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Ochratoxin A production by *Aspergillus niger*: Effect of water activity and a biopreserver formulated with *Lactobacillus plantarum* CRL 778

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ABSTRACT

Some filamentous fungi of the genera *Aspergillus* (*A.*) are of high impact in food safety due to the production of a toxic secondary metabolite, the ochratoxin A (OTA). The present study was carried out to evaluate the effect water activity (a_w) on the growth and the OTA production by the strain *Aspergillus niger* 13D both *in vitro* assays and in bread slices. High values of a_w favoured growth and OTA production by *A. niger* 13D in Czapek Yeast extract agar medium (CYA) with the optimum at 0.995. In bread slices, fungi growth rate (μ_{max}: 0.90–1.07 cm d^{-1}) and OTA production (5.13–10.79 ng g^{-1}) were lower respect to CYA medium (μ_{max}: 1.09–1.81 cm d^{-1}, OTA concentration: 26.02–133.28 ng g^{-1}) independently to a_w. Furthermore, efficacy antifungal of biopreserver formulated with *Lactobacillus plantarum* CRL 778 (SL778) for packed bread was evaluated. Our result evidenced the inhibitory activity of SL778 against growth of ochratoxigenic *A. niger* 13D in bread making at different a_w. In addition, this biopreserver demonstrated is principally antimycotoxigenic, observing a reduction 60% of OTA at 0.995 and 0.971 a_w in bread added with SL778.

1. Introduction

The production of mycotoxins e.g., ochratoxin A (OTA) by filamentous fungi manly *Aspergillus* and *Penicillium* is of high impact in food safety. OTA is nephrotoxic, immunosupressive, teratogenic, and has been classified as a possible carcinogen (Aish, Rippon, Barlow, & Hattersley, 2004; Felizardo & Câmara, 2013; Murphy, Hendrich, Landgren, & Bryant, 2006). The presence of OTA occurs in a variety of foods, as well as in cereal grains, wine, grape juice, coffee, legumes, beer, nuts, yerba mate, meat, etc (Castrillo, Horianski, & Jerke, 2013; Clark & Snedeker, 2006). In these different food, the OTA production by fungal is influenced by intrinsic and extrinsic factors such as pH, composition of substrate, relative moisture or temperature, but water activity is one of the most important (Kapetanakou, Kollias, Drosinos, & Skandamis, 2012; Magan & Aldred, 2007; Valero, Farre, Sanchis, Ramos, & Marin, 2006).

In the last years authors reported the contamination of bread with high (6–19.6 ng g^{-1}) levels of OTA (Bento, Pena, Lino, & Pereira, 2009; Cengiz, Oruç, Uzunoğlu, & Sonal, 2007; Duarte, Bento, Pena, & Lino, 2009; Duarte, Pena, & Lino, 2010; González-Osnaya, Soriano, Moltó, & Mañes, 2007; Juan, Pena, Lino, Moltó, & Mañes, 2008). The presence of OTA in unpolluted bread comes from the wheat flour and is only partly destroyed during the bread making process (Subirade, 1996). In addition, the OTA occurrence in bread can be is the result of direct contamination with ochratoxigenic moulds. The contaminated bread is to be a risk for human health either directly, as a result of people eating mouldy bread, or indirectly, as a result of consumption of products of animals fed with mouldy bread (Osborne, 1980). Although the likelihood of people eating spoiled bread is very low in developed countries, the incidence in developing countries may be greater. As the bread is an excellent energy source (rich in starch and has reasonable protein content) is a common practice the use of mouldy bread to feed animals, and so decrease economic losses. This practice is special interest because OTA can be accumulated in the meat of animals intended consumed human (Perši, Pleadin, Kovačević, Scortichini, & Milone, 2014).

In bakery products, preservatives (salts of propionic and sorbic acids) are added to prevent growth of spoilage fungi. However, in

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recent years, there has been consumer pressure to reduce the use of such preservatives. Previous studies have suggested that the use of suboptimal concentrations of these preservatives may stimulate the growth and OTA production of some spoilage fungi of bread (Arroyo, Aldred, & Magan, 2005). Thus, suboptimal doses could pose a problem and allow mould spoilage to occur. In the last decade, however, has increased the interest for biological methods to prevention of the fungal growth using lactic acid bacteria (LAB) (Gerez, Carbajo, Rollán, Torres Leal, & Font de Valdez, 2010; Ryan, Dal Bello, & Arendt, 2008; Ryan et al., 2011). Although studies on antifungal LAB against black *Aspergilli* (*A*) and ochratoxigenic fungi are comparatively less. Recently, Kapetanakou et al. (2012) reported a study in which a mix of six LAB strains was tested as potential inhibitor of *Aspergillus carbonarius* both growth and OTA production in culture media and beverages without positive results.

In a previous work, *Lactobacillus* (*L.*) *plantarum* CRL 778 and others lactobacilli proved to be effective in inhibiting the growth of spoilage molds, such as *Aspergillus* (*A.*) niger, *Penicillium* (*P.*) sp. and *Fusarium* (*F.*) *graminearum*, previously isolated from contaminated bread; and *P. digitatum* and *Geotrichum* (*G.*) *citri-aurantii*, isolated from a commercial citrus fruit packing industry (Gerez, Torino, Rollán, & Font de Valdez, 2009; Gerez, Torres, Font de Valdez, & Rollán, 2013). The antifungal effect being related to the production of lactic, acetic, phenyllactic acids (PLA) and hydroxyphenyllactic acid (OH-PLA). From these result, a ready-to-use biopreserver (SL778) for packed bread was developed. The supplementing traditional doughs with biopreserver SL778 significantly improved the shelf life of packaged bread (Gerez, Torino, Obregozo, & Font de Valdez, 2010). The present study was carried out to evaluate the effect water activity (a_w) on the growth and the OTA production by the strain *A. niger* 13D both *in vitro* assays and in bread slices. Furthermore, efficacy of SL778 to control of *A. niger* 13D in bread making at different a_w was evaluated.

2. Materials and methods

2.1. Microorganisms

L. plantarum CRL 778 was isolated from homemade wheat sourdough and belongs to the culture collection of Centro de Referencia para Lactobacilos (CERELA-CONICET, Tucumán, Argentina). For the assays, 16-h old cultures in Man, Rogosa and Sharpe broth (MRS, Oxoid) at 37 °C were centrifuged (8936 *g*, 10 min) and the cells obtained washed twice with sterile potassium phosphate buffer 0.1 M (pH 6.5) and suspended in the same buffer.

The ochratoxigenic *Aspergillus* (*A.*) *niger* 13 D was obtained from the culture collection of National University of Río Cuarto (UNRC), Córdoba, Argentina, and stored at −20 °C in 40% glycerol. The mould strain was grown at 25 °C for 7 days on malt extract agar (MEA; Difco Laboratories, Detroit, Michigan, USA) plates. The conidia were collected with sterile soft agar (Tween 80, 0.5 g L^{-1} and agar, 1.0 g L^{-1}) and counted at the microscope in a haemocytometer chamber to adjust the concentration to 10^4 conidia ml^{-1}.

2.2. In vitro assays

The effect of a_w on *A. niger* 13 D was evaluated in plates containing Czapek Yeast extract agar medium (CYA) (Pitt, 1973) adjusted to $a_w = 0.955$, 0.964, 0.971, 0.982, and 0.995 by addition of known amounts of glycerol (Dallyn & Fox, 1980). The a_w was checked with an a_w meter (AquaLab Series 3, Decagon Devices Inc., Pullman, WA, USA). CYA agar plates were inoculated centrally with a needlepoint of the *A. niger* 13 D spore suspension. Petri dishes of the same a_w were stacked in clean polythene bags together with a jar open containing glycerol/water solutions of the same a_w value (Ramos, Labernia, Marina, Sanchis, & Magan, 1998). All plates were incubated during 7 days at 30 °C. The growth fungus was determined as lag phase and the mycelial extension according to Mitchell, Parra, Aldred, and Magan (2004). Temporal mycelial extension rates were determined daily in two directions at right angles to each other until the medium was fully colonized. The radial extension rates were plotted against time and the growth rates calculated using linear regression (mm day^{-1}).

2.3. Bread assays

L. plantarum CRL 778 was inoculated (15% v/v) in a mixture of (g/l): 232 wheat flour, 100 quinua flour, 6.6 monohydrated dextrose, 4.1 Na-citrate, 2.6 KH_2PO_4 and 1 L tap water. This slurry was fermented under stirring at 30 °C for 24 h and samples were taken to evaluate: cell viability, and total titratable acidity (TTA), pH, and organic acids. This fermented slurry (S778) was used for wheat bread manufacture.

The dough (DS778) was prepared as follows: 1000 g wheat flour, 12 g dried yeast (Levex, Chile), 20 g NaCl, 30 g fat (baking margarine, Danica Dorada, Argentina), 30 g dried skim milk (Veronica, Argentina) and 0.5 L of tap water. To incorporate the slurry, tap water was partially replaced (40%) in the dough preparation by equal amounts of the slurry fermented by *L. plantarum* CRL 778 (S778). Doughs without S778 were used as control (Dc). The doughs were individually placed in aluminum pans for fermentation (2 h, 37 °C) and samples were taken at the end this period to determine pH (pHmeter PT-10 model, Sartorius AG, Goettingen, Germany) and organic acids.

After fermentation, the doughs were baked in an oven (180 °C). Cooked breads were cooled in a laminar flow cabinet to prevent contamination. Breads obtained from dough Dc and DS778 were named as Bc and B778, respectively. The bread Bc and B778 were cut in slices and inoculated centrally with a needlepoint of the spore suspension. The slices were put into sterile Petri dishes and stacked in clean polythene bags together with a jar open containing glycerol/water solutions of the a_w values (Ramos et al., 1998). Quadruplicate sets of each condition were incubated at 30 °C for 7 days. The a_w of slices were controlled with an a_w meter. The lag phase and mycelial extension were determined as described.

2.4. Organic acids determination

In fermented doughs, samples (10 g) were homogenized with distilled water (90 ml) in a homogenizer (The Virtis Company, Gardiner, New York 12525) and centrifuged (8000 *g* for 10 min) to obtain the supernatant. Proteins were removed from all samples by mixing (500 μl) with trichloroacetic acid (500 μl) in vortex and incubating at 4 °C for 30 min. The treated samples were centrifuged (12,000 *g*, 15 min, 5 °C), filtered (0.22 μm filters; Ministart high flow, Sartorius, Goettingen, Germany). The organic acids present in these samples were determined by High Performance Liquid Chromatography (HPLC) using an ion-exclusion Aminex HPX-87H column (300 mm × 7.8 mm, Bio Rad, USA) under the following conditions: mobile phase H_2SO_4 (5 mmol l^{-1}) at a flow rate of 0.6 ml/min and column temperature of 45 °C. A refractive index detector was used to identify lactate and acetate while an UV detector set at 210 nm was used to identify PLA and OH-PLA. Both detectors were connected to the software Peak Simple II (Knauer Company, Berlin, Germany) for data analysis. Organic acid were quantified on the basis of the detector response compared with that of a range of standards (Sigma). Organic acids concentrations were expressed in mg g^{-1}.

Table 1
Growth parameters and OTA production by *A. niger* 13D in CYA agar medium at different a_w.

	Water activity (a_w)				
	0.995	0.982	0.971	0.964	0.955
Lag phase (h)	12.16 ± 0.88[b]	14.36 ± 0.84	16.65 ± 2.11	18.29 ± 1.61	22.35 ± 0.96
μ_{max} (cm d^{-1})	1.81 ± 0.03	1.47 ± 0.02	1.35 ± 0.11	1.18 ± 0.07	1.09 ± 0.02
OTA concentration (ng g^{-1})[a]	133.28 ± 1.51	85.56 ± 10.14	26.03 ± 2.87	9.86 ± 1.35	8.87 ± 1.13

[a] Concentration of OTA in CYA agar medium after 7 days incubation.
[b] Mean values ± standard deviation of three replicates is presented.

2.5. Ochratoxin A determination

The extraction of OTA was performed after 7 days incubation according to Bragulat, Abarca, and Cabañes (2001) with modifications. On each sampling, three agar plugs (diameter 5 mm) were removed from the inner, middle and outer area of each colony. Plugs were weighed and introduced into 3-ml vial. Methanol (1 ml) was added, and the vials were shaken for 2 h (Autovortex SA6, Surrey, UK). After the extracts were centrifuged at 14,000 *g* for 10 min, filtered (Syringe filtres, 17 mm, 0.45 μm, nylon membranes, TITAN) and stored at 5 °C until using. The OTA concentration was determined by reversed phase HPLC with fluorescence detection (λexc 330 nm; λem 460 nm) using a C18 column (Grace, Research AG S.A., USA; 250 × 4.6 mm, 5 μm particles) connected to a pre-column (Supelguard LC-ABZ, Supelco; 20 × 4.6 mm, 5 μm particle size). The mobile phase (Scudamore & MacDonald, 1998, with modifications) was acetonitrile–water–acetic acid, 40:60:2 pumped at 1.0 ml min^{-1}. The injection volume was 50 μl and the retention time was around 17 ± 0.2 min. OTA was quantified on the basis of the HPLC fluorimetric response compared with that of a range of OTA standards (Sigma). The detection limit of the analyses was 5 ng g^{-1}.

The concentration of OTA from bread samples (7 days incubation) was evaluated using commercial ELISA kit (R1311 Ridascreen®Ochratoxin A 30/15, Germany). Three agar plugs (diameter 5 mm) were removed from each colony on slices, weighed and introduced into a small glass of precipitation. These samples were homogenized with ultra-turrax homogenizer (T-18; Ika) and the extraction of OTA was carried out as described in the kit. Sample were diluted were necessary to keep within the range of detection and quantification (limit of detection 1.25 ng g^{-1}).

2.6. Statistical analysis

Results of two independent assays are presented as mean values ± standard deviation (SD). Data were analysed by ANOVA and Tukey's test. The statistical analysis was carried out with the Statistica 5.5 program (Statsoft, Tulsa, OK, USA). Results were considered significantly different at $p < 0.05$.

3. Results

3.1. Growth and OTA production by A. niger 13D in vitro assays

Results obtained are showed in Table 1. Growth of *A. niger* 13D in CYA medium took place at all a_w conditions assayed. On the whole, the specific growth rate increased at higher a_w values while the opposite occurred for the lag phase. The maximum growth rate (μ_{max}:1.81 ± 0.03 cm d^{-1}) and minimum lag phases (12.16 ± 0.88 h) was observed at 0.995 a_w. OTA concentration increased at high a_w, being the optimum condition at 0.995 a_w for OTA production (133 ± 1.5 ng/g). No different significant in the concentration at both 0.955 and 0.964 a_w was observed.

3.2. Effect of biopreserver SL778 on growth and OTA production by A. niger 13D in bread making

Doughs were prepared with the commercial yeast alone (control dough, Dc) and with the addition of *L. plantarum* CRL 778 (DS778). Results are presented in Table 3. After 2 h fermentation, doughs DS778 reached lower (4.6) pH compared to DC control (pH 5.25) due to manly to lactic acid production. The antifungal metabolites of the lactobacilli e.g., acetic acid, PLA and OH-PLA were also produced in DS778.

Breads slices (Bc and B778) elaborated with the corresponding doughs Dc and DS778, respectively, were inoculated with *A. niger* 13D by central puncture and maintained at different a_w values. Results are presented in Table 2. *A. niger* 13D was as able to develop in control bread (Bc) at all a_w values. The highest growth rates (μ_{max} = 1.07 ± 0.11 cm d^{-1}) was observed at a_w 0.995, 0.982 and 0.971. In the bread B778, the fungi displayed a decrease maximum growth (7 days) and growth rate, in addition a slight lengthening in the lag phase. The greater (24.3%) inhibition was found at 0.995 a_w.

The minimum (5.1 ng g^{-1}) and maximum (10.8 ng g^{-1}) OTA production was found at 0.982 and 0.971 a_w, respectively in control bread Bc (Fig. 1). So, optimal condition for growth (a_w 0.995) did not coincide with the ones for OTA formation (a_w 0.971) in bread. In B778 bread, the OTA production was lower observing a reduction 60% at 0.995 and 0.971 a_w respect to Bc. No significant statistical differences ($p \leq 0.05$) at 0.964 and 0.955 a_w were found.

4. Discussion

Growth of fungi can be influenced by extrinsic factors such as a_w. *A. niger* 13D was able to grow in CYA medium over a wide range of a_w from 0.955 to 0.995. In addition, the results showed a significant influence of a_w on not only growth but also on OTA production. High values of a_w favoured growth and OTA production by *A. niger* 13D in CYA medium with the optimum at 0.995. In contrast, Esteban, Abarca, Bragulat, and Cabañes (2006) working with *A. niger* strains reported different a_w conditions for optimal growth (high a_w, 0.995) and OTA production (low a_w, 0.960). The amounts of OTA obtained in CYA medium were greater (487 ng g^{-1}) than the amounts obtained in our study (up to 133.28 ng g^{-1}).

Numerous studies have described the effect of a_w on growth of black aspergilli in culture medium but only few addressed the impact of a_w in natural substrates (Astoreca, Barberis, Magnoli,

Table 2
Production of organic acids and pH value of control doughs (Dc) and doughs with *L. plantarum* CRL 778 (DS778) after 2 h fermentation at 37 °C.

	Dc	DS778
pH	5.25 ± 0.04	4.61 ± 0.06
Lactic acid	ND	4.25 ± 0.41 (mg g^{-1})
Acetic acid	0.01 ± 0.00 (mg g^{-1})	0.44 ± 0.15 (mg g^{-1})
Phenyllactic acid	ND[a]	0.013 ± 0.002 (mg g^{-1})
Hydroxy-phenyllactic acid	ND	0.010 ± 0.001 (mg g^{-1})

[a] ND: not detected.

Table 3
Growth parameters of *A. niger* 13D in bread slices maintained at different a_w for 7 days. Control bread (Bc) and bread with *L. plantarum* CRL 778 (B778).

	Bread slices	a_w				
		0.995	0.982	0.971	0.964	0.955
Lag phase (h)	B	31.74 ± 3.47	34.03 ± 3.32	31.03 ± 3.49	30.81 ± 8.75	32.39 ± 1.98
	B778	34.65 ± 4.34	34.89 ± 2.68	32.22 ± 6.18	32.24 ± 6.36	32.40 ± 3.31
μ_{max} (cm d^{-1})	Bc	1.07 ± 0.11	1.07 ± 0.03	0.97 ± 0.02	0.92 ± 0.02	0.90 ± 0.02
	B778	0.81 ± 0.07	0.82 ± 0.06	0.81 ± 0.10	0.82 ± 0.06	0.76 ± 0.05
Growth (7 days)	Bc	5.38 ± 0.13	5.10 ± 0.12	4.60 ± 0.40	4.65 ± 0.50	4.52 ± 0.05
	B778	3.95 ± 0.29	4.22 ± 0.17	4.13 ± 0.12	4.33 ± 0.05	4.13 ± 0.30

Bc and B778 correspond to bread prepared with doughs DC and DS778, respectively.

Combina, & Dalcero, 2009; Bellí, Marín, Coronas, Sanchís, & Ramos, 2007; Marín, Hodžić, Ramos, & Sanchís, 2008). To corroborate this statement the growth of and OTA production by *A. niger* 13D was also determined on bread slices as natural substrate. Some results were similar to those obtained for *A. niger* 13D in CYA medium, i.e., growth and OTA production took place over a wide range of a_w (0.995–0.955) which corresponded to a_w of commercial bread (Ayub, Wahab, & Durrani, 2003). However, fungi growth rate (μ_{max}: 0.90–1.07 cm d^{-1}) and OTA production (5.13–10.79 ng g^{-1}) were lower in bread slices respect to CYA medium (μ_{max}: 1.09–1.81 cm d^{-1}, OTA concentration: 26.02–133.28 ng g^{-1}) independently to a_w. These results suggest that the nutrient sources affect the fungal growth and that studies on artificial sub-sutrates not accurately represent the real capacity of fungi to grow and produce OTA. In addition, the greatest (10.79 ng g^{-1} bread) amount of OTA was produced at 0.971 a_w in bread assays instead of at 0.995 a_w as in CYA medium.

Physical and chemical methods have been developed to control the occurrence of these microorganisms and their toxins, but no efficient strategy has yet been proposed to reduce the presence of mycotoxins. Moreover, some moulds have acquired the ability to resist chemical treatments and some preservatives used in bakery (Davidson, 2001; Nielsen & de Boer, 2000). The reduction of such moulds in food production is thus of primary importance and there is great interest in developing efficient and safe strategies for this purpose. On the other hand, with the increasing interest in food safety throughout the world, LAB cultures with high antifungal and antimycotoxigenic potential could be of outmost importance in limiting mycotoxin exposure. Biological control has been proposed as an alternative to chemicals to reduce the incidence of ochratoxigenic molds and the OTA contamination of foods (Djossou et al., 2011; Rouse, Harnett, Vaughan, & van Sinderen, 2008; Schillinger & Villarreal, 2010). However, none of these studies considered the effect of LAB on mycotoxin production. Recently, Kapetanakou et al. (2012) reported negative results for six LAB strains evaluated both in culture media and beverages as inhibitors of *A. carbonarius*. Our result confirmed the inhibitory activity of *L. plantarum* CRL 778 against growth of ochratoxigenic *A. niger* 13D in bread making at different a_w. In addition, this LAB strain demonstrated is principally antimycotoxigenic, observing a reduction 60% of OTA at 0.995 and 0.971 a_w in bread B778. The present work indicated the possibility of using *L. plantarum* CRL 778 in biological control of OTA in bread. Further studies on the effects of this LAB on other OTA producing fungi present in bread are needed. The mechanisms behind the antagonist activity of that LAB need to be clarified.

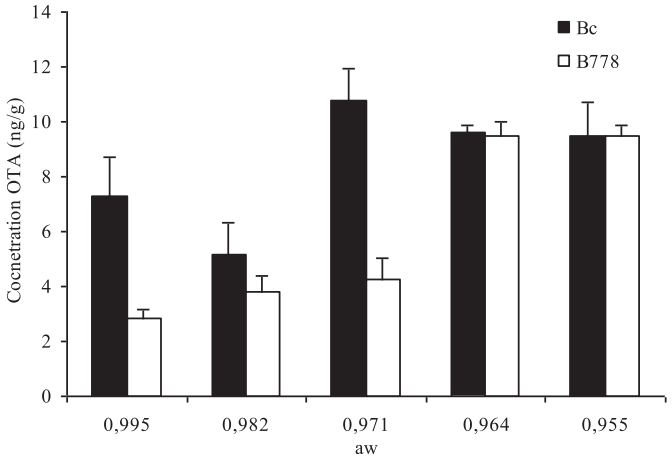

Fig. 1. Production of OTA by *A. niger* 13D in bread maintained at different a_w. Control (Bc) bread and bread with *L. plantarum* CRL 778 (B778).

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