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Occurrence, distribution and contamination levels of heat-resistant moulds throughout the processing of pasteurized high-acid fruit products

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ABSTRACT

Heat-resistant moulds (HRMs) are well known for their ability to survive pasteurization and spoil high-acid food products, which is of great concern for processors of fruit-based products worldwide. Whilst the majority of the studies on HRMs over the last decades have addressed their inactivation, few data are currently available regarding their contamination levels in fruit and fruit-based products. Thus, this study aimed to quantify and identify heat-resistant fungal ascospores from samples collected throughout the processing of pasteurized high-acid fruit products. In addition, an assessment on the effect of processing on the contamination levels of HRMs in these products was carried out. A total of 332 samples from 111 batches were analyzed from three processing plants (=three processing lines): strawberry puree (n = 88, Belgium), concentrated orange juice (n = 90, Brazil) and apple puree (n = 154, the Netherlands). HRMs were detected in 96.4% (107/111) of the batches and 59.3% (197/332) of the analyzed samples. HRMs were present in 90.9% of the samples from the strawberry puree processing line (1–215 ascospores/100 g), 46.7% of the samples from the orange juice processing line (1–200 ascospores/100 g) and 48.7% of samples from the apple puree processing line (1–84 ascospores/100 g). Despite the high occurrence, the majority (76.8%, 255/332) of the samples were either not contaminated or presented low levels of HRMs (< 10 ascospores/100 g). For both strawberry puree and concentrated orange juice, processing had no statistically significant effect on the levels of HRMs ($p > 0.05$). On the contrary, a significant reduction ($p < 0.05$) in HRMs levels was observed during the processing of apple puree. Twelve species were identified belonging to four genera - *Byssochlamys*, *Aspergillus* with *Neosartorya*-type ascospores, *Talaromyces* and *Rasamsonia*. *N. fumigata* (23.6%), *N. fischeri* (19.1%) and *B. nivea* (5.5%) were the predominant species in pasteurized products. The quantitative data (contamination levels of HRMs) were fitted to exponential distributions and will ultimately be included as input to spoilage risk assessment models which would allow better control of the spoilage of heat treated fruit products caused by heat-resistant moulds.

1. Introduction

Spoilage incidents of thermally processed high-acid fruit products by heat-resistant moulds (HRMs) have been reported for over 80 years (Beuchat, 1998; Filtenborg et al., 2004; Olliver and Rendle, 1934) and they are still cause for great concern to the fruit processing industry, influencing both import and export markets.

The most important species of heat-resistant fungi isolated from food products belong to the genera *Paecilomyces* (*Byssochlamys* morph), *Aspergillus* (*Neosartorya* morph), *Talaromyces* and *Penicillium* (*Eupenicillium* morph) (Dijksterhuis, 2007; Pitt and Hocking, 2009; Samson et al., 2010). HRMs have already been detected in various fruits and fruits products, such as strawberries, blueberries, lemon cells and apple juice (Aragão, 1989; Kikoku et al., 2008; Salomão et al., 2014;

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Tranquillini et al., 2017). Besides their occurrence in raw materials, they have also been isolated from cardboard materials used in aseptic packaging (Delgado et al., 2012). The presence of HRMs in raw materials and processing environments may compromise the microbial stability of heat-treated fruit products. The spoilage risk is not only due to their great resistance to thermal processing and chemical compounds (e.g. chlorine, alcohol and hydrogen peroxide) (Dijksterhuis and Teunissen, 2004; Silva and Gibbs, 2004; Tournas, 1994), but also due to their ability to grow under low pH (pH < 4.0) and limited headspace oxygen levels (Nielsen et al., 1989; Taniwaki et al., 2009; Tournas, 1994). The (asco)spores of many species are activated after a sub-lethal temperature or pressure trigger (Dijksterhuis and Teunissen, 2004; Reyns et al., 2003). Moreover, once present, even in low numbers, some HRMs can germinate during storage at room temperature and spoil the products (Sant'Ana et al., 2010a), causing large economic losses. Besides the spoilage risk, many species of heat-resistant fungi may also pose a health risk due to mycotoxin production (Frąc et al., 2015; Houbraken et al., 2006; Sant'Ana et al., 2010b; Tournas, 1994).

In order to increase the microbial stability of high-acid fruit products, different tools can be used in the food process chain. These tools include the adoption of good manufacturing practices (GMPs) which are crucial to prevent entry of microbial contaminants into food processing environments and inactivation of potentially present ascospores by thermal (Kikoku et al., 2008; Sant'Ana et al., 2009; Scaramuzza and Berni, 2014; Souza et al., 2017) and non-thermal (Evelyn and Silva, 2015; Evelyn and Silva, 2017; Ferreira et al., 2009) processes. In addition, prevention of growth of HRMs is an equally important tool to increase the microbial stability of high-acid fruit products (Berni et al., 2017; Panagou et al., 2010; Taniwaki et al., 2009; Tremarin et al., 2015). However, the exact impact of these measures in reducing the probability of spoilage of high-acid fruit products by HRMs has not yet been adequately quantified.

Quantitative microbial risk assessment (QMRA), which is mostly used as a tool to determine and manage the risks of foodborne pathogens, has recently been used to assess the risk of spoilage of food by fungi (Gougouli and Koutsoumanis, 2017). This is a very valuable approach which incorporates the variability related to the occurrence, inactivation and growth of target spoilage microorganisms along the food chain. Despite the vast amount of information regarding the inactivation of ascospores, few data (e.g. Aragão, 1989; Salomão et al., 2014; Tranquillini et al., 2017) are currently available regarding their occurrence in fruit products. Moreover, were data is available, only a low number of samples were analyzed leading to scarcity in quantitative data. Therefore, there is a need for more data in order to determine the distribution of contamination levels of HRMs and their variability, not only in the raw material but also throughout the processing steps and in pasteurized products. Likewise, these data can be very useful to assess the spoilage risk of high-acid fruit products by heat-resistant moulds. Thus, the major objectives of this study were to determine (i) the incidence and distribution of contamination levels of fungal ascospores in three fruit processing lines: strawberry puree, orange juice and apple puree; (ii) the identity at genus and species levels of detected HRMs; and (iii) the effect of processing on the contamination levels of ascospores.

2. Material and methods

2.1. Sampling

A total of 332 samples (each ca. 150 g) were aseptically collected from 111 batches of three processing plants: strawberry puree (Belgium), concentrated orange juice (Brazil) and apple puree (the Netherlands). The process flow diagrams are shown in Fig. 1. With regards to the strawberry puree processing, frozen strawberries are received and stored at −18 °C until they are used. After thawing, the strawberries are crushed and sieved and then held in a buffering tank until they are pasteurized and packaged. Samples of strawberry puree (n = 29 batches, 88 samples) were collected during May to September 2016 at the follow stages: after crushing (n = 29), after sieving (n = 29) and after pasteurization (n = 30). In the orange juice processing line, fresh oranges are received, washed and screened according their sizes/variety. The juice is then extracted and filtered before pasteurization in a heat exchanger. Orange juice concentrate samples (n = 30 batches, 90 samples) were collected in August and September 2016 at the follow stages of processing: at extraction output (n = 30), at evaporator inlet (n = 30) and after pasteurization (n = 30). With regards to the apple puree processing line, apples are received, washed and screened. The apple puree is obtained after extraction/sieving followed by pasteurization. Samples of apple puree (n = 52 batches, n = 154) were collected in October and November 2016 at the following stages of processing: raw material-apples (n = 52), after sieving (n = 52) and after pasteurization (n = 50). Pasteurization was in all cases performed for a few seconds. With regards to the relative intensity of the pasteurization applied, strawberry puree was subjected to the least intense pasteurization process, whereas apple puree was subjected to the most intense pasteurization process. All the samples were stored at −20 °C until the analysis were performed.

2.2. Quantification of heat-resistant fungal ascospores

Hundred grams (100 g) of sample was analyzed for heat-resistant fungal ascospores. Firstly, the samples were aseptically transferred to stomacher bags and diluted in 150 mL of sterile distilled water. The diluted samples were then homogenized in a stomacher (Lab Blender 400, Seward Laboratory, London, UK) for 2 min and heat-sealed half way along their length to avoid the presence of air bubbles. After sealing, the bags were heat treated in a temperature-controlled water bath (Memmert, WB 10, Germany) at 80 ± 1 °C for 30 min. Thereafter, the samples were aseptically transferred to Schott bottles containing 250 mL of molten double strength Malt Extract Agar (MEA) (Oxoid, Basingstoke, UK) supplemented with chloramphenicol (200 mg/L, Oxoid, Basingstoke, UK) and tempered to 55 °C. Subsequently, the heat-treated samples were thoroughly mixed with the agar and distributed into seven 140 mm diameter Petri dishes. The dishes were placed in plastic bags and then incubated at 30 °C for up to 30 days and visually checked for growth every 7 days. The results obtained after 30 days were expressed as ascospores/100 g.

2.3. Isolation and morphological identification

All colonies recovered from analyzed samples were picked with a sterile inoculation loop, streaked onto MEA plates and incubated at 30 °C for 7 days. This was repeated until pure cultures (isolates) were obtained, i.e., when identical macroscopic characteristics (e.g. color, size, colony appearance) were observed. The pure isolates were then cultured on different media and incubated at different temperatures according to the identification keys of Pitt and Hocking (2009) and Samson et al. (2010).

2.4. Molecular identification

Gene sequencing was used to identify the HRMs at the species level. The sequencing was performed in three stages: DNA extraction, DNA amplification and DNA sequencing. Firstly, the isolates were grown for 14 days at 25 °C in 3 mL of potato dextrose broth (PDB) (Oxoid, Basingstoke, UK) in 12-well microplates. The resulting mycelial mats were harvested in Eppendorf tubes after centrifugation at 8000 × *g*, 5 min and 4 °C. The pellets obtained were then lyophilized at −80 °C for 20 h to produce a fine powder. Subsequently, the genomic DNA was obtained using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instructions. After extraction, the DNA was then amplified using the universal primers ITS4 (