species.

2. Materials and methods

2.1. Samples

Seven samples of frozen chicken nuggets were analyzed. The samples had been returned by customers to the nugget industries in Brazil since they were moldy at the time of food preparation. None of the products had passed the expiration date.

2.2. Determination of filamentous fungi

The samples were thawed at room temperature before being analyzed by dilution plating. Under aseptic conditions, 25 g of each sample was weighed and 225 mL of 0.01% sterile peptone water was added. After homogenization, aliquots of the serial dilutions were prepared and inoculated on plates containing potato dextrose agar (PDA) supplemented with chloramphenicol. The plates were incubated at 25 °C for 7 days and 5 °C for 21 days. The results were expressed in colony-forming-units per gram of sample (CFU/g), according to Pitt and Hocking's (2009) methodology.

The medium PDA was selected after comparative tests carried out with 18% Glycerol Agar Dichloran (DG18) and Rose Bengal Agar Dichloran (DRBC), both supplemented with chloramphenicol. All media showed similar recoveries when incubated at 25 °C, but the recoveries at low temperatures by PDA were significantly higher when compared to DRBC and DG18 which mostly presented no growth of fungi at all.

2.3. Identification of filamentous fungi

After incubation, the plates were examined and all the fungal species were first isolated on Petri dishes containing Czapek yeast autolysate agar (CYA) to be later identified by specific protocols for each genus.

The isolated *Aspergillus* sp. and *Penicillium* sp. were grown in CYA and Malt Extract Agar (MEA). The genus *Penicillium* was identified according to Pitt (1979) and Samson and Frisvad (2004) and identification of the genus *Aspergillus* was performed according to Pitt and Hocking (2009). Briefly, the isolates of *Aspergillus* sp. and *Penicillium* sp. were inoculated at three points on CYA and MEA plates and incubated at 25 °C and also on CYA at 5 °C and 37 °C for 7 days.

The identification of fungi was based both on macroscopic (colony diameter, color, exudate, and soluble pigment production) and microscopic characteristics when growing under different temperatures, confirmed by analyses of the extrolite profile of the most prevalent species recovered by each sample.

2.4. Determination of extrolites produced by some Penicillium spp

Extrolites were examined by HPLC as described by Smedsgaard (1997). The fungal isolates were inoculated at 3 points in CYA and yeast extract agar and sucrose (YESA) and cultured for 7 days at 25 °C. After the incubation time, 5 plugs from each colony were cut out, placed in a 1.5 mL glass vial, and extracted. Extraction was performed using 500 μL of a solution of ethyl acetate/ dichloromethane/ methanol (3: 2: 1, v/ v/ v) with 1% (v/ v) formic acid and ultrasonicated for 10 min. The organic solvent was transferred to another vial and evaporated. The eluted product was transferred to another vial, the solvents evaporated at 1 mbar in a Rotavapor centrifuge evaporator and the dried extract re-dissolved in 500 μL methanol. After filtering through a 0.45 μm PFTE filter, 3 μL was injected in an Agilent 1100 HPLC (Waldbron, Germany) equipped with a diode array and a fluorescence detector. The separation of compounds was performed on a 50 mm × 2 mm id, 3 μm Luna C18 (II) column (Phenomenex, USA), equipped with a Security Guard pre-column. A linear gradient of water with 0.05% trifluoracetic acid (TFA) and acetonitrile with 0.05% TFA was used as mobile phase going from 15% acetonitrile to 100% acetonitrile in 20 min and then maintained for 5 min before returning to start conditions. The diode array detector sampled UV spectra from 200 to 600 nm every 0.7 s. Chromatograms at 210 and 280 nm were used for detection. For fluorescence detection, the excitation wavelength was 230 nm and the emission wavelength was 450 nm. The extrolites were identified by their UV spectra. Authentic analytical standards were employed for retention time and retention index comparison with the extrolites detected (Nielsen & Smedsgaard, 2003).

3. Results and discussion

Table 1 shows the results of the total count of filamentous fungi in the seven samples of frozen chicken nuggets. The recovery of contamination ranged from 10^1 to 10^8 CFU/g, with similar counts at both incubation temperatures tested. The sample presenting 10^1 CFU/g showed a high yeast count.

Because yeasts and molds are ubiquitous, their existence in frozen food products is neither surprising nor unusual. Enumeration and identification of psychrophilic fungi encountered in frozen food products is important in understanding the sources and kinds of mold damage encountered (Kuehn & Gunderson, 1963). The ability of psychrophiles to survive and proliferate at low temperatures implies that they have

Table 1
Determination and identification of filamentous fungi that spoiled frozen chicken nuggets using different incubation temperatures in potato dextrose agar (PDA).

Sample	Fungal count (CFU/g)		Fungal species isolated
	5 °C	25 °C	
A	8.0×10^7	3.8×10^6	*Penicillium corylophilum, Penicillium implicatum, Penicillium manginii*[a]
B	5.6×10^8	2.6×10^6	*Penicillium commune, Penicillium glabrum*[a]
C	1.2×10^8	7.2×10^8	*Penicillium glabrum, Penicillium polonicum*[a]
D	1.0×10^5	1.47×10^5	*Aspergillus ustus, Cladosporium* sp., *Penicillium glabrum, Penicillium polonicum*[a]
E	2.0×10^1	1.0×10^1	*Aspergillus fumigatus, Aspergillus versicolor, Penicillium crustosum*[a], *Penicillium fellutanum*
F	1.0×10^6	2.3×10^6	*Penicillium chermesinum, Penicillium funiculosum, Penicillium glabrum*[a], *Penicillium solitum*
G	2.0×10^4	2.6×10^5	*Penicillium glabrum*[a]

CFU/g: colony-forming units per gram of chicken nugget.
[a] Most prevalent species in the sample.

overcome key barriers inherent to cold environments (D'Amico, Collins, Marx, Feller, & Gerday, 2006). Meat products that have a high water content and are stored at room temperature or by refrigeration tend to deteriorate by the action of bacteria and yeasts due to their shorter generation time. On the other hand, spoilage by filamentous fungi occurs more easily when the surface of the product becomes dry or when this product is stored at lower temperatures (Lowry & Gill, 1984; Sonjak, Ličen, Frisvad, & Gunde-Cimerman, 2011). The psychrophilic fungi and their role in the deterioration of frozen foods has been little studied (Kuhen & Gunderson, 1962).

Only three fungal genera were present in the dilutions considered, with the *Penicillium* genus most frequently isolated, followed by *Aspergillus* and *Cladosporium*. *Penicillium* sp. and *Aspergillus* sp. are among the most frequent genera of fungi in foods, and *Cladosporium* is an important spoiler of foods conserved at low temperatures (Pitt & Hocking, 2009). The predominance of *Penicillium* sp. and *Aspergillus* sp. was also observed in a study conducted by Hegazy and Agami (2011) in frozen chicken nuggets from Egypt, with a predominance of *Aspergillus* spp. Unlike the samples analyzed in our study, the samples analyzed by Hegazy and Agami (2011) were not spoiled. It is known that, unlike *Aspergillus, Penicillium* has a greater number of species capable of growth at temperatures below 5 °C (Pitt & Hocking, 2009). Thus, although present in the processed product, the population of *Aspergillus* sp. should suffer little change during the storage period, whereas *Penicillium* sp. tends to rise if the food is not it is kept below −10 °C, leading to deterioration of the product.

The four predominant species involved in the deterioration of the nuggets were *Penicillium glabrum, Penicillium polonicum, Penicillium manginii*, and *Penicillium crustosum* (Table 1). Scholte, Samson, and Dijksterhuis (2002) assert that *Penicillium* species are often isolated from spoiled meat substrates, including processed products. In general, *Penicillium* species inhabit the soil, fruits, and grain but because they produce a large amount of spores they can easily spread through the air and settle in reservoirs such as humans, dust, raw materials, sewage, and surfaces for equipment and manipulation (Scholte et al., 2002).

Ingredients added during the preparation of chicken nuggets may be a source of contamination, since *P. polonicum* is a species frequently isolated from maize and wheat from tropical countries (Samson & Frisvad, 2004). These cereals are present in the breading flour of nuggets (Barbut, 2002). The adequate selection of raw materials and sanitary improvements in processing environment could minimize the presence of fungi. Once psychrophilic fungi have already contaminated the food, the occurrence of abuses could allow for their multiplication, leading to product spoilage.

According to Scholte et al. (2002), the factors that determine the fungal cultures that develop in meat products are the quality of raw material, cold chain maintenance, sanitary facilities (equipment), physical characteristics, and biochemical products and processing practices. Failures in systems for maintaining the cold chain can increase storage temperatures to values near 0 °C, despite the fact that frozen food should be kept at least to below −12 °C (Kennedy et al., 2005; Mürmann et al., 2004; Saccomori, 2013). In addition, the existence of psychrophilic fungi contaminating the frozen foods may make them microbiologically unstable.

Saccomori (2013) investigated chicken nuggets inoculated with *Penicillium* spp. and stored at low temperatures and showed that *P. polonicum* was able to form visible colonies after 120 days of incubation at −5 °C. The same author also evaluated *P. glabrum*, but the limiting temperature for colony formation of this species was 0 °C after 63 days of incubation. Both fungi tested remained viable during the period tested and could be recovered in culture media even when nuggets were incubated at −18 °C. Hence, the major problem related to spoilage of frozen nuggets appears to be the storage of nuggets contaminated by psychrophilic fungi in temperatures above those recommended by the manufacturer. No studies were found evaluating the production of mycotoxins by these *Penicillium* species under low temperatures.

Table 2 shows the extrolites produced by the predominant *Penicillium* spp. isolated from the samples of frozen chicken nuggets. Isolates of *P. commune, P. crustosum, P. manginni*, and *P. polonicum* were able to produce at least one mycotoxin. Isolates of *P. glabrum* and *P. solitum* were the only ones that did not produce mycotoxins, although other metabolites with known biological activity were synthesized. An extensive study of extrolites produced by *Penicillium* subgenus *Penicillium* was published by Frisvad, Smedsgaard, Larsen, and Samson (2004), showing also the metabolites usually produced by the main species present in our study.

The mycotoxin producing ability displayed by most of the isolates from this study is a disturbing fact. It is widely known that there is an active metabolism and dissemination of hyaline fungal hyphae inside substrates before the formation of visible colonies on the surface of the food. A level of contamination near 10^6 CFU/g of frozen chicken nuggets is required for the fungal colonies to become visible on the product surface (Saccomori, 2013). In the interstitial period, there is a risk of consumer exposure to mycotoxins. This fact highlights the importance of ensuring that the products are kept under the temperature recommended by the manufacturer to prevent spore germination and mycelial formation. Studies evaluating the ability of psychrophilic *Penicillium* to produce mycotoxins under low temperatures should be carried out.

It is important to highlight the production of two neurotoxins: citreoveridin by the *P. manginii* and verrucosidin by the *P. polonicum* isolates tested. The neurotoxicity of verrucosidin was reported in animals (Wilson, Byerly, & Burka, 1981) and demonstrated by experimental tests carried out with rats (Fink-Gremmels, Henning, & Leistner, 1991). Citreoviridin is attributed with the triggering of Keshan disease, which presents a higher prevalence in China, and acute heart disease and beriberi, with several cases reported in Japan (Hou, Li, & Qi, 2006; Nishie, Cole, & Dorner, 1988; Ueno, 1974). Verrucosidin is a potent teratogenic agent in rats (Morrissey & Vesonder, 1986).

Other neurotoxins produced by the evaluated isolates were Penitrem A and Roquefortine C, produced by *P. crustosum*. These mycotoxins are considered tremorgenic, inducing muscle tremors, ataxia, and convulsion (Boysen et al., 2002). Some studies have reported cases of poisoning by these toxic metabolites in dogs (Boysen et al., 2002; Walter, 2002; Young et al., 2003). Clinical signs of these animals have been confused with those related to strychnine poisoning, but the

Table 2
Extrolites produced by the prevalent *Penicillium* spp. isolated from moldy frozen chicken nuggets.

Isolate	Identification	Extrolites produced
01/12 NGT	*Penicillium manginii*	Citreoviridin[M], citreomontanin
16/12 NGT	*Penicillium commune*	**1.1** Cyclopaldic acid[P], cyclopiazonic acid[M], FKI-3389, chromanols
23/12 NGT	*Penicillium polonicum*	Anacin, cyclopenol, cyclopenin, dehydrocyclopeptin, verrucosidin[M], deoxyverrucosidin, viridicatol, 3-methocyviridicatin, verrucofortine
29/12 NGT	*Penicillium glabrum*	Asterric acid, geodin, citromycetin, asperflavin, questin
39/12 NGT	*Penicillium glabrum*	Citromycetin, geodin, questinol
33/12 NGT	*Penicillium polonicum*	Anacin, cyclopenol, cyclopenin, dehydrocyclopeptin, verrucosidin[M], deoxyverrucosidin, viridicatol, 3-methoxyviridicatin, verrucofortine
30/12NGT	*Penicillium solitum*	Compactin[P], solistatin[P], viridicatin, viridicatol, cyclopeptin, cyclopenol, cyclopenin, and andrastrin A
35/12 NGT	*Penicillium glabrum*	Asterric acid, geodin, questinol, questin
51/12 NGT	*Penicillium crustosum*	Terrestric acid, thomitrem A & E, penitrem A[M], roquefortine C[M], cyclopenin, viridicatol, viridicatin

M = mycotoxin; P = potential or actual-pharmaceutical (according to Frisvad et al., 2004).

analysis of intestinal content from animals revealed the presence of these mycotoxins associated with the ingestion of moldy foods, such as cereal flour, dairy products, and pasta.

Cyclopiazonic acid, produced by *P. commune*, is also a very relevant mycotoxin. It induces degenerative necrosis of the liver, pancreas, spleen, kidney, salivary gland, muscle, and myocardium in rats (Morrisey, Norred, Cole, & Dorner, 1985). It has also been associated with human poisoning (Rao & Husain, 1985), such as moldy corn toxicosis, known as "Kodua poisoning" (Bhide, 1962). This non-lethal disease in humans is characterized by sleeplessness, tremors, and giddiness (Rao & Husain, 1985). Moreover, cyclopiazonic acid is known for its immunosuppressive action even at low dosages (Kamalavenkatesh, Vairamuthu, Balachandran, Manohar & Raj, 2005).

4. Conclusion

The growth of fungi in frozen foods is a problem that has become evident with the popularization of domestic freezing systems and remains present in the food industry, especially in tropical countries, where the occurrence of temperature abuse is more common. In addition, the constant exposure of foods to low temperatures could allow the development of psychrophilic microbiota. Great progress has been made in the study of the bacteriological aspects of food spoilage, but the role of molds and yeasts has not been studied with equal thoroughness. Considering the occurrence of fungal spoilage in frozen food and the potential hazard involved, more studies on psychrophilic fungi growth in foods stored at low temperatures are necessary. Industries should facilitate access to spoiled products to enable further investigation, since it is generally difficult for researchers obtain these products.

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