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Lactic acid bacteria against post-harvest moulds and ochratoxin A isolated from stored wheat

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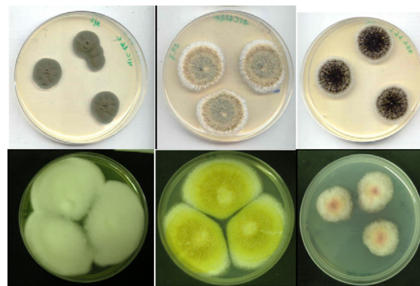
HIGHLIGHTS

- *Lactobacillus plantarum* exhibited a large antifungal spectrum.
- Lactic bacteria showed high inhibitory effect against ochratoxigenic fungal strain.
- Temperature, pH and bacterial biomass has a significant effect on fungal inhibition.
- Temperature, pH and bacterial biomass has a significant effect on OTA production.
- Selected LAB may be exploited as a potential OTA detoxifying agent.

GRAPHICAL ABSTRACT



Mould strains isolated from stored durum wheat.

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ABSTRACT

A total of 54 lactic acid bacteria (LAB) were isolated from stored wheat samples sourced from grain silos in North Tunisia. Fifteen representative isolates were identified by 16S rDNA sequencing as *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus graminis*, *Lactobacillus coryniformis* and *Weissella cibaria*. These isolates were screened for antifungal activity in dual culture agar plate assay against eight post-harvest moulds (*Penicillium expansum*, *Penicillium chrysogenum*, *Penicillium glabrum*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus carbonarius*, *Fusarium graminearum* and *Alternaria alternata*). All LAB showed inhibitory activity against moulds, especially strains of *L. plantarum* which exhibited a large antifungal spectrum. Moreover, LAB species such as *L. plantarum* LabN10, *L. graminis* LabN11 and *P. pentosaceus* LabN12 showed high inhibitory effects against the ochratoxigenic strain *A. carbonarius* ANC89. These LAB were also investigated for their ability to reduce *A. carbonarius* ANC89 biomass and its ochratoxin A (OTA) production on liquid medium at 28 and 37 °C and varied pH conditions. The results indicated that factors such as temperature, pH and bacterial biomass on mixed cultures, has a significant effect on fungal inhibition and OTA production. High percentage of OTA reduction was obtained by *L. plantarum* and *L. graminis* (>97%) followed by *P. pentosaceus* (>81.5%). These findings suggest that in addition to *L. plantarum*, *L. graminis* and *P. pentosaceus* strains may be exploited as a potential OTA detoxifying agent to protect humans and animals health against this toxic metabolite.

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1. Introduction

Eukaryotic and prokaryotic organisms like moulds, yeasts and bacteria are natural contaminating microorganisms of cereal grains. Nevertheless, some filamentous fungi may develop at different phases of seeds handling especially when agricultural practices, storage conditions and processing stage are not well controlled. These moulds can cause irreversible damages and losses of quality product as well as the production of toxic metabolites in grain.

Ochratoxin A (OTA) is a fungal secondary metabolite produced by species belonging to *Aspergillus* and *Penicillium* genera, mainly *Aspergillus niger*, *A. ochraceus*, *Aspergillus carbonarius* and *Penicillium verrucosum*. This mycotoxin causes several negatives effects on animals and humans including nephrotoxic, teratogenic, embryotoxic, immunotoxic, genotoxic, and neurotoxic effects (JECFA, 2001; Wangikar et al., 2005). Moreover OTA is considered to be a possible human carcinogen and is classified as Group 2B by the International Agency of Research on Cancer (IARC, 2002). Ochratoxin A has been found in human food and animal feed like cereals, wine, cocoa, dried vine fruits, olives, coffee, beer, and spices (JECFA, 2001; Birzele et al., 2000; Fazekas et al., 2002; Roussos et al., 2006). Thus, their production in such commodities can be influenced by several factors, including temperature, water activity, pH, nutrients availability and competitive growth of other microorganisms. The contamination of food by mycotoxins can represent a direct source of human exposure by direct consumption or an indirect source through the consumption of derived products from animals fed with contaminated feed.

Due to the importance of wheat in the human diet and considering the impact of this mycotoxin on health, preventive measures for the reduction or the elimination of fungal growth and mycotoxins have received much attention in recent years. Safe strategies for this purpose were investigated by the use of microorganisms such as yeasts, filamentous fungi and bacteria as antimicrobial and mycotoxins detoxification agents (Sharma et al., 2009). In fact, the requirements of biological products with low impact on the environment, on human health and with ability to bind such toxic metabolites take increasing attention. The use of lactic acid bacteria (LAB) which are generally recognized as safe have received significant attention as a novel approach to the control of pathogens in foods by the production of antimicrobial compounds (Klaenhammer, 1993; Settani et al., 2005; Gerez et al., 2013) but only few studies have been investigated for the LAB efficiency in matter of OTA removal from foods (Skrinjar et al., 1996; Turbic et al., 2002; Piotrowska and Zakowska, 2005). Studies about this topic are necessary since wheat is the most important cereal crop in many countries including Tunisia. In this study, a series of bacterio-fungal culture experiments were carried out to: (i) analyze the development states of co-occurred fungus (*A. carbonarius*) and LAB under different temperatures and pH conditions. (ii) Analyze negative aspects of bacterial effects on fungal OTA production and biomass formation. For that, isolated LAB from stored durum wheat were initially identified. These investigations were made for the first time for *Lactobacillus graminis* and *Pediococcus pentosaceus*.

2. Materials and methods

2.1. Isolation of lactic acid bacteria

Lactic acid bacteria (LAB) were obtained from stored wheat samples sourced from grain silos in North Tunisia. According to the enrichment method described by Chen et al. (2005), durum wheat samples were inoculated in 10 ml MRS broth and incubated under anaerobic conditions for 2–3 days at 30 °C. After incubation, cultures obtained were serially diluted and spreaded in duplicate on the surface of MRS agar media usually used to isolate LAB associated to food matrices. In order to avoid fungal and yeast growth cycloheximide (0.01%) was added to the media. Petri dishes were anaerobically incubated for 2 days at 30 °C. Colonies arising on plates were picked on MRS media by successive sub-culturing for purification. The isolates were then characterized for morphological characteristics, Gram-positive, catalase-negative, non-mobile, cocci and rods colonies. Only isolates that showed negative reaction were maintained at −80 °C in glycerol stocks and subjected to further molecular identification.

2.2. DNA extraction and molecular identification of LAB

Genomic DNA was extracted according to CTAB/NaCl method described by Wilson (1987) and modified by using lysozyme (1 mg/ml) for cell lysis. Bacterial strains were characterized genotypically by 16S rDNA gene PCR amplification using universal primer pair s-d-bact-0008-a-S-20/s-d-bact-1495-a-A20 according to Daffonchio et al. (1998). PCR was performed on a thermocycler (BioRad) with initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C (45 s), annealing at 55 °C (1 mn), elongation at 72 °C for 2 min and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis through 1.5% (w/v) agarose gel containing ethidium bromide (0.5 mg/ml) and the DNA was detected by UV trans-illumination (UV spectrophotometer). 16S rDNA PCR amplicons were purified with Exonuclease-I and Shrimp Alkaline Phosphatase (Exo-Sap, Fermentas, Life Sciences). DNA sequencing was performed in an automated capillary DNA sequencer (Applied Biosystems 3130XL) using a Big Dye Terminator cycle sequencing Kit V3.1 (Applied Biosystems). Identification to the closet relative taxa of the isolates was achieved using BLAST analysis tool in the GenBank DNA database (www.ncbi.nih.gov). Phylogenetic analysis of 16S rRNA gene sequences was conducted with MEGA-5 software (Tamura et al., 2011). Phylogenetic tree was constructed by using neighbor-joining method (Saitou and Nei, 1987).

2.3. Nucleotide sequence accession numbers

The sequences of the 16S rDNA gene of LAB isolates have been submitted to the GenBank databases under accession numbers from KF554129 to KF554141.

2.4. Screening for antifungal activities against post-harvest moulds

2.4.1. Fungal strains and growth conditions

A total of eight post-harvest moulds were isolated from stored durum wheat in previous studies (Belkacem-Hanfi et al., 2013). Fungal isolates belonging to four genera of *Penicillium* (*Penicillium expansum* ANP234, *Penicillium chrysogenum* ANP67 and *Penicillium glabrum* ANP521), *Aspergillus* (*Aspergillus flavus* AFF94, *A. niger* ANN131 and *A. carbonarius* ANC89), *Fusarium* (*Fusarium graminearum* ANF14) and *Alternaria* (*Alternaria alternata* ANA212) were used for next antifungal experiment. Among these species, *A. carbonarius* ANC89 was selected as OTA producer reference strain. Spore suspensions of each fungal strain were prepared then grown on PDA medium for 7 days at 28 °C in 250 ml flask. In a next step, 100 ml of sterilized distilled water supplemented with Tween80 (0.01%) were added. Finally, spores were counted with Malassez-cell and suspensions were adjusted to about 10^5 spores/ml.

2.4.2. Antifungal growth activities of LAB isolates in dual culture

All LAB isolates were tested for their antifungal activities against post-harvest moulds using dual culture technique as described by Whipps (1987) with little modification: culture medium used for the test was MRS modified medium containing for

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