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Reduction *in vitro* of the minor *Fusarium* mycotoxin beauvericin employing different strains of probiotic bacteria

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ABSTRACT

The interaction between the minor *Fusarium* mycotoxins BEA and 13 bacterial strains characteristic of the gastrointestinal tract as *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium adolescentes*, *Lactobacillus rhamnosus*, *Lactobacillus casei-casei*, *Lactobacillus plantarum*, *Eubacterium crispatus*, *Salmonella fecalis*, *Salmonella termofilus*, *Lactobacillus ruminis*, *Lactobacillus casei* and *Lactobacillus animalis* was studied.

The fermentations were carried out in the liquid medium of MRS during 4, 12, 16, 24 and 48 h at 37 °C, under anaerobic conditions.

Levels of BEA in the fermentation liquid, on the cell walls and on the internal part of the cells were determined using liquid chromatography coupled to the mass spectrometry detector (LC-MS/MS). Results showed that the bacteria reduced the concentration of the BEA present in the medium, part of the mycotoxin was adsorbed by cell wall and part internalized by the bacteria. All the bacteria analyzed in this study showed a significant BEA reduction during the fermentation process, in particular the mean diminution resulted variable from 66 to the 83%.

1. Introduction

Beauvericin (BEA) is a cyclic hexadepsipeptide mycotoxin which was originally isolated from *Beauveria bassiana* and later from *Paecilomyces fumosoroseus* and *Fusarium lateritium* (Meca et al., 2010). BEA has strong antibacterial, antifungal, and insecticidal activities (Castlebury, Sutherland, Tanner, Henderson, & Cerniglia, 1999), and has also shown significant cytotoxic activity toward various human cancer cell lines. The notable antimicrobial and cytotoxic activities of BEA have attracted research interest in its application as a potential antibiotic and anticancer agent for human health care (Jow, Chou, Chen, & Tsai, 2004; Zhang, Yan, Zhang, Huang, & Chen, 2007).

BEA is most widely produced by many entomopathogenic *Fusarium* fungal species (Fotso, Leslie, & Smith, 2002). In addition to BEA, enniatins, which belong to another class of cyclicpeptides having entomopathogenic activities, are also produced by many of these *Fusarium* species. Nevertheless, BEA is the most common and abundant mycotoxin, and plays a major role in the insecticidal activities of these *Fusarium* species (Moretti, Mule, Ritieni, & Logrieco, 2007).

As regard the antibacterial activity of the BEA, Castlebury et al. (1999) studied the action of this bioactive compound on bacteria typically isolated from mammalian intestinal tract to understand the interaction of the BEA with the normal intestinal microbiota that has an important role in nutrition, physiology, and colonization resistance to invasive pathogens. Also, Fotso and Smith (2003) evaluated the BEA mutagenicity, utilizing the Ames test on *Salmonella typhimurium* standard tester strain.

BEA has exhibited minimum inhibitory concentration (MIC) values of 0.8–1.6 μg/mL against *Mycobacterium tuberculosis*, and IC_{50} values of 1.3–2.4 μg/mL against *Plasmodium falciparium* (Nilanota, Isaka, Kittakoop, & Trakulnaleamsai, 2002).

The potential toxic role of BEA is exemplified by results from *in vitro* studies using cell lines. For instance, BEA induces significant cell deaths in insect, murine, and human tumor cell lines (Calo et al., 2003; Mazziotti & Perlmutter, 1998). Furthermore, BEA is a potent and specific cholesterol acyltransferase inhibitor in rat liver microsomes (Tomoda et al., 1992).

In mammalian cell lines, cell deaths caused by BEA have been suggested to involve a Ca^{2+} dependent pathway, in which BEA induces a significant increase in intracellular Ca^{2+} concentration

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that leads death cell as a result of a combination of both apoptosis and necrosis (Jow et al., 2004; Lin et al., 2005; Logrieco et al., 1998; Nilanota et al., 2002; Ojcius, Zychlinsky, Zheng, & Young, 1991).

BEA is considered a normal contaminant of cereals and also of products composed by cereals (Zinedine, Meca, Mañes, & Font, 2011). In particular, the presence of BEA in food commodities has been recently reported during the last decade in some European countries (Finland, Norway, Spain, Slovakia, Croatia, Switzerland and Italy), in USA, in South Africa (Jestoi, 2008; Meca, Zinedine, Blesa, Font, & Mañes, 2010; Munkvold, Stahr, Logrieco, Moretti, & Ritieni, 1998; Ritieni et al., 1997; Shephard, Sewram, Nieuwoudt, Marasas, & Ritieni, 1999; Zinedine et al., 2011) in higher concentrations (milligrams per kilograms) respect to the classical legislate *Fusarium* mycotoxins as the fumonisins or the trichothecenes.

As regards the methodologies employed for the reduction of this contaminant in food, in the scientific literature is present only a US patent (Duvick & Rod, 1998) on the biological detoxification of the minor *Fusarium* mycotoxin BEA. In particular the authors, employing as detoxification agent a strain of *Nocardia Glubera*, reduced the contamination by BEA in wheat kernels of 50% considering an initial contamination of the mycotoxin of 1000 mg/L.

The biological detoxification strategies for the reduction of the other *Fusarium* mycotoxins were studied by many authors. In particular Young, Zhou, Yu, Zhu, and Gong (2007) evaluated the degradation of 12 trichothecenes by chicken intestinal microbes observing a complete conversion to the deepoxy metabolites of the non-acylated trichothecenes and a deacetylation of the monoacetyl trichothecenes.

Guan et al. (2009), evaluated the transformation of several trichothecenes mycotoxins by microorganism isolated by fish digesta, evidencing a completely transformation of the mycotoxin deoxynivalenol (DON) to deepoxy DON (dE-DON) at 15 °C in full medium after 96 h incubation. The authors evidenced also that most of the other trichothecenes were transformed to deacetyl and/or deepoxy products.

Islam, Zhou, Young, Goodwin, and Pauls (2011) studied the aerobic and anaerobic deepoxydation of mycotoxin DON by bacteria originating from agricultural soil. In particular the bacteria isolated by the authors and related to the family of *Serratia*, *Clostridium*, *Citrobacter*, *Enterococcus*, *Stenotrophomonas*, *Streptomyces* produced through an enzyme process the de-epoxydized DON after 60 h of incubation. Bacterial deepoxydation of DON occurred also in the pH range 6.0–7.5, and a wide array of temperatures (12–40 °C).

Considering the lack of data on the biological detoxification of the minor *Fusarium* mycotoxins the aim of this study was to evaluate the reduction *in vitro* of the minor *Fusarium* mycotoxin BEA employing different strains of probiotic bacteria as detoxification agents.

2. Material and methods

2.1. Chemicals

A stock standard solution of BEA (98% of purity) (Sigma–Aldrich, St. Luis, USA) was prepared by dissolving 1 mg of standard in 1 mL of pure methanol, obtaining a 1 mg BEA/mL (1000 μg/mL) solution. This stock solution was then diluted with methanol in order to obtain the appropriated work solutions with concentrations of 1, 10 and 100 mg/L. All BEA solutions were stored in darkness at 4 °C until the LC-MS/MS analysis. Acetonitrile, methanol, water, ethyl acetate (all of LC grade) and acetic acid were purchased from Merck (Whitehouse Station, NJ, USA).

2.2. Strains and methodology

The study was carried out using 13 strains probiotic bacteria, named *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium adolescentes*, *Lactobacillus rhamnosus*, *Lactobacillus casei-casei*, *Lactobacillus plantarum*, *Eubacterium crispatus*, *Salmonella fecalis*, *Salmonella termofilus*, *Lactobacillus ruminis*, *Lactobacillus casei* and *Lactobacillus animalis*.

The strains were obtained at the Spanish Type Culture Collection (CECT Valencia, Spain), in sterile 18% glycerol.

For longer survival and higher quantitative retrieval of the cultures, they were stored at −80 °C. When needed, recovery of strains was undertaken by two consecutive subcultures in appropriate media prior to use.

The microbes were cultured in 15 mL sterile plastic centrifuge tubes utilizing as growth medium 10 mL of DeMan-Rogosa-Sharpe (MRS broth, Oxoid Madrid, Spain) and incubated at 37 °C in an anaerobic atmosphere with 95% CO_2 and 5% H_2 during 48 h. After that each bacterial suspensions at concentrations of 10^8 CFU/mL was added to a fresh 10 mL of MRS contaminated with 5 mg/L of the mycotoxin BEA, incubated at 37 °C in an anaerobic atmosphere with 95% CO_2 and 5% H_2 during 4, 12, 16, 24 and 48 h.

The mediums were analyzed in order to determinate the residual concentration of BEA present in the growth medium, the toxin concentration adsorbed by bacteria on the cells wall, and also the BEA amount internalized in the cells.

2.3. BEA extraction from fermented mediums

The fermentation tubes were centrifuged at 4000 rpm (Centrifuge 5810R, Eppendorf, Germany) during 5 min at 4 °C in order to separate the fermented medium from the cells. BEA contained in fermented medium was extracted as follows (Jestoi, 2008). Five mL of fermented MRS were introduced in a 20 mL plastic tube, and extracted three times with 5 mL of ethyl acetate using a vortex VWR international (Barcelona, Spain) during 1 min. After that the mixtures were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 4000 rpm for 10 min at 4 °C. The organic phases were completely evaporated by a rotary evaporator (Buchi, Switzerland) operating at 30 °C and 30 mbar pressure, resuspended in 1 mL of methanol, filtered with 0.22 μM filters (Pheomenex, Madrid, Spain) and analyzed by LC-MS/MS (Meca, Blaiotta, & Ritieni, 2010; Meca, Zinedine, et al., 2010).

2.4. BEA extraction from cells wall

The BEA adsorbed on the cell walls was extracted suspending the pellet, separated from the fermented medium, in 1 mL of saline solution (0.90% NaCl, w/v) in a 1.5 mL vial. The extraction was carried out by agitation with an orbital shaker (IKA Ks 260 basic, Stanfen, Germany) for 12 h, then the samples were centrifuged at 4000 rpm (Centrifuge 5810R, Eppendorf, Germany) for 10 min at 4 °C and the upper layer was filtered with 0.22 μM filters (Pheomenex, Madrid, Spain) and analyzed by LC-MS/MS (Meca, Blaiotta, et al., 2010; Meca, Zinedine, et al., 2010).

2.5. BEA extraction from cells

The BEA contained in the cells was determined as follows: cells were sonicated in a saline solution (0.90% NaCl, w/v) for 30 min. The pellet was suspended in 5 mL of saline solution, and 5 mL of ethyl acetate was added. After mixing with a vortex for 1 min and centrifuging (4000 rpm for 10 min at 4 °C, Centrifuge 5810R, Eppendorf, Germany), the upper layer, 5 mL approximately, were