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ORIGINAL ARTICLE

Antifungal and antimycotoxigenic effect of *Lactobacillus plantarum* CRL 778 at different water activity values

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Abstract Ochratoxin A (OTA) is a mycotoxin produced by filamentous fungi with high impact in food safety due to its toxicity. In the last decade, the presence of OTA was widely reported in different foods. In this study, the ability of *Lactobacillus* (*L.*) *plantarum* CRL 778 to control growth and OTA production by *Aspergillus* (*A.*) *niger* 13D strain, at different water activity (a_w) values (0.955, 0.964, 0.971, 0.982, and 0.995) was determined *in vitro*. Both parameters were significantly ($p < 0.05$) reduced by the lactobacilli and the effect depended on a_w. Greatest growth rate inhibition (46.9%) was obtained at $a_w = 0.995$, which is the most suitable value for growth and production of antifungal metabolites (lactic acid, acetic acid, phenyllactic and hydroxyl-phenyllactic acids) by *L. plantarum* CRL 778. Besides, morphological changes and inhibition of melanin synthesis were observed in colonies of *A. niger* 13D in presence of *L. plantarum* CRL 778 at a_w ranged between 0.971 and 0.995. In addition, maximum reduction (90%) of OTA production took place at $a_w = 0.971$, while inhibition of fungi growth was more evident at $a_w = 0.995$. These findings suggest that *L. plantarum* CRL 778 could be used for control of ochratoxigenic fungal growth and OTA contamination in different fermented foods with a_w values between 0.971 and 0.995.

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PALABRAS CLAVE
Bacterias lácticas;
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Ocratoxina A;
Actividad del agua

Efecto antifúngico y antimicotoxigénico de *Lactobacillus plantarum* CRL 778 a diferentes valores de actividad de agua

Resumen Ocratoxina A (OTA) es una micotoxina producida por hongos filamentosos con un alto impacto en la seguridad alimentaria debido a su toxicidad. En la última década se ha reportado ampliamente a nivel mundial, la presencia de OTA en diversos alimentos. En este estudio se evaluó *in vitro*, la capacidad de *Lactobacillus (L.) plantarum* CRL 778 de controlar el crecimiento y la producción de OTA por *Aspergillus (A.) niger* 13D, a diferentes valores de actividad de agua (a_w): 0.955, 0.964, 0.971, 0.982 y 0.995). La cepa láctica redujo significativamente ($p < 0.05$) ambos parámetros, siendo el efecto dependiente del valor de a_w. La mayor inhibición del crecimiento (46.9%) se obtuvo a $a_w = 0.995$, valor más adecuado para el crecimiento y producción de metabolitos antifúngicos (ácido láctico, ácido acético, ácidos fenil-láctico e hidroxi-fenil láctico) por la cepa láctica. Además, se observaron cambios morfológicos en las colonias de *A. niger* 13D, crecidas en presencia de *L. plantarum* CRL 778 a valores de a_w de 0.971 y 0.995. El porcentaje máximo de reducción en la producción de OTA (90%) por la cepa láctica se observó a un valor de $a_w = 0.971$, mientras la inhibición del crecimiento fúngico fue mayor cuando $a_w = 0.995$. Estos hallazgos sugieren que *L. plantarum* CRL 778 podría emplearse para el control de la contaminación por hongos ocratoxigénicos en alimentos con valores de a_w comprendidos entre 0.971-0.995.

Introduction

Ochratoxin A (OTA) is a mycotoxin produced by filamentous fungi with nephrotoxic, immunotoxic, teratogenic and carcinogenic effects[27,31,33]. In the last decade, its presence has been extensively reported worldwide in common foodstuff and beverages. This has aroused significant public concern and also constitutes a major economic problem[2,3,26].

The OTA production by fungi of genera *Aspergillus* (*A.*) and *Penicillium* is influenced by environmental and nutritional factors such as pH, temperature, water activity (a_w) and nitrogen and carbon sources[17–19]. At present, several physical and chemical methods have been reported to prevent the ochratoxigenic fungi growth and OTA detoxification in products for human and animal consumption; however none of these strategies were completely successful[10,16]. Currently, some molds acquired resistance to some chemical additives such as potassium sorbate, sorbic and benzoic acids, which were considered effective preservatives in the past[29]. Besides, current legislation restricts the use of some preservatives for food manufacture. Moreover, consumer increasingly demand reduction of chemical compounds in food or feed and production of natural and healthy products. In this context, in the last decade researchers have shown interest for biological methods, being lactic acid bacteria (LAB) the most powerful prokaryotes when it comes to antifungal potential[6,11,14]. Nevertheless, there is a lack of publications on antifungal LAB against black *Aspergilli* and ochratoxigenic fungi. Kapetanakou et al.[17] reported that none of the six LAB strains tested as potential inhibitors of *A. carbonarius* growth and OTA production had positive results. More recently, de Melo Pereira et al.[16] reported that *Lactobacillus* (*L.*) *brevis* LPBB03 was able to inhibit the ochratoxigenic strain *A. westerdijkiae in vitro*. However, this study did not evaluate the antimycotoxin effect of the LAB.

In previous works, *L. plantarum* CRL 778 and other lactobacilli proved to be effective in inhibiting the growth of spoilage molds, isolated from contaminated bread and commercial citrus fruits[13,14]. The antifungal effect was related to the production of lactic, acetic, and phenyllactic acids. From these results, a ready-to-use biopreserver (SL778: fermented mixture of wheat flour, sucrose, skimmed milk and water) for packed bread was developed and its technologies properties were evaluated[12]. It was observed lower growth and OTA production by *A. niger* in breads made with SL778 and, remarkably, the effect was dependent on storage water activity[11]. The aim of the present study was to determine *in vitro*, the ability of *L. plantarum* CRL 778 to control OTA production and the growth of *A. niger* 13D at different values of water activity, in order to propose it inclusion in different fermented food.

Materials and methods

Microorganisms

L. plantarum CRL 778, isolated from homemade wheat sourdough, was obtained from the culture collection of Centro de Referencia para Lactobacilos (CERELA-CONICET, Tucumán, Argentina). For the assays, 16-h old cultures in MRS broth (Oxoid, Ltd., Basingstoke, England) at 37 °C were centrifuged (8936 × *g*, 10 min, 4 °C) and cells obtained were washed twice with sterile potassium phosphate buffer 0.1 M (pH 6.5) and suspended in the same buffer. The ochratoxigenic *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 mold strain was grown on malt extract agar (MEA; Difco Laboratories, Detroit, Michigan, USA) plates at 25 °C for 7 days. Conidia were collected in sterile soft agar (Tween 80, 0.5 g/l and agar, 1.0 g/l) and counted microscopically in a hemocytometer chamber to adjust its concentration to 10^4 conidia per milliliter in sterile water.

In vitro inhibition assays

The inhibitory effect of *L. plantarum* CRL 778 on *A. niger* 13 D was evaluated on Petri plates (90 mm diameter × 15 mm) containing yeast extract agar medium (CYA)[28]. *L. plantarum* cell suspension (3×10^9 CFU/ml) was inoculated (2%, v/v) in plates containing molten and cooled (45 °C) CYA with a_w adjusted to 0.955, 0.964, 0.971, 0.982, and 0.995. Different amounts of glycerol:water were used to modified a_w values; they were checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA). These a_w values correspond to those found in different foods as cheddar cheese, vegetable, milk and fresh meat, among other. Inoculated Petri dishes (CYA-*L. plantarum* CRL 778) were sealed in polyethylene bags and incubated 24 h at 30 °C. After this, Petri dishes were inoculated with spore suspension of *A. niger* 13 D by central puncture. Plates containing CYA agar medium were adjusted to different a_w values, inoculated with *A. niger* 13 D and used as control. All dishes were sealed in closed plastic containers, each one containing a glycerol: water solution at a determined a_w[30]. Quadruplicate sets of each assay were incubated 7 days at 30 °C and the lag phase and the mycelial extension were used as growth fungal parameters[22]. Temporal mycelial extension rates were determined daily in two directions at right angles to each other until the medium was fully colonized. Radial extension rates were plotted against time and the growth rates were calculated using linear regression (mm/day) at each a_w. The extraction of OTA was performed after 7 days of incubation of Petri dishes at 30 °C. On each sampling, three agar plugs were removed from different points of the mycelium growth and extracted with 1 ml of methanol. The mixture was centrifuged at 14,000 × *g* during 10 min, evaporated to dryness and kept at 5 °C until use. The OTA concentration was determined by reversed phase HPLC according to Gerez et al.[11]

Effect of a_w on the growth and production of organic acid by *L. plantarum* CRL 778

Growth and organic acids production by *L. plantarum* CRL 778 were determined in CYA broth (200 ml) adjusted to different a_w values as described above. After incubation (30 °C for 24 h), samples were withdrawn from cell cultures and bacterial growth was ascertained by optical density at 580 nm (Spectrophotometer 1000 series, Cecil, Cambridge, England). The pH values (pHmeter Altronix-TPX1 Ph, mV-Meter, Sartorius, Goettingen, Germany) and organic acids [lactate, acetate, phenyllactic acid (PLA) and hydroxyphenyllactic acid (OH-PLA)] concentrations were also determined. The organic acids were determined by High Performance Liquid Chromatography[14] (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) 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 acids were quantified on the basis of detector response compared with the respective standards (Sigma–Aldrich).

Statistical analysis

Results of two independent assays are presented as mean values ± standard deviation (SD). Data were analyzed 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 $p < 0.05$.

Results

Inhibitory effect of *L. plantarum* CRL 778

A. niger 13D was able to grow in CYA medium at all a_w assayed (Table 1). In general, in the absence of *L. plantarum* CRL 778, the specific growth rate increased at higher a_w values, while the lag phase showed the opposite behavior. On the whole, the inhibitory effect of *L. plantarum* CRL 778 depended on the a_w value of the medium. In fact, in the presence of CRL 778 strain (13D-CRL 778), a decrease

Table 1 Lag phase and growth rate (μ_{max}) of *A. niger* 13D in CYA agar with and without *L. plantarum* CRL 778 at different a_w

	Culture	Water activity (a_w)				
		0.955	0.964	0.971	0.982	0.995
Lag phase(h)	13D	$22.35 \pm 0.96^{1,a}$	$18.29 \pm 1.61^{1,b}$	$16.65 \pm 2.11^{1,c}$	$14.36 \pm 0.84^{1,c,d}$	$12.16 \pm 0.88^{1,d}$
	13D-CRL778	$23.61 \pm 1.75^{1,a}$	$21.23 \pm 1.73^{2,a}$	$22.54 \pm 5.28^{2,a}$	$25.05 \pm 2.59^{2,a}$	$22.34 \pm 2.94^{2,a}$
Fungal growth rate (cm/d)	13D	$1.11 \pm 0.04^{1,a}$	$1.18 \pm 0.07^{1,a}$	$1.35 \pm 0.11^{1,b}$	$1.47 \pm 0.02^{1,b}$	$1.81 \pm 0.03^{1,c}$
	13D-CRL778	$1.25 \pm 0.05^{1,a}$	$1.38 \pm 0.02^{1,a,b}$	$1.02 \pm 0.25^{2,a,c}$	$1.09 \pm 0.06^{2,a,c}$	$0.96 \pm 0.08^{2,c}$

13D = *A. niger* 13D; 13 D-CRL778 = *A. niger* 13D with *L. plantarum* CRL 778. Fungal growth rate (μ_{max}): cm of colony/days. Means with the same superscript letter in the same row and the same number in the same column show no significant differences between them ($p < 0.05$) by the Tukey test.

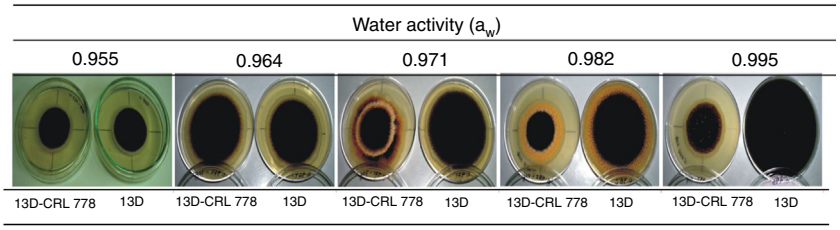

Figure 1 Growth of *A. niger* 13 D in CYA medium agar adjusted to different a_w after 7 days. 13D: *A. niger* 13 D; 13D-CRL778: *A. niger* 13 D plus *L. plantarum* CRL 778.

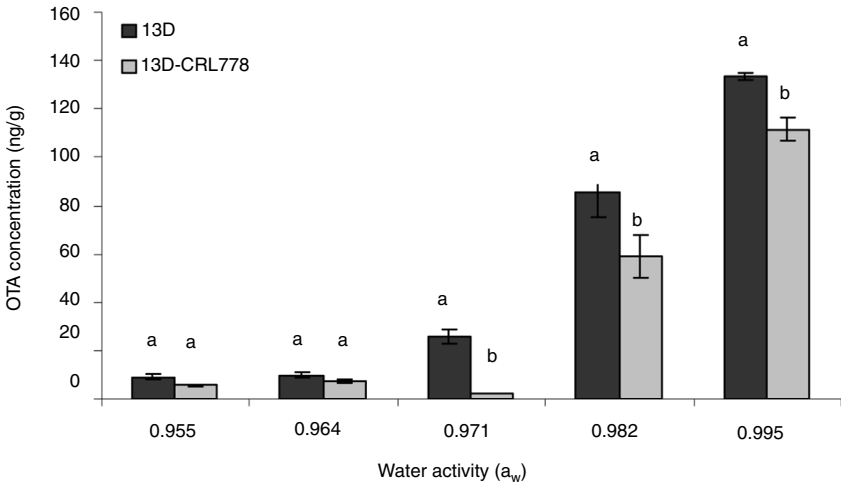

Figure 2 Effect of *L. plantarum* CRL 778 on OTA production by *A. niger* 13D in CYA agar after 7 days at 30 °C. OTA concentration was determined by reversed phase-HPLC.

in the *A. niger* 13D growth rate and a lengthening of the lag phase were observed, specially at $a_w = 0.995$ and 0.982. In addition, colonies of *A. niger* 13D growing in the presence of *L. plantarum* CRL 778 exhibited morphological variations compared to the control (13D), such as visible loss of conidia melanization in their outline (0.971–0.995 a_w) (Fig. 1).

Production of OTA (at 7 days) by *A. niger* 13D alone and mixed with *L. plantarum* CRL 775 (13D-CRL778) in CYA agar at different a_w is shown in Figure 2. In general, production of OTA by *A. niger* (13D) was dependent on a_w, *i.e.* at higher water activity, an increment in OTA concentration was observed, being 0.995 a_w the optimal condition for OTA production (133 ± 1.5 ng/g). Mycotoxin concentration was inhibited (16–90%) by *L. plantarum* CRL778 (13D-CRL778) in most of a_w value tested. The maximum reduction (90%) was observed at 0.971 a_w, in which toxin was almost negligible. On the contrary, no significant differences in the concentration at both 0.955 and 0.964 a_w were observed.

Effect of a_w on growth and antifungal acids production by *L. plantarum* CRL 778

Taking into account that antifungal and antimycotoxigenic activity of CRL 778 varied significantly with a_w levels, growth and antifungal metabolite production of the LAB strain were studied in the same conditions. As shown in Figure 3, *L. plantarum* CRL 778 was able to grow at all a_w tested, with a maximum ($A_{580\,nm} = 1.39$) at $a_w = 0.995$ and minimum ($A_{580\,nm} = 0.40$) at $a_w = 0.955$. Production of lactic, acetic, phenyllactic (PLA) and hydroxy-phenyllactic (OH-PLA) acids (antifungal metabolites) increased as increased a_w levels and were also greater (5.3, 4.6, 1.9 and 2.2 times, respectively) at $a_w = 0.995$.

Discussion

Fungal growth and OTA production can be influenced by intrinsic factors of food matrixes, such as a_w. Our results demonstrated that *A. niger* 13D was able to grow and also produce OTA over a wide range of a_w (0.955–0.995) in CYA medium. These observations could explain why *A. niger* is considered the most common *Aspergillus* species responsible for food contamination and is also among the most frequently fungi isolated from dried products[1]. It is important to note that both, fungus growth and mycotoxin production were affected by a_w; being the highest a_w assayed (0.995), the optimal condition for both parameters. Other authors also showed a positive influence of this abiotic factor on growth and OTA accumulation of the *A.* section Nigri strains and others ochratoxigen fungi[4,15,21]. In fact, higher water activities seem to favor both parameters. On the contrary, Esteban et al.[9] reported that optimal conditions for growth were different from those for OTA production in some *A. niger* strains tested in CYA medium.

Some biostrategies have been proposed as alternatives to chemicals in order to reduce growth of ochratoxigenic

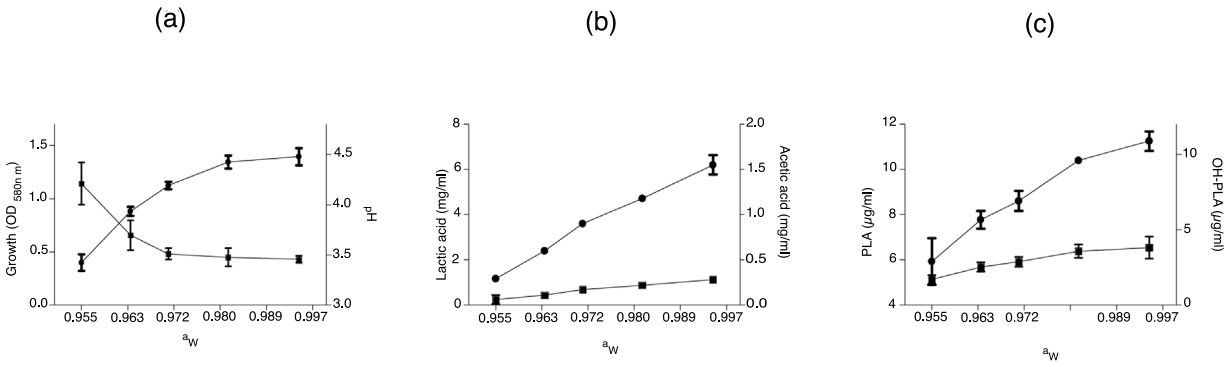

Figure 3 Growth of *L. plantarum* CRL 778 in CYA medium adjusted to different a_w after 24 h incubation at 30 °C. a – growth (circles) and pH (squares), b – lactic acid (circles) and acetic acid (squares), c – PLA (circles) and OH-PLA (squares). Organic acids concentrations were determined by HPLC.

mold and OTA contamination of foods. However, relevant use of antifungal LAB requires thorough knowledge of the parameters involved in modulating antifungal properties. In this study, growth of *A. niger* 13 D was significantly reduced by *L. plantarum* CRL 778 in CYA medium, being the inhibition related to a_w. Thus, the highest inhibitory effect (46.9% reduction in growth rate) was observed at $a_w = 0.995$, optimum condition for growing and producing antifungal metabolites (lactic and acetic acids, PLA and OH-PLA) by *L. plantarum* CRL 778. In addition, morphological and melanization effects were observed in colonies of *A. niger* 13D growing in presence of *L. plantarum* CRL 778 at $a_w = 0.971–0.995$ (Fig. 1). Also, inhibition of sporulation and slower radial growth were observed in these colonies. Synthesis of melanin would play a fundamental role in the resistance of certain fungi to antifungal compounds[32]. In fact, it was reported that melanin could provide structural support to resist osmotic changes as well as protection from oxidative damage and antifungal metabolites in many fungal genera[24]. Hence, inhibition of melanin synthesis by CRL 778 strain would be related to its antifungal effect.

In general, a decrease in the mycelium formation leads to less production of toxins. However, several researches have shown that there is not always correlation between restriction of growth and potential inhibition of mycotoxin production. Our results showed that *L. plantarum* CRL778 (13D-CRL778) was also able to inhibit the OTA production, observing the maximum percentage reduction (90%) at $a_w = 0.971$. Differences observed between optimum growth conditions and OTA production inhibition may be due to other antagonistic mechanisms generated by the LAB, besides acids production. In fact, it was reported that during cell growth and lysis, bacteria may release potentially inhibitory compounds against fungal growth and mycotoxin production[5]. Moreover, some authors reported that certain *Lactobacillus* and *Lactococcus*, were able to bind certain mutagenic compounds such as mycotoxins to their cell wall[7].

Although there are several reports regarding antifungal potential of LAB, they are mostly focused on growth inhibition but not on the mycotoxin production[8,20,25]. In this sense, Kapetanakou et al.[17] reported that none of six LAB strains tested as potential inhibitors of *A. carbonarius* were able to inhibit both, growth and OTA production, in culture media and beverages. On the contrary, four *Lactobacillus* strains showed. *in vitro*, interesting antifungal activity on ochratoxin-producing *Aspergillus* species (*A. carbonarius*, *A. niger*, *A. ochraceus*, *A. westerdijkiae*)[6,23], however, the later study did not evaluate antimycotoxin effect. In the present study, *L. plantarum* CRL 778 proved to be efficient in inhibiting the OTA-producing *A. niger* 13 D strain in CYA medium at different a_w values (0.971–0.995), besides its antifungal activity. In addition, the maximum reduction (90%) of OTA production took place at $a_w = 0.971$, while fungi growth inhibition was more evident at $a_w = 0.995$. These results agree with those reported by Esteban et al.[9], who found that optimal growth condition was different from those of OTA production in some *A. niger* strains tested in CYA medium. On the other hand, these results confirm previous study, in which a biopreservative with *L. plantarum* CRL 778 was effective to control OTA production and growth of *A. niger* 13D in bread[11]. Although additional studies are needed, our results strongly suggest that *L. plantarum* CRL 778 could be used to control ochratoxigenic fungi growth and OTA contamination in others food with a_w values 0.971–0995, such as cheddar cheese, vegetable and fresh meat, among other.

Conflicts of interest

No potential conflict of interest was reported by the authors.

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