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Biodegradation of ochratoxin A by *Pediococcus parvulus* isolated from Douro wines

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ABSTRACT

Lactic acid bacteria (LAB) are a promising solution to reduce exposure to dietary mycotoxins because of the unique mycotoxin decontaminating characteristic of some LAB. Ochratoxin A (OTA) is one of the most prominent mycotoxins found in agricultural commodities. The present work reports on the ability of *Pediococcus parvulus* strains that were isolated from Douro wines that spontaneously underwent malolactic fermentation to detoxify OTA. These strains were identified and characterised using a polyphasic approach that employed both phenotypic and genotypic methods. When cultivated on OTA-supplemented MRS media, OTA was biodegraded into OTα by certain *P. parvulus* strains. The presence of OTα was confirmed using LC–MS/MS. The conversion of OTA into OTα indicates that the OTA amide bond was hydrolysed by a putative peptidase. The rate of OTA biodegradation was found to be dependent on the inoculum size and on the incubation temperature. Adsorption assays with dead *P. parvulus* cells showed that approximately 1.3% ± 1.0 of the OTA was adsorbed onto cells wall, which excludes this mechanism in the elimination of OTA by strains that degrades OTA. Under optimum conditions, 50% and 90% of OTA were degraded in 6 and 19 h, respectively. Other LAB strains that belonged to different species were tested but did not degrade OTA. OTA biodegradation by *P. parvulus* UTAD 473 was observed in grape must. Because some *P. parvulus* strains have relevant probiotic properties, the strains that were identified could be particularly relevant to food and feed applications to counteract the toxic effects of OTA.

1. Introduction

Lactic acid bacteria (LAB) are generally thought to be beneficial microorganisms and are widely studied because of their probiotic properties (Naidu et al., 1999). They are commonly used for the production of fermented food products, such as yogurt, cheese, fermented milks, meat, fish, vegetables (sauerkraut and pickles), certain beer brands and wines (Stiles, 1996). For the production of wine, LAB are involved in malolactic fermentation (MLF). MLF is a secondary fermentation process that is used to improve the quality of some wines. MLF reduces acidity (via the conversion of L — malic acid to L — lactic acid), increases microbial stability and improves the aroma, flavour and mouthfeel (du Toit et al., 2010; Kunkee, 1967).

While not widely known, some LAB are able to control potential health hazards that are linked to mycotoxins (Abrunhosa et al., 2010; Dalié et al., 2010; Salminen et al., 2010; Shetty and Jespersen, 2006). Mycotoxins are acutely toxic fungal secondary metabolites that can be found in agricultural commodities. Many mycotoxins are carcinogenic, mutagenic, teratogenic, neurotoxic and immunosuppressive (Bennett and Klich, 2003). Therefore, the levels of mycotoxins in food and feed products should be reduced to the lowest technologically possible levels. Accordingly, several physical, chemical and biological methods are currently available to control the levels of mycotoxins (EFSA, 2009). Nevertheless, the demand for innovative solutions continues to overcome the limitations of the current strategies for reducing mycotoxin levels. In particular, new microorganisms that are actively able to detoxify mycotoxins are needed.

In this context, LAB are of particular interest to researchers, and several strains that bind aflatoxins (El-Nezami et al., 1998), trichothecenes (H.S. El-Nezami et al., 2002), zearalenone (H. El-Nezami et al., 2002), ochratoxin A and patulin (Fuchs et al., 2008) have been identified in model systems. Some strains were also successfully tested in vivo. The LC-705 *Lactobacillus rhamnosus* strain was shown to reduce the intestinal adsorption of aflatoxin B_1 by 74% in chickens (El-Nezami et al., 2000). Some commercial products that are based on this and

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other LAB strains are already available (EFSA, 2009; Salminen et al., 2010). In most reports, the binding of mycotoxins by LAB was determined to be the main mechanism for detoxification (Dalié et al., 2010; El-Nezami et al., 2004). Exopolysaccharides and peptidoglycans are considered to be the most plausible binding molecules (Dalié et al., 2010). However, the involvement of metabolic conversion was suggested in some studies but the full mechanistic pathways was not fully elucidated (Fuchs et al., 2008; Niderkorn et al., 2007).

Ochratoxin A (OTA) is an important mycotoxin that is found in agricultural commodities. It is produced by some *Penicillium* and *Aspergillus* species and is mainly found in cereals, coffee beans, cocoa beans, grapes, raisins, wine, figs, pork meat and spices (Jørgensen, 2005). OTA is nephrotoxic, hepatotoxic, immunotoxic, teratogenic and carcinogenic (category 2B) (Bennett and Klich, 2003). Chemically, it is composed of an isocoumarin moiety (known as ochratoxin α) and a L-β-phenylalanine molecule that are linked by an amide bond (van der Merwe et al., 1965). Ochratoxin α (OTα) is considered to be non-toxic and has an elimination half-life that is 10-times less than that of OTA (Li et al., 1997). In animals and humans, the conversion of OTA into OTα via hydrolysis of the amide bond constitutes the predominant detoxification pathway of OTA (Wu et al., 2011). Several proteolytic enzymes (Abrunhosa et al., 2006, 2010), microorganisms (Abrunhosa et al., 2010; Varga et al., 2010) and plant cells (Ruhland et al., 1996) have been shown to hydrolyse this amide bound to detoxify OTA. In the present work, we investigate the capacity of LAB isolated from Douro wines to biodegrade OTA through this detoxification pathway.

2. Materials and methods

2.1. Identification and characterisation of LAB

The LAB strains that were used in the present study were obtained from the UTAD-LAB collection. This collection had been previously isolated from red wines of the Douro region that had spontaneously undergone MLF. Several species of *Lactobacillus*, *Oenococcus oeni*, *Pediococcus parvulus* and *Leuconostoc mesenteroides* were identified (Inês, 2007). The isolates were also screened for enzymes considered to be valuable for obtaining a high quality wine, as for example, the presence of β-glucosidase and malolactic enzymes, and absence of histidine, tyrosine decarboxylases and enzymes from the arginine deaminase pathway. The strains were verified phenotypically on adequate culture media and were verified genotypically by identifying the genes that encoded the enzymes of interest (Inês et al., 2008). The following 19 strains used in the present study were randomly picked from different genera: 9 strains of *O. oeni*, 6 strains of *P. parvulus* and 4 strains of *Lactobacillus plantarum* (listed in Table 1). The re-identification of the strains that had the ability to biodegrade OTA was performed using the simplified key for phenotypic differentiation of species within the genera *Pediococcus* and *Tetragenococcus* as proposed by Holzapfel et al. (2006) and validated by a BLAST analysis of partial sequences of the 16S rRNA gene (497-836 nt). A phylogenetic tree based on the partial sequences was constructed by applying Kimura 2-parameter (Kimura, 1980) and Neighbor-Joining methods (Saitou and Nei, 1987) as grouping method. The tree was rooted by using *Bifidobacterium bifidum* sequence as outgroup. Bootstrap values were calculated from 1000 replications (Felsenstein, 1985). Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The reference strains *O. oeni* CECT 217^T, *P. parvulus* CECT 7350^T and *L. plantarum* CECT 748^T were used as controls and were purchased from the Spanish Type Culture Collection (CECT, University of Valencia, Spain).

2.2. Screening for OTA biodegradation

Strains were cultured from frozen aliquots onto Petri dishes with MRS agar. The plates were then incubated at 30 °C in 2.5 L anaerobic jars that were prepared with AnaeroGen™ sachet (AN0025, Oxoid) and the anaerobic indicator (BR0055, Oxoid). Unless stated otherwise, strains were always cultivated under anaerobic conditions. Subsequently, strains were cultured in sterile microcentrifuge tubes with 1 mL of MRS broth (Oxoid) at 30 °C for 2 days. The inoculum for each strain was propagated at 30 °C for 7 days in falcon tubes that contained 5 mL of MRS broth. The inoculum concentration was determined by counting CFU in MRS agar plates by serially diluting the inoculums. The concentration was adjusted as needed using sterile MRS broth. To study the ability of LAB strains to degrade OTA, a MRS broth medium that was

Table 1
The elimination of OTA by bacterial strains when cultivated in MRS media supplemented with 1 μg of OTA/mL for 7 days at 30 °C. Values are the average of three independent replicates ± standard deviation.

LAB species	Strain	OTA (μ/mL)	OTα (μ/mL)	OTA eliminated (%)
Controls	–	1.173 ± 0.033	0	0
Lactobacillus plantarum	CECT 748^T	1.013 ± 0.004	0	14 ± 0.4
Oenococcus oeni	CECT 217^T	0.936 ± 0.034	0	20 ± 2.9
Pediococcus parvulus	CECT 7350^T	0.986 ± 0.028	0	16 ± 2.4
Lactobacillus plantarum	UTAD 346	1.051 ± 0.007	0	10 ± 0.6
	UTAD 350	1.023 ± 0.006	0	13 ± 0.5
	UTAD 460	1.059 ± 0.004	0	10 ± 0.4
	UTAD 461	1.041 ± 0.007	0	11 ± 0.6
Oenococcus oeni	UTAD 224	0.988 ± 0.004	0	16 ± 0.3
	UTAD 228	0.997 ± 0.003	0	15 ± 0.3
	UTAD 244	0.997 ± 0.006	0	15 ± 0.5
	UTAD 279	1.008 ± 0.009	0	14 ± 0.7
	UTAD 295	0.990 ± 0.009	0	16 ± 0.8
	UTAD 296	0.999 ± 0.005	0	15 ± 0.4
	UTAD 474	0.993 ± 0.005	0	15 ± 0.4
	UTAD 602	1.009 ± 0.006	0	14 ± 0.5
	UTAD 653	1.008 ± 0.010	0	14 ± 0.9
Pediococcus parvulus	UTAD 111B	0.328 ± 0.139	0.635 ± 0.117	72 ± 11.8
	UTAD 168*	0.133 ± 0.072	0.810 ± 0.051	89 ± 6.2
	UTAD 333	0.037 ± 0.007	0.949 ± 0.001	97 ± 0.6
	UTAD 334*	0.070 ± 0.059	0.863 ± 0.035	94 ± 5.1
	UTAD 335	0.025 ± 0.001	0.957 ± 0.007	98 ± 0.1
	UTAD 473*	0	0.922 ± 0.034	100 ± 0

* *P. parvulus* UTAD 168, *P. parvulus* UTAD 334 and *P. parvulus* UTAD 473 are now deposited in the Spanish Type Culture Collection (CECT, University of Valencia, Spain) with the following codes, respectively: *Pediococcus parvulus* CECT 7950, CECT 7951 and CECT 7952.

supplemented with 1.0 μg OTA/mL (MRS-OTA) was prepared as follows. A stock solution at a concentration of 25 μg of OTA/mL was prepared in toluene/acetic acid (99:1, v/v) using commercial standard OTA (O1877, Sigma) and stored at −20 °C until use. The appropriate volume of the OTA stock solution was added to an Erlenmeyer flask and was evaporated to dryness at 50 °C using a gentle stream of nitrogen. The appropriated volume of MRS broth (Oxoid) was then added to the flasks. The flasks were then placed in an ultrasonic bath for 15 min to assist the solubilisation of the OTA. The media was autoclaved at 121 °C for 15 min and cooled. Subsequently, 5 mL aliquots were transferred into sterile 15 mL conical tubes using aseptic technique. For each strain, 5 mL of MRS-OTA was inoculated in triplicate with 0.1 mL of the prepared inoculum at a concentration of 10^9 CFU/mL. Three negative controls were also prepared using 0.1 mL of sterile MRS in the place of the inoculum. Tubes were incubated at 30 °C for 7 days in 2.5 L anaerobic jars as previously described. The culture media and bacterial cells were extracted by the addition of 5 mL of acetonitrile/acetic acid (99:1, v/v) to the culture tubes, which were then vortexed for 1 min. A 2 mL sample was then collected and filtered into a clean 2 mL amber borosilicate glass vial using syringe-fitted PP filters with 0.45 μm pores (Merck). This sample was preserved at 4 °C until HPLC analysis.

2.3. Characterisation of the OTA biodegradation

The effects of 3 factors in the biodegradation of OTA by *P. parvulus* UTAD 473 were studied. The factors evaluated were the inoculum size, OTA concentration in MRS medium and the incubation temperature. Experiments were conducted in triplicate following the procedure that has been described previously. To evaluate the effect of inoculum size, tubes with 5 mL of MRS-OTA were inoculated to achieve a cell concentration of 10^3, 10^6 and 10^9 CFU/mL and incubated at 30 °C. To evaluate the effect of OTA concentration, tubes with 5 mL of MRS supplemented with 0.01, 0.1 and 1.0 μg OTA/mL were inoculated to achieve 10^9 CFU/mL and incubated at 30 °C. To evaluate the effect of temperature, tubes with 5 mL of MRS supplemented with 1.0 μg OTA/mL were inoculated to achieve 10^9 CFU/mL and incubated at 20, 30 and 37 °C. OTA and OTα levels were analysed over time by extracting and analysing three replicates on a daily basis until a maximum of 7 days of incubation, as previously described. Samples were preserved at 4 °C until HPLC analysis was performed. Three negative controls using 0.1 mL of sterile MRS instead of inoculum were prepared for each of the evaluated factors. The negative controls were extracted after 7 days of incubation. All the experiments were conducted in 2.5 L anaerobic jars.

2.4. Evaluation of OTA adsorption to cell walls

To investigate if OTA was adsorbing to cells walls of *P. parvulus*, dead cells from *P. parvulus* UTAD 473 were used. The inoculum was prepared as described previously. The tubes were then autoclaved at 121 °C for 15 min. Dead cells were added to 5 mL of MRS-OTA in triplicate to a final concentration of 10^9 CFU/mL, and the tubes were vortex-mixed for 30 s. The mixtures was vortexed every 15 min for 1 h. Finally, tubes were centrifuged (9000 ×*g* for 20 min), and the supernatant was collected and filtered into clean tubes using syringe-fitted PP filters with 0.2 μm pores (Merck). The extraction of the ochratoxins from the supernatants was performed as previously described. The cell pellets were extracted by adding 2 mL of acetonitrile/water/acetic acid (99:99:2, v/v/v) to them. After vortexing for 1 min, the extracts were filtered into clean glass vials and preserved at 4 °C until HPLC analysis was performed.

2.5. Analytical techniques

Samples were analysed by HPLC with fluorescence detection, as described elsewhere with some modification (Abrunhosa and Venâncio, 2007). Briefly, the HPLC system was comprised of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector (λ_{exc} = 333 nm and λ_{em} = 460 nm; gain = 100) and a Jones Chromatography 7971 column heater that was maintained at 30 °C. The instrument and the chromatographic data were managed by a Varian 850-MIB data system interface and a Galaxie chromatography data system, respectively. The chromatographic separation was performed on a C_{18} reversed-phase YMC-Pack ODS-AQ analytical column (250 × 4.6 mm I.D., 5 μm) that was fitted with a pre-column with the same stationary phase. The compounds were eluted using a flow rate of 0.8 mL/min for a 21 min isocratic run. The injection volume was 50 μL. The mobile phase was a mixture of acetonitrile/water/acetic acid (99:99:2, v/v/v) that was filtered and degassed with a 0.2 μm membrane filter (GHP, Gelman). OTA was identified by comparing the retention time of the peak samples with the standards. Recorded retention times for OTα and OTA were approximately 7.5 and 19.8 min, respectively. Standards with 0.5, 1.0, 2.5, 5.0 and 7.5 μg/mL were prepared by serially diluting the primary OTA stock solution (25 μg/mL). The standards were used to elaborate the calibration curve, which was regularly checked. OTA quantification was performed by measuring the peak area and comparing it to the respective OTA calibration curve. OTα was quantified in equivalents of OTA.

2.6. Ochratoxin α confirmation

The *P. parvulus* UTAD 473 samples described in Table 1 were used to confirm the presence of OTα using LC–MS/MS. Analyses were performed using a Thermo Finnigan LXQ linear ion trap mass spectrometer that was coupled to a Finnigan surveyor HPLC system. Separation was performed on a Hydro-RP 100A phenomex column (100 × 3.0 mm I.D., 2.5 μm) using a mixture of acetonitrile/water/acetic acid (40:60:1, v/v/v) at a flow rate of 0.20 mL/min for 40 min. A 20 μL sample was injected. Negative electrospray ionisation (ESI) was used for ion preparation. The conditions had been determined previously with a tune process to optimise the efficiency of the analysis. The operating conditions were as follows: sheath gas flow, 10 lb/in.^{2}; sweep gas flow, 30 lb/in.^{2}; auxiliary gas flow, 30 lb/in.^{2}; source voltage 5 kV; capillary voltage, −45.00 V; tube lens, −95.87 V.

MS/MS analysis was performed using two segments for precursor ions of 255.5 and 402.0. To identify the compounds the following parent-to-fragments were monitored: *m/z* 402.0→358.0 for OTA and *m/z* 255.0→211.0 for OTα. The collision energy for the products ions *m/z* 358.0 and 211.0 was 35 eV. The conditions of the MS/MS analyses were as follows: Q, 0.25; act. time, 30 ms; act. type, CID.

2.7. Data analysis

The OTA concentration over time data were fitted using a logistic model adapted from recommendations of the FOCUS work group report on pesticide degradation kinetics (FOCUS, 2006). Data were fitted to the model using a nonlinear least squares fit regression using GraphPad Prism vers. 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com and Eq. (1).

$$S = S_0 * \left[\frac{a_{max}}{a_{max} - a_0 + a_0 * e^{(r*t)}}\right]^{\frac{a_{max}}{r}} \quad (1)$$

S is the percentage of OTA present at time t, S_0 is the initial percentage of OTA present in the assay, a_{max} is the maximum value of the degradation constant, a_0 is the initial value of the degradation constant, r is the microbial growth rate and t is the time (FOCUS, 2006).

To perform the regression analysis, OTA data were converted to a 0–100% scale. S_0 was constrained to 100% and the initial values of a_{max}, a_0 and r were set to 0.02, 0.01 and 0.03, respectively. The a_{max}, a_0 and r parameters were estimated using a fitted equation to calculate the DT50 and DT90 (the time required for 50% and 90% of the OTA to be degraded) according to Eqs. (2) and (3), respectively.

$$DT50 = \frac{1}{r} * \ln\left[1 - \frac{a_{max}}{a_0} * \left(1 - 2^{\frac{r}{a_{max}}}\right)\right] \quad (2)$$

$$DT90 = \frac{1}{r} * \ln\left[1 - \frac{a_{max}}{a_0} * \left(1 - 10^{\frac{r}{a_{max}}}\right)\right] \quad (3)$$

2.8. Evaluation of OTA biodegradation by P. parvulus in food matrices

Inoculum of *P. parvulus* UTAD 473 was prepared by cultivating the strain in 30 mL MRS (Oxoid) at 30 °C for 5 days. Then the biomass was transferred to 200 mL of adaptation media (MRS, 50 g/L; glucose, 20 g/L; fructose, 40 g/L; malic acid, 4 g/L; Tween 80, 1 g/L; ethanol, 6%; pH 4.6) (Lerm, 2010) and incubated at 25 °C for 5 days. CFU were determined by plating serial decimal dilutions in MRS and inoculum concentration adjusted to 10^{11} CFU/mL. To evaluate if *P. parvulus* UTAD 473 was able to biodegrade OTA in food matrices, synthetic wine and grape must supplemented with 7 μg OTA/L were used. Synthetic wine was prepared according to Mateo et al. (2010) with an ethanol content of 12.5%. Grape must was prepared with grapes from the Red Globe variety as follows: grapes were blended, the juice was filtered using filter paper (G595$^{1/2}$, Merck), supplemented with 2 g/L of yeast extract (Difco) and sterilized at 115 °C during 25 min. Experiments were conducted in Erlenmeyer flasks with 100 mL of synthetic wine or grape juice in triplicate. Flasks were inoculated with 1 mL of inoculum to reach 10^9 CFU/mL and incubated at 25 °C for 30 days. Samples were collected over time to determine OTA (as previously described), pH and biomass by measuring optical density (OD) at 600 nm.

3. Results

3.1. LAB identification and characterisation

The *P. parvulus* strain identification was reconfirmed and all the strains of interest had the ability to ferment galactose, grew at pH 7.0, grew at 35 °C and did not ferment lactose. The identification was also validated by a BLAST analysis of partial sequences of the 16S rRNA gene that were obtained for the three strains, which presented an elevated capacity for OTA detoxification (Fig. 1). The three strains (*P. parvulus* UTAD 168, *P. parvulus* UTAD 334 and *P. parvulus* UTAD 473) are deposited in CECT with the following code numbers: *P. parvulus* CECT 7950, CECT 7951 and CECT 7952, respectively. Their 16S ribosomal RNA gene nucleotide sequences are deposited in the National Center for Biotechnology Information (NCBI) under the accession numbers KF886568, KF886569 and KF886570, respectively.

3.2. Screening for OTA biodegradation

The percentage of OTA that was eliminated by strains after a 7 day cultivation period in MRS-OTA is presented in Table 1. As mentioned previously, the biodegradation of OTA into OTα was only performed by *P. parvulus* strains that were isolated from Douro wines. In assays that involved the other bacterial strains, no OTα was detected even when some OTA was eliminated from the culture media (between 11% and 20%, depending on the strain). Furthermore, when the chromatograms for the non-*P. parvulus* strains were compared to those of the controls (data not show), no other peaks that may have been the result of an alternative metabolisation pathway were detected. The slight elimination of OTA likely resulted from the inability of the extraction method to solubilise the OTA that was adsorbed onto the cell walls.

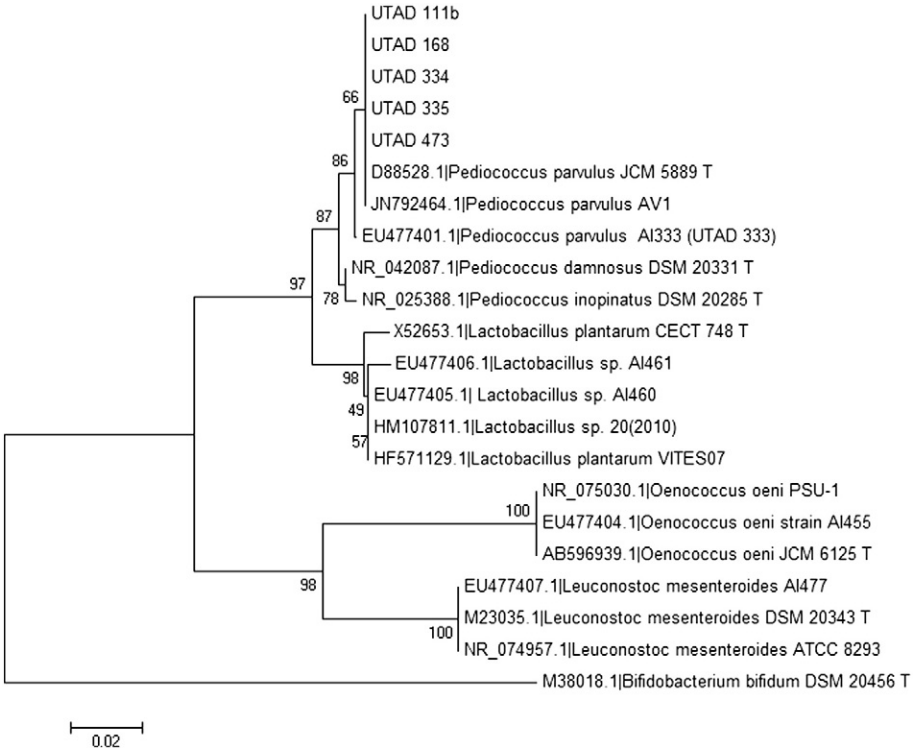

Fig. 1. Phylogenetic tree based on partial 16S rRNA gene sequences analysis (497–836 nt), showing the taxonomic position of the strains that revealed highest activity for OTA detoxification.

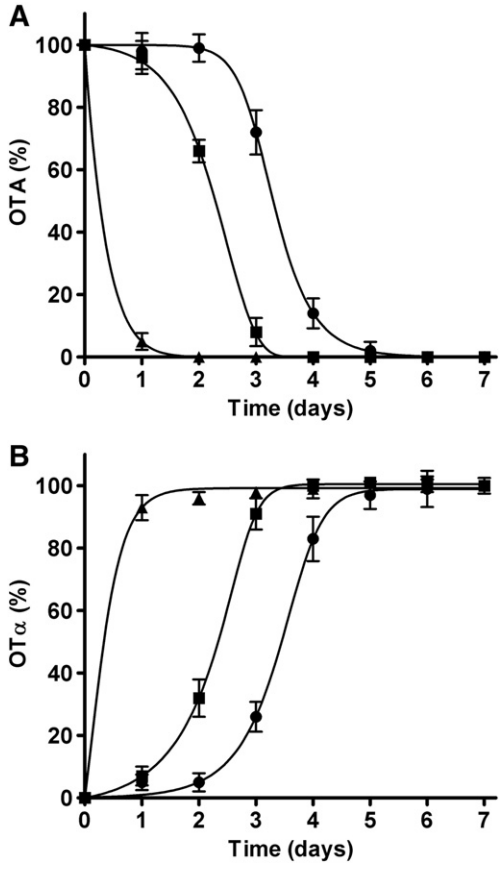

Fig. 2. Effect of the inoculum size on the biodegradation of OTA by *P. parvulus* UTAD 473 when cultivated in MRS-OTA medium. (A) OTA and (B) OTα concentration in the culture media over time. Assays were performed with (●) 10^3 CFU/mL, (■) 10^6 CFU/mL and (▲) 10^9 CFU/mL. Data fitted using GraphPad Prism.

The highest biodegradation capacity was observed for the *P. parvulus* UTAD 473 strain. This strain completely eliminated OTA from the culture medium. Interestingly, the reference strain, *P. parvulus* CECT 7350^T, only eliminated 16% of the OTA from the culture medium and did not biodegrade OTA into OTα. Therefore, the observed ability of *P. parvulus* to degrade OTA may be specific to the strains that were isolated from the Douro wines. Further investigation of the ability of *P. parvulus* UTAD 473 to biodegrade OTA was conducted by evaluating the effect of the concentration of the inoculum, the effect of OTA concentration in the culture media, and the effect of the incubation temperature. Additionally, the adsorption of OTA to the cell wall of the *P. parvulus* UTAD 473 strain was investigated.

Table 2
Effect of inoculum size, OTA concentration and temperature on the capacity of *P. parvulus* UTAD 473 to biodegrade OTA. The calculated values for DT50 and DT90 in hours are displayed.

	DT50 (h)	DT90 (h)
Inoculum size		
10^3 CFU/mL	79	100
10^6 CFU/mL	55	70
10^9 CFU/mL	6	19
OTA concentration		
0.01 μg/mL	9	30
0.1 μg/mL	6	25
1.0 μg/mL	6	25
Temperature		
22 °C	32	63
30 °C	9	31
37 °C	6	24

3.3. Characterisation of OTA biodegradation by *P. parvulus*

The effect of the inoculum size on the biodegradation of OTA is depicted in Fig. 2. It can clearly be observed that the conversion rate of OTA into OTα increased when the inoculum size was increased from 10^3 to 10^9 CFU/mL. Additionally, a longer lag phase was observed in assays conducted with inoculums that were less concentrated. Therefore, a longer time was required to initiate the biodegradation of OTA and to achieve the total conversion of OTA into OTα in these cultures. Potentially, the cells required more time to adapt to the MRS-OTA medium and to synthesise the enzyme needed to degrade OTA. The effect of the inoculum size on OTA degradation was also observed when DT50 and DT90 were compared (Table 2). A decrease of 92% and 81%, respectively, was observed for DT50 and DT90 when the inoculum size was increased from 10^3 to 10^9 CFU/mL. When the most concentrated inoculum was used, the time required for 50% and 90% OTA degradation was 6 and 19 h, respectively.

OTA biodegradation by *P. parvulus* UTAD 473 is also dependent on the incubation temperature (Table 2). Specifically, at 22 °C the DT50 and DT90 obtained were significantly longer compared to cultures at

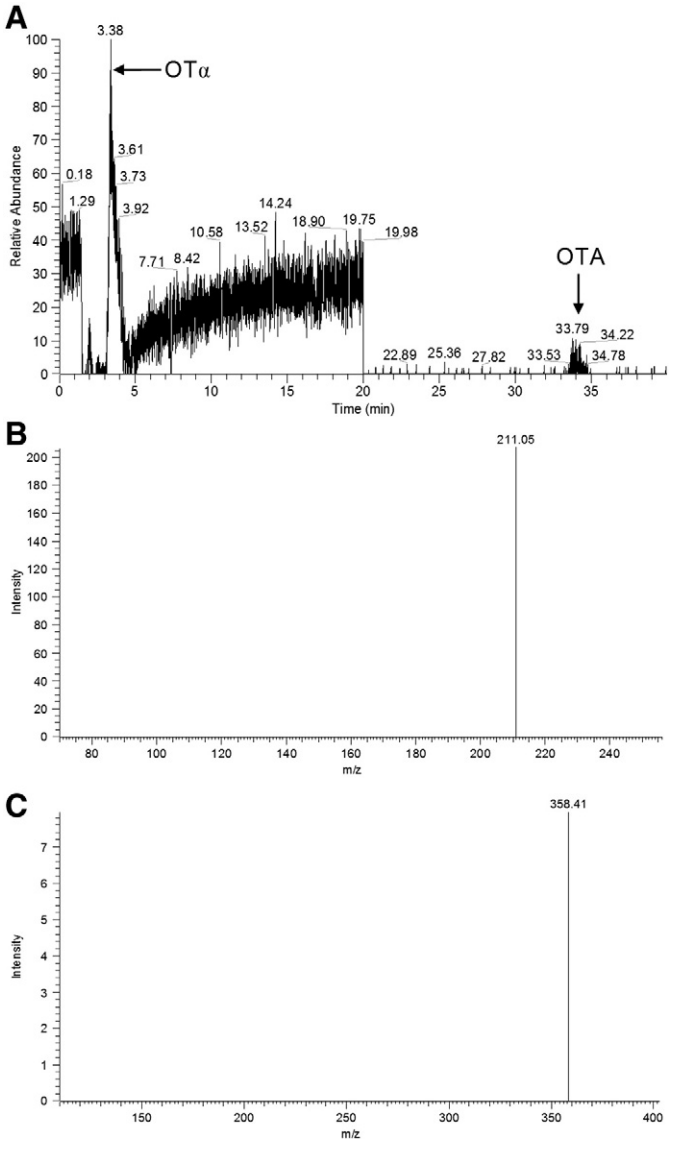

Fig. 3. Presence of OTA and OTα was confirmed by LC–MS/MS. (A) TIC chromatogram for *P. parvulus* UTAD 473 monitored from 0 to 20 min at *m/z* 255 and from 20 to 40 min at *m/z* 402. (B) Mass spectrum after fragmentation of the OTα peak at 3.38 min in chromatogram A. (C) Mass spectrum of OTA peak at 34.00 min in chromatogram A.

30 and 37 °C. This indicated that OTA biodegradation rates decreased as a result of slower cell growth rates and slower enzymatic reaction rates. The difference in the DT50 and DT90 for the 30 and 37 °C cultures was small. However, a reduction in the DT50 and DT90 parameters was observed for the 37 °C culture compared to the 30 °C culture, which indicates a more rapid degradation of OTA at 37 °C. At 37 °C DT50 and DT90 were 6 and 24 h, respectively.

In contrast to the previous factors, the concentration of OTA in culture media did not influence the biodegradation rate of the OTA. A slight increase in the DT50 and DT90 was observed for the 0.01 μg/mL OTA culture. This may suggest that OTA is required to induce the synthesis of the enzyme that is responsible for the biodegradation of OTA. The DT50 and DT90 that were identified for the 0.1 and 1.0 μg/mL OTA cultures were identical and were 6 and 25 h, respectively, for each concentration of OTA.

For the assays that were conducted to evaluate the adsorption of OTA onto the *P. parvulus* cells, 98.7% ± 1.6 of the OTA remained in the culture media, and only 1.3% ± 1.0 of the OTA was adsorbed onto the bacterial cell walls. Therefore, the binding mechanism for the elimination of OTA is negligible.

3.4. Ochratoxin α confirmation

The LC–MS/MS analysis of the *P. parvulus* UTAD 473 sample indicated the presence of a peak with a retention time of 3.38 min that had the same mass transition characteristics (*m/z* 255.0–211.0) of OTα. A peak with a retention time of 34.00 min had the same mass transition characteristics (*m/z* 402.0–358.0) of OTA (Fig. 3). These data unequivocally identified OTα as the metabolite that resulted from OTA biodegradation by the *P. parvulus* strains that were isolated from the Douro wines.

3.5. Evaluation of OTA biodegradation in food matrices by P. parvulus

OTA biodegradation by *P. parvulus* UTAD 473 was observed in grape must but not in synthetic wine. The concentration of OTA and OTα detected over time in both matrices is shown in Fig. 4. In grape must, a 50% and 80% reduction in the levels of OTA was observed after 3 and 6 days of incubation, respectively. In the wine cultures, no reduction in the levels of OTA was observed and no OTα was detected. Nonetheless, as it can be seen in Fig. 5, an increase of biomass (expressed by measuring OD at 600 nm) was observed in both matrices, even if the bacteria grew better in must than in wine. The lack of elimination of OTA in synthetic wine may, eventually, be due to the presence of ethanol, which may have inhibited the enzyme responsible for the hydrolysis of OTA. The pH had no effect in this respect since it remained approximately the same in both matrices during the incubation period (Fig. 5).

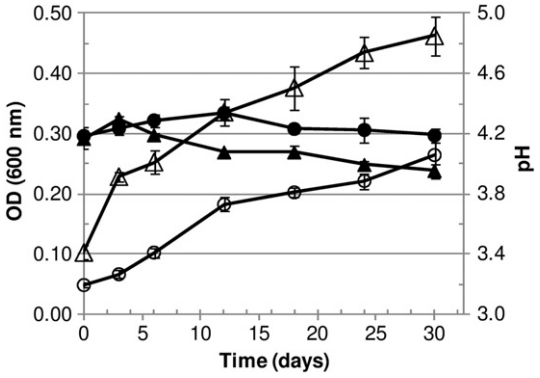

Fig. 5. Optical density (open symbols) and pH (closed symbols) over time of synthetic wine (circles) and grape must (triangles) supplemented with 7.0 μg of OTA/L and inoculated with *P. parvulus* UTAD 473.

4. Discussion

OTA is one of the most important mycotoxins that is found in food and feed products. Thus, research has been conducted to develop new and better strategies to reduce the negative impact of OTA to human and animal health. Several microorganisms that can biodegrade OTA into OTα have been identified (Abrunhosa et al., 2010; Varga et al., 2010). However, to the best of our knowledge, no reports clearly demonstrate the biodegradation of OTA by lactic acid bacteria. The existing studies either conclude that OTA was adsorbed onto the cell wall of LAB or did not allow the mechanism of elimination of OTA to be elucidated.

One of the first reports that studied the elimination of OTA from milk used the *Streptococcus salivarius* subsp. *thermophilus*, the *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Bifidobacterium bifidum* (Skrinjar et al., 1996). OTA was observed to be completely eliminated at low levels (0.05 and 0.1 μg/mL), but no biodegradation products were observed and no mechanisms for degradation were described. Similarly, Bohm et al. (2000) reported that several lactobacilli were effective at removing OTA from MRS media. *L. bulgaricus* 259/2 was observed to eliminate up to 94% of OTA. However, the authors did not determine whether the elimination of OTA was a result of biodegradation or adsorption. More recently, the elimination of OTA from culture media using oenological LAB species was also studied by Del Prete et al. (2007). In this study, the degradation of OTA was found to reach a

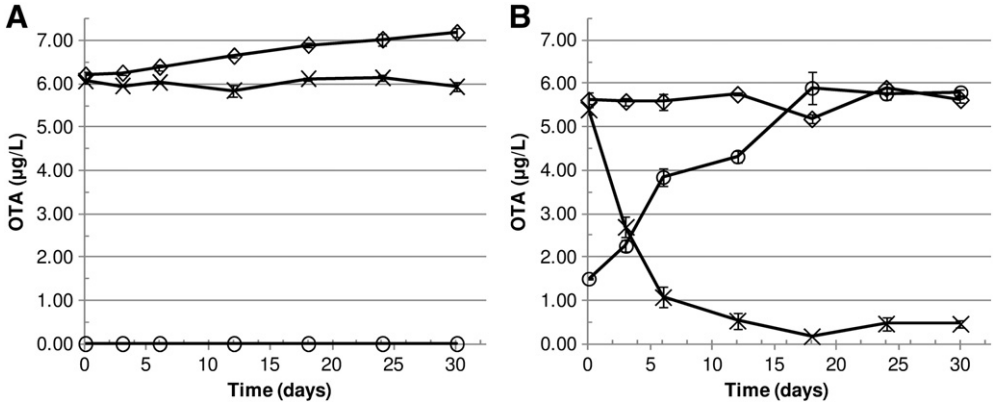

Fig. 4. OTA and OTα levels detected over time in (A) synthetic wine and (B) grape must that were supplemented with 7.0 μg of OTA/L and inoculated with *P. parvulus* UTAD 473. (◇) OTA in controls, (×) OTA in wine or grape must, and (○) OTα in wine or grape must.

maximum of only 28%, and no degradation products were detected. Thus, the authors concluded that a cell-binding phenomenon was most likely involved in the elimination of OTA. This finding is corroborated by our data because we obtained similar levels of elimination of OTA by the *L. plantarum* and *O. oeni* wine strains (elimination of up to 20% of the OTA in culture media) without having detectable OTα or other biodegradation products. Using a different approach, Fuchs et al. (2008) studied the capacity of LAB to eliminate OTA in buffer solutions. *Lactobacillus acidophilus* VM20 was identified to be the most active strain. This strain was able to remove greater than 95% of the OTA from the buffer solutions. In this study, the authors found that OTA was more efficiently removed by viable cells then by dead cells. Thus, the authors concluded that some metabolic degradation was involved, but they were not able to elucidate the mechanism of the elimination of OTA.

The present work is the first study that clearly describes the ability of LAB strains to biodegrade OTA. Specifically, *P. parvulus* strains were identified to be able to degrade OTA. This species is a Gram-positive and catalase negative LAB from the *Lactobacillaceae* family (Gunther et al., 1962). *P. parvulus* is a facultative anaerobe cocci that is strictly homofermentative and forms pares or tetrads. The bacteria can be associated with fermented vegetables, meat, fermented alcoholic beverages, dairy products and silage (Bennik et al., 1997; Holzapfel et al., 2006; Li et al., 2008; Maifreni et al., 2004; Mesas et al., 2011; Simpson et al., 2002; Tohno et al., 2012). *P. parvulus* also has some important probiotic properties: cholesterol-lowering effects as a result of exopolysaccharides that are produced (Martensson et al., 2005), immunomodulatory properties based on in vitro evidence, resistance to gastrointestinal stress and adherence to intestinal cells (de Palencia et al., 2009). Additionally, the bacteria have inhibitory effects on microorganisms as a result of produced pediocins (Immerstrand et al., 2010), and it has potential utility in the production of functional foods (Garai-Ibabe et al., 2010). The use of *P. parvulus* has been successfully applied to sausage fermentation, and contributes to enhance the hygienic quality of sausage by inhibiting non-lactic acid bacteria (Li et al., 2008). This species has been described to have an important role in the fermentation of certain Belgian acidic beers (Martens et al., 1997) and has been shown to have some antifungal effects (Magnusson et al., 2003).

The pathway to biodegrade OTA that is used by the *P. parvulus* strains isolated from the Douro wines is the same pathway that has been identified in other microorganisms (Abrunhosa et al., 2010). Specifically, OTA is degraded via the hydrolysis of the OTA amide group and the subsequent release of the OTα and L-β-phenylalanine moieties. It is known that this hydrolysis is mediated at varying degrees of efficiency by some peptidases. The carboxypeptidase A enzyme from the bovine pancreas (Pitout, 1969), a commercial lipase (Stander et al., 2000), several commercial proteases and an OTA-hydrolytic enzyme isolated from *Aspergillus niger* (Abrunhosa et al., 2006), and carboxypeptidase Y from *Saccharomyces cerevisiae* (Abrunhosa et al., 2010), all can mediate the hydrolysis reaction. The hydrolysis of the amide bond of OTA is thought to mediate the detoxification of OTA because OTα is nontoxic.

In conclusion, because of the ability of the *P. parvulus* strains isolated from Douro wines to degrade OTA, one can appreciate their potential biotechnological application to reduce the health hazards associated with OTA. Because the strains mediate OTA biodegradation under anaerobic conditions, they may be suitable as silage inoculants or as feed additives. They may be of particular interest for swine nutrition because swine are the most susceptible livestock to the toxic effects of OTA. Typically, OTA levels that are detected in feed are significantly higher than the level found in food products. Therefore, applications for use in animal nutrition may be more valuable. Nevertheless, *P. parvulus* strains may have potential in human nutrition as observed in the assays performed in grape must. Some *P. parvulus* strains have already been found to be useful for sausage and beer fermentation, and others have shown properties that are relevant for probiotic applications. OTA-biodegrading *P. parvulus* may also be useful to eliminate OTA from wines. One potential strategy may involve the inoculation of wines with *P. parvulus* before alcoholic fermentation since it eliminates OTA in grape must. Inoculation of wines with malolactic bacteria and yeast at the same time has been advocated by some researchers because the bacteria have a better chance of growing and acclimatising to the media in the absence of ethanol and when nutrients are abundant (Bou et al., 2005). This strategy may be possible for the *P. parvulus* strains that degraded OTA. Even if some researchers associated *P. parvulus* with the ropiness of wine, others do not establish any direct association. For example, Fugelsang and Edwards (2007) consider in their book that it is possible that the growth of pediococci in wine may not adversely affect quality and may actually add desirable flavours and aromas under certain circumstances. Further studies should also be conducted to evaluate the contribution of these strains to the sensory characteristic and quality of wines.

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