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Toxicity reduction of ochratoxin A by lactic acid bacteria

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by the metabolism of fungus belonging to the genus *Aspergillus* and *Penicillium*. In this paper we report, the capacity of different cultures of lactic acid bacteria (LAB) to degrade OTA present in MRS broth at both pH 3.5 and 6.5. A study of OTA reduction during gastrointestinal digestion carried out with the LAB was also performed. Taking into account the two reduction mechanisms of OTA studied in this work as the enzymatic one and the adsorption on the cell wall, as well as at pH 3.5 and 6.5 the reduction values of OTA were in a range of 30–99%, being the strains with greater reduction (97% and 95%) *Lb. rhamnosus* CECT 278T and *Lb. plantarum* CECT 749 respectively. In the experiments carried out digesting the OTA in MRS medium with LAB, the highest bioaccessibility reduction was observed by the strain of *Lb. johnsonii* CECT 289, showing a mean reduction around all the gastrointestinal digestion process of 97.4%. The mass spectrometry associated to the linear ion trap method identified ochratoxin alpha (OTα) m/z = 256.1 and phenylalanine (Phe) m/z = 166.1 as the major metabolites of OTA degradation in LAB cultures.

1. Introduction

Ochratoxin A (Fig. 1) (OTA) is a mycotoxin produced by secondary metabolism of many filamentous species belonging to the genera *Aspergillus* and *Penicillium*.

OTA is a potent nephrotoxic mycotoxin in nature and also displays other adverse effects such as hepatotoxicity, teratogenicity, and immunosuppression (Pfohl-Leszkowicz et al., 1998). OTA has been proven to be carcinogenic in kidney and liver. It has been classified as a group 2B human carcinogen by the International Agency for Research on Cancer (IARC), and World Health Organization (WHO) (IARC, 1993). OTA has been putatively implicated in the etiology of Balkan endemic nephropathy (BEN) and recognized to be related to urinary tract tumors in animals (Paul et al., 2002; Pfohl-Leszkowicz et al., 2002).

Regarding the alimentary aspect, OTA is a common contaminant of grains such as barley, corn, rye, wheat, and oats, with cereal-based products typically accounting for 50–80% of the average consumer intake of the mycotoxin (Jorgensen and Jacobsen, 2002). OTA has also been reported in other plant products including coffee beans, spices, nuts, olives, grapes, beans, and figs (O'Callaghan et al., 2006). In addition, OTA can survive many typical food-processing procedures, and has been reported in bread made from contaminated wheat (Scudamore et al., 2003), in beer and wine (Odhav and Naicker, 2002).

Many countries and international organizations have regulated the OTA content in several commodities. The European Commission, 2006 has enforced the limits of OTA in cereals and cereal products with the following levels: 5.0 ng/g for raw cereal grains, 3.0 ng/g for cereals and cereal products intended for human consumption, 0.5 ng/g for baby food and cereal-based food intended for young children. For the dried vine fruits, soluble coffee and some dried fruits, the European commission has set a maximal permissible limit for OTA at 10.0 ng/g.

OTA is a moderately stable molecule, which can survive most food processings, such as roasting, brewing and baking, to some extent (Scott, 1996). Several chemical and physical methods such as hypochlorite treatment (Refai et al., 1996), ammoniation and heat treatment have been developed to detoxify OTA in animal feed or alcoholic beverages. Other detoxification methods suggest the use of ozone, alkaline hydrogen peroxide treatment and gamma irradiation in cereals and derivates (Janos et al., 2000).

Yeast, including *Saccharomyces* sp., *Rhodotorula* sp., *Cryptococcus* sp., and *Trichosporon mycotoxinivorans*, can hydrolyze the amide bond of OTA to produce nontoxic products phenylalanine (Phe) and ochratoxin alpha (OTα) (Abrunhosa et al., 2010).

A bacterium, *Phenylobacterium immobile*, which can use Phe as a sole carbon source, converted OTA to OTα and three other metabolites resulting from the breakdown of the phenyl moiety of Phe (Wegst and Ligens, 1983).

The aims of the study were to evaluate a) the OTA elimination by different strains of lactic acid bacteria in MRS medium, b) the OTA reduction during a simulated gastrointestinal digestion in presence of lactic acid bacteria, using an *in vitro* dynamic model and c) the OTA degradation products formed during the fermentations through the

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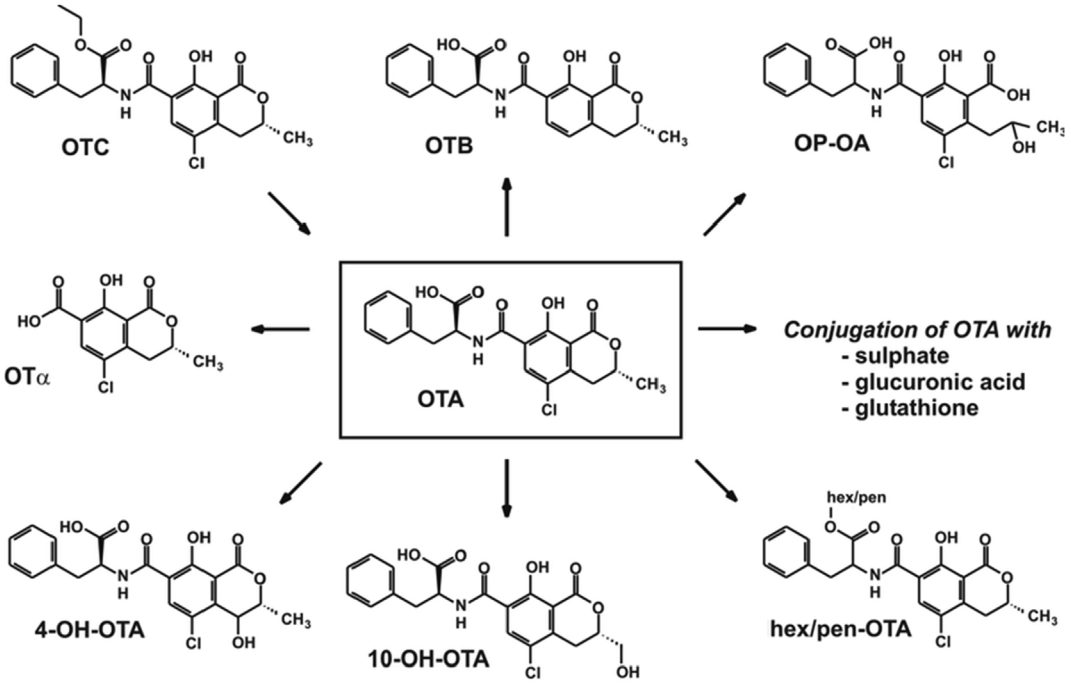

Fig. 1. Chemical structure of the OTA and of the main conjugation and degradation products.

technique of the Liquid chromatography coupled to the mass spectrometry with linear ion trap.

2. Materials and methods

2.1. Chemicals

A stock standard solution of OTA (Sigma–Aldrich, St. Luis, USA) was prepared by dissolving 1 mg of OTA standard in 1 mL of pure methanol, obtaining a 1 mg OTA/mL (1000 μg/mL) solution. This stock solution was diluted with methanol in order to obtain the appropriated work solutions (1, 10 and 100 mg/L). OTA solutions were stored in amber vials at 4 °C until the liquid-chromatography coupled a fluorescence detector (LC-FLD) analysis. Acetonitrile, methanol, water, ethyl acetate (all of LC grade) and acetic acid were purchased from Merck (Whitehouse Station, NJ, USA).

2.2. Assay for proteolytic activity

Proteolytic activity was assessed by an agar diffusion method. Milk agar plates containing 1.6% (w/v) skim milk and 1.5% (w/v) agar were prepared. Ten microliter of each strain at a final concentration of 10^8 CFU/mL was inoculated onto plates. After incubation at 37 °C for 48 h, absence of an inhibitory zone was observed. Each plate was examined for clear zones (Cho et al., 2015).

2.3. Strains and methodology

Twenty-seven commercial probiotic strains were used in the *in vitro* tests to evaluate their capacity to reduce OTA in MRS. In particular, *Bf. adolescentis* CECT 5781T, *Bf. bifidum* CECT 870T, *Bf. breve* CECT 4839T, *Bf. longum* CECT 4551, *Lb. casei* CECT 475T, *Lb. casei* CECT 4040, *Lb. casei*-CECT 4045, *Lb. delbrueckii bulgaricus* CECT 4005, *Lb. fermentum* CECT 562, *Lb. johnsonii* CECT 289, *Lb. paracasei* CECT 4022, *Lb. plantarum* CECT 220, *Lb. plantarum* CECT 221, *Lb. plantarum* CECT 222, *Lb. plantarum* CECT 223, *Lb. plantarum* CECT 224, *Lb. plantarum* CECT 225, *Lb. plantarum* CECT 226, *Lb. plantarum* CECT 748, *Lb. plantarum* CECT 749, *Lb. reuteri* CECT 725, *Lb. rhamnosus* CECT 278T, *Lb. rhamnosus* CECT 288, *Lb. sakei* CECT 906T, *Lb. salivarius* CECT 4062, *Lb. salivarius* CECT 4305, *Lc. mesenteroides* CECT 219T, *Lc. mesenteroides* CECT 215 and *Lc. mesenteroides* CECT 394 were obtained from the Spanish Type Culture Collection (CECT Valencia, Spain), in sterile 18% glycerol.

The microbes were cultured in 15 mL sterile plastic centrifuge tubes utilizing as growth medium 10 mL of De Man-Rogosa-Sharpe (MRS broth, Oxoid Madrid, Spain) for *Bifidobacterium* and *Lactobacillus*. The tubes were incubated at 37 °C under anaerobic conditions (Anaerocult A, Merk-Darmstadt, Germany) before experiments. Then, the suspensions of each strain at concentrations of 10^8 CFU/mL were added to a fresh 10 mL of MRS adjusted to pH 3.5 and 6.5 contaminated with 0.6 μg/mL of OTA and incubated at 37 °C during 24 h.

The mediums were analyzed in order to determinate the residual concentrations of OTA present in the growth medium and also to identify the possible degradation products.

2.4. OTA extraction and analysis from MRS medium

The fermentation tubes were centrifuged at 4000 rpm (Centrifuge 5810R, Eppendorf, Germany) for 5 min at 4 °C in order to separate the fermented medium from the cells. OTA in the fermented medium was extracted as follows (Abrunhosa et al., 2014). Five milliliters of fermented MRS were putted in a 20 mL test tube, and extracted three times with 5 mL of ethyl acetate utilizing a vortex (VWR International, Barcelona, Spain) for 1 min. The resulting extracts were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 4000 rpm for 10 min at 4 °C. The organic phases were completely evaporated using a rotary evaporator (Buchi, Switzerland) operating at 30 °C and 30 mbar pressure; the remaining was resuspended in 1 mL of methanol, filtered with 0.22 μm filters (Pheomenex, Madrid, Spain) and analyzed by LC–FLD (Shimadzu, Kyoto, Japan). Specifically, chromatographic separation was performed under gradient conditions at flow rate of 1.2 mL/min. The fluorescence detector was set up at λex 333 nm and λem 460 nm. The instrument was equipped with a Hamilton syringe (Reno, Nev.) and a conventional C18 column was used. The mobile phases were composed of two eluents, both containing 1% acetic acid, the eluent A was water and the eluent B acetonitrile. The elution gradient was established initially with 30% eluent B, increased to 70% in 5 min. The