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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.