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# Susceptibility of *Aspergillus* spp. to acetic and sorbic acids based on pH and effect of sub-inhibitory doses of sorbic acid on ochratoxin A production

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ABSTRACT

The use of organic acids as preservatives in foods is an alternative for restricting the development of moulds in food and subsequently reducing the possibility of mycotoxin contamination. The objective of this study was to investigate the influence of acetic and sorbic acids on the growth of species of *Aspergillus* in culture media at different pH values, besides determining the influence of sub-inhibitory concentrations of sorbic acid on the production of ochratoxin A. To assess the sensitivity of fungal isolates to acids, factorial arrangement was used with nine isolates of *Aspergillus*, four pH levels (4.5, 5.0, 5.5, and 6.0) and two types of acids in the concentrations: acetic acid (0; 12.5; 25; 50; 100; 200; 400 and 800 mM) and sorbic acid (0; 0.5; 1; 2; 4; 8; 16; 32 mM). The experiments were conducted in triplicate, with incubation for six days at 25 °C. A 2 × 2 factorial arrangement with two ochratoxigenic isolates and two pH values (4.5 and 5.0) was used to determine the inhibitory concentration (IC) and the influence of sorbic acid on the production of ochratoxin A. The sorbic acid concentrations used for ochratoxin A production studies were the same used for testing susceptibility. This experiment was conducted in duplicate, with incubation for seven days and determination of the IC for each isolate, where the controls and two concentrations below IC (25% and 50% of the IC) were selected for the analysis of ochratoxin A. Ochratoxin A was quantified by high performance liquid chromatography with fluorescence detection. There was variability in the sensitivity of the species tested for the acids assessed. *Aspergillus carbonarius*, *Aspergillus parasiticus* and *Aspergillus flavus* were more sensitive to the tested acids while *Aspergillus niger* and *Aspergillus tubingensis* were the most resistant when compared with the other isolates. A direct relationship between the pH values and the inhibitory concentration was observed and, in general, each increase of 0.5 in the pH value doubled the acid concentration necessary to inhibit the growth of a same isolate. It was also observed that sorbic acid under-dosing, in addition to not preventing fungal growth, can stimulate the production of ochratoxin A in isolates of *A. carbonarius* and *A. niger*.



## 1. Introduction

The development of moulds in foods and the subsequent risk of their contamination by mycotoxins are a public health concern (Hussein & Brasel, 2001). Ochratoxin A is among the most important mycotoxins, presenting nephrotoxic action and carcinogenic properties (Charmeley, Rosenberg, & Trenholm, 1994). Reducing the fungal growth and consequently preventing the formation of this toxin are the most efficient way to reduce human exposure in the diet (Kuiper-Goodman, 1996).

Traditionally, many preservatives have been used to prevent fungal growth in food. The use of organic acids has been highlighted due to the status of many of these organic acids of GRAS (Generally Recognized as Safe) by the American Food and Drug Administration (FDA) (Ricke, 2003). However, these acids must be used in proper concentrations to be effective, and to determine the most effective concentrations of a given organic acid, it is important to consider a number of factors, among which pH is considered a primary determinant of effectiveness (Lund, George, & Franklin, 1987).

Several organic acids (e.g., acetic, citric and lactic acids) can be used quantum satis in the formulation of a food product, although some, such as acetic acid, may provide adverse sensory changes in the foods when used in high doses (Taniguchi et al., 1998). Thus, it is essential for the food industry to define the concentrations of acetic acid needed to control deterioration without compromising the acceptability of a product.

On the other hand, there are organic acids, such as sorbic acid, which have a certain acceptable daily intake (ADI). In these cases, it is very

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important to use caution in the definition of the concentrations required to inhibit fungal growth. Additionally, there is evidence that the use of inadequate concentrations of sorbic acid and its salts may pose an indirect risk to the health of consumers by promoting ochratoxin A production (Arroyo, Aldred, & Magan, 2005), since it induces tolerance of moulds to this preservative, besides stimulating their growth (Marin, Abellana, Rubinat, Sanchis, & Ramos, 2003).

It is well known that the form of a weak acid presentation (dissociated or undissociated) will influence antimicrobial power. However, there are no studies demonstrating the effect of pH variation at constant concentrations of organic acids on the antifungal activity of these, simulating buffering situations that commonly occur in food. Nor have we found studies evaluating the effect of sub-inhibitory doses of sorbic acid on the production of ochratoxin A in isolated *Aspergillus* section Nigri.

Thus, the aim of this study was to investigate the susceptibility of the genus *Aspergillus*, mainly section *Nigri*, to acetic and sorbic acids at different pH values, and to determine the influence of under-dosing of sorbic acid on the production of ochratoxin A by *Aspergillus carbonarius* and *Aspergillus niger*.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Isolates

Nine isolates of *Aspergillus* spp. were used for this study (Table 1). They have been previously identified according to the methodology described by Pitt and Hocking (2009) and their identity was confirmed through analysis of the profile of secondary metabolites, using the methodology described by Smedsgaard (1997).

The fungal isolates were inoculated at 3 points in Czapek Yeast Extract Agar (CYA) and Yeast Extract and Sucrose Agar (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. 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).

**Table 1**
Isolates from *Aspergillus* used in the experiments, according to identification, origin, substrate and ability to produce ochratoxin A.

| Strain | Identification | Section | Location | Substrate | Production of ochratoxin |
|---|---|---|---|---|---|
| ITAL 792cc | *A. carbonarius* | *Nigri* | Brazil | Cocoa | Positive |
| ITAL 1375cc | *A. carbonarius* | *Nigri* | Brazil | Cocoa | Positive |
| ITAL 325 F | *A. luchuensis* | *Nigri* | Spain | Date | Negative |
| ITAL 331 F | *A. niger* | *Nigri* | Spain | Date | Positive |
| ITAL 1240cc | *A. niger* | *Nigri* | Brazil | Cocoa | Positive |
| ITAL 500 F | *A. niger* | *Nigri* | Mexico | Date | Negative |
| ITAL 277 F | *A. tubingensis* | *Nigri* | Spain | Date | Negative |
| ATCC 2384 | *A. flavus* | *Flavi* | – | – | Negative |
| ITAL 79cc | *A. parasiticus* | *Flavi* | Brazil | Cocoa | Negative |

#### 2.1.2. Organic acids

The organic acids tested were acetic acid (Impex 99.7%) and sorbic acid (Vetec 99%).

### 2.2. Propagation of the moulds

The mould culture was propagated and preserved in tubes with approximately 10 mL CYA medium, incubated at a temperature of 25 °C ± 1 °C for` six days.

### 2.3. Inoculum preparation

The conidial suspension was prepared by adding 10 mL of sterile Tween 80 solution at a concentration of 0.01% in each of the previously inoculated tubes. Then the surface was scraped with a sterile loop, homogenizing in a vortex and then filtering with sterile gauze to remove the hyphae and conidiophores. The conidia were counted using a Neubauer chamber, adjusting the inoculum concentration of $10^5$ conidia/mL when necessary (Stratford, Plumridge, Nebe-Von-Caron, & Archer, 2009).

### 2.4. Assessment of fungal susceptibility to organic acids

A factorial arrangement was used, containing: nine fungal isolates (Table 1), four levels of pH (4.5; 5.0; 5.5 and 6.0) and two types of acids in different concentrations: acetic acid (0 mM (0 ppm); 12.5 mM (751 ppm); 25 mM (1501 ppm); 50 mM (3003 ppm); 100 mM (6005 ppm); 200 mM (12,010 ppm); 400 mM (24,020 ppm) and 800 mM (48,040 ppm) and sorbic acid (0 mM (0 ppm); 0.5 mM (56.7 ppm); 1 mM (112.1 ppm); 2 mM (224.3 ppm); 4 mM (448.5 ppm); 8 mM (897 ppm); 16 mM (1794 ppm); 32 mM (3588 ppm) and 64 mM (7176 ppm)). For the experiment, the methodology proposed by Stratford et al. (2009) was used with some modifications, using a basal culture medium described by León et al. (2012). The basal medium containing, per litre of distilled water: 10 g of malt extract and 20 g of yeast extract, autoclaved at 121 °C for 15 min. As soon as the medium reached 45 °C, it was homogenized with each of the acids in the concentration to be tested and the final pH was aseptically adjusted with a sterile solution of KOH (5 M) or HCl (5 M). Subsequently, 4 mL of this mixture was transferred to sterile test tubes.

A culture medium without acids and pH adjusted with HCl or KOH was used as a negative control. All test tubes were inoculated with a suspension of 10 μl of the spores ($10^5$ conidia/mL) previously prepared, giving a final concentration of $10^3$ conidia/tube, and incubated statically at 25 °C for six days. After the incubation period, they were visually assessed following a dichotomous scale (grown or not grown) and the inhibitory concentration (IC) was defined as the lowest concentration of a tested acid where there was no fungal growth. The experiments were conducted in triplicate.

### 2.5. Calculating the dissociated and undissociated fraction of the acid

For each concentration tested, the portion of undissociated acid was calculated using the formula proposed by Henderson–Hasselbalch (Eq. (1)).

$$pH = pK_a + \log([A]/[HA]) \quad (1)$$

where, $pK_a$ is the dissociation constant of the acid; [A] is the concentration of molecules of the dissociated acid and [HA] corresponds to the total concentration of undissociated acid.