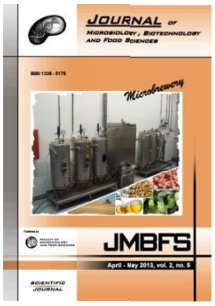

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CELL-SURFACE BINDING OF DEOXYNIVALENOL TO *Lactobacillus paracasei* subsp. *tolerans* ISOLATED FROM SOURDOUGH STARTER CULTURE

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ABSTRACT

Deoxynivalenol (DON) and fumonisin B_1 (FB_1) are two contaminant-mycotoxins frequently found in food commodities produced under poor conditions. Several methods have been suggested for the detoxification of such mycotoxins. Among the proposed methods, biological detoxification seems to be the most promising and cost-efficient. This study explores the capability of one strain of lactic acid bacteria, identified as *Lactobacillus paracasei* subsp. *tolerans*, to bind both DON and FB_1 in liquid cultures. Here we report the ability of heat-inactivated cells to significantly reduce concentrations of DON in liquid cultures. Further mechanistic investigation showed that the detoxification process is a result of the physical binding of such mycotoxins to the cell wall of this bacterium.

Keywords: *Lactobacillus*, deoxynivalenol, fumonisin, detoxification

INTRODUCTION

Lactic acid bacteria (LAB) have been used throughout history for the production of value-added fermented food commodities (**Brashears *et al.*, 2005**). In addition, this group of microorganisms constitutes a major part of healthy intestinal microbiota (**Damelin *et al.*, 1995; Lindgren and Dobrogosz, 1990**). One of the most interesting applications of incorporating lactic acid bacteria in diet is the antifungal capacity of such strains (**Batish *et al.*, 1997; Bullerman *et al.*, 2006; Gourama and Bullerman, 1997; Hassan and Bullerman, 2008a, b; Magnusson and Schnurer, 2001**). Lactic acid bacteria strains isolated from different sources show promising abilities of inhibiting mold spoilage and improving the shelf-lives of final processed products (**Corsetti *et al.*, 2000; Topisirovic *et al.*, 2006; Valerio *et al.*, 2004; Vaughan *et al.*, 1994**). Numerous studies imply the capacity of certain isolates of LAB to bind and remove mycotoxins (**El-Nezami *et al.*, 2002a; Mokoena *et al.*, 2005**). The same fact was also observed in materials rich in this group of microorganisms such as rumen liquids and animal feces (**Kiessling *et al.*, 1984; Swanson *et al.*, 1988**).

Recently, our lab reported the isolation of *Lactobacillus paracasei* subsp. *tolerans* from sourdough starter culture that demonstrated a strong antifungal activity against different *Fusarium* species including *Fusarium proliferatum* and *Fusarium graminearum* (**Hassan and Bullerman, 2008a, b**). The objective of our current study was to investigate the ability of this particular isolate to reduce fumonisin B_1 and deoxynivalenol concentrations in liquid-cultures, thus providing value-added fermented/malted products.

MATERIAL AND METHODS

Lactic acid bacteria isolation and storage

Lactobacillus paracasei subsp. *tolerans was* isolated from a traditional home-style sourdough bread culture as described earlier (**Hassan and Bullerman, 2008a**). The frozen stock was kept in 20% glycerol at -20°C. The isolate was reactivated by streaking on deMan, Rogosa, and Sharpe (MRS) agar plates and grown for 24-36 hours at 37°C (**Hassan and Bullerman, 2008b**).

Testing for mycotoxin detoxification activity

Commercial Lactobacilli MRS broth (Becton Dickinson, Franklin Lakes, New Jersey; Cat. # 288130) was used as the testing medium. Stock solutions of fumonisin B_1 (Sigma-Aldrich, St. Louis, MO; Cat. # F1147) and deoxynivalenol (Sigma-Aldrich, St. Louis, MO; Cat. # D0156) were reconstituted to obtain a 2.5 µg/ml final concentration. Inoculums (10 µl) of activated *Lactobacillus paracasei* subsp. *tolerans* were added and tubes were kept overnight at 37°C on an orbital shaker (150 rpm). Growth of the bacterial cultures was monitored and normalized by OD_{600}. The overnight cultures were centrifuged at 10,000 rpm for 10 min. and resulting supernatants were filtered through 0.22 µm filters (Millipore, Billerica, Massachusetts; Cat. # SLGS033SS). These supernatants were used later for mycotoxin analyses as described below. In order to determine the amount of mycotoxin that was binding to the bacterial cell-walls, the collected pellets above were washed with sterile phosphate buffered saline (PBS) under vigorous shaking conditions in order to release any bound mycotoxins. Mycotoxin concentrations were also determined in these washing steps.

Mycotoxin binding of heat-inactivated bacteria

To distinguish between cell-wall binding and enzyme-mediated degradation of the studied mycotoxins, we first heat-inactivated LAB cells and then introduced them to the MRS broth containing fumonisin B_1 and deoxynivalenol (2.5 µg/ml final concentration). A total of 400 ml's of MRS broth was inoculated with *Lactobacillus paracasei* subsp. *tolerans* and grown for 48 hours at 37°C. The medium containing the viable bacteria was then autoclaved (30 min. at 121° C) and centrifuged at 10,000 rpm for 15 min. The supernatant was discarded and the pellet was re-introduced into sterile MRS broth containing 2.5 µg/ml of each mycotoxins separately at an optical density (OD_{600}) of 2.25. The mix was kept on orbital shaker for one hour before proceeding to mycotoxin analysis. The OD_{600} normalization step was essential to make valid comparisons between heat-inactivated and actively-growing bacteria. The selected optical density value (2.25) reflect a similar level of LAB growth after 18 hours of incubation with mycotoxin/broth mixtures. *Escherichia coli* (a kind gift from Dr. Andrew Benson, Department of Food Science and Technology, University of Nebraska-Lincoln) was included also in the above experiments to determine if gram-negative cell walls possess a similar ability to remove mycotoxins. In all trails, *E. coli* was grown overnight in commercial nutrient broth (Becton Dickinson, Franklin Lakes, New Jersey) containing FB_1 and DON (2.5 µg/ml) at 37°C on an orbital shaker (150 rpm) before cell pellet removal and mycotoxin determination.

Enzyme linked immunosorbant assay (ELISA) measurement of mycotoxins

Neogen-Veratox quantitative fumonisin-high sensitivity (Neogen Corporation, Lansing, Michigan; Cat. # 8832) and deoxynivalenol-high sensitivity (Neogen Corporation, Cat. # 8332) enzyme linked immunosorbant assay kits; EL301 Microwell Reader (Neogen Corporation, Cat. # 9301); and the

Neogen's Veratox software were used to quantitatively measure mycotoxin concentrations. In essence, a 100 µl portion of the cell-free supernatant/washing buffer was serially diluted using 900 µl of sterilized distilled water to reach the linear range of the testing kit. Subsequent mycotoxin determinations were conducted according to the manufacture's protocol. The manufacturer's provided Neogen-Veratox software contains a programmed algorithm that converts absorbance values to concentrations expressed as parts per billion (ppb) or parts per million (ppm) which were later converted into ng/ml scale taking the dilution factors into account. StatView package (Abacus, Cupertino, California; version 4.57) was used to verify any significant differences between controls and treatments. All the reported studies were conducted in triplicates.

RESULTS AND DISCUSSION

The aim of this study was to examine the ability of *Lactobacillus paracasei* subsp. *tolerans* isolate of detoxifying both DON and FB_1 from liquid media. This isolate is an efficient acid producer and it shows a promising ability of inhibiting wide spectrum of *Fusarium* species (**Hassan and Bullerman, 2008a, b**). In this study, heat-inactivated cells demonstrated a significant ability to reduce DON in MRS broth (Figure 1).

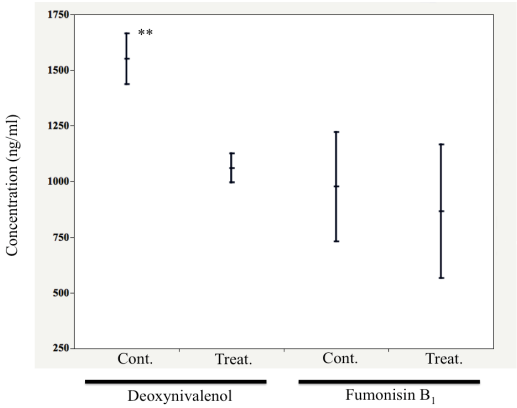

Figure 1 Reduced levels of deoxynivalenol and fumonisin B_1 were observed after one hour incubation with heat-inactivated bacterial cells of *Lactobacillus paracasei* subsp. *tolerans*

Under the same conditions, heat-inactivated cells were able also to decrease FB_1 concentrations in culture broth, but this reduction was not statistically significant (Figure 1). Similar abilities of heat-inactivated lactic acid bacteria to bind mycotoxins present in solution and liquid cultures have been reported earlier (**El-Nezami *et al.*, 2000; El-Nezami *et al.*, 2002b; Franco *et al.*, 2011; Peltonen *et al.*, 2001; Pierides *et al.*, 2000**). Conversely, viable cells of this isolate were able to slightly lower the concentration of DON and FB_1 concentrations in liquid cultures but the reduction was not statistically significant (Tab. 1).

Table 1 Fumonisin B_1 and deoxynivalenol concentrations (ng/ml) in culture media after 18 hours incubation with *Lactobacillus paracasei* subsp. *tolerans* at 37° C

Mycotoxins	Group	Number of samples	Mean	SEM
FB_1	Control	3	527 ± 506	292.3
	Treatment	3	513 ± 304	175.7
DON	Control	3	1797.7 ± 137.7	79.5
	Treatment	3	1793.7 ± 55.6	32.1

The ability of heat-inactivated bacterial cells to bind DON and significantly lower its concentrations compared to weak binding of DON to viable cells is explained by the pH effect. When viable cells grow actively in the media they ferment present carbohydrates, lowering the pH of the medium, which in turn affects how DON is adsorbed onto cell surface. Our results in this regard are in agreement with the previously published observation by **Shehata *et al.* (2000**) who showed that DON adsorption ranged from 47 to 99% depending on the pH value of the medium. On the other hand, the weak binding of FB_1 to the actively growing cells is explained by the reduced stability of complexes formed between this mycotoxin and cell surface. Tab. 2 clearly shows that complexes formed between DON and bacterial cell walls are more stable, hence the minimum observed release of DON into the PBS washing buffers.

Table 2 Fumonisin B_1 and deoxynivalenol concentrations (ng/ml) in the PBS-washing buffers

Mycotoxin	Number of samples	Mean	SEM
FB_1	3	550 ± 230	133.17
DON	3	43.7 ± 8.7	5.04

On the other hand, the weak association between FB_1 and LAB leads to almost a complete release of all bound FB_1 into washing buffers. These findings are similar to those of **Haskard *et al.* (2001**) who showed that mycotoxin binding was a reversible process and the stability of complexes formed depend at large on mycotoxin structure, bacterial strain, treatment, and surrounding environmental conditions.

Collectively, our results suggest that biding, rather than enzyme-mediated degradation, was responsible for the lowering of deoxynivalenol in treatment samples compared to controls. Similar observations were reported recently with much speculation about the actual binding site and the role of each cellular component in this binding (**Bueno *et al.*, 2007; Fuchs *et al.*, 2008**). In order to examine the involvement of bacterial cell walls in binding, we incorporated gram-negative *E. coli* cells as controls in all mycotoxin-binding trials under similar growth conditions and mycotoxin concentrations. No reductions in mycotoxin concentrations were observed when *E. coli* was used (data not shown), thus confirming the hypothesis that gram-positive cell walls are involved in the reported binding process. This observation aligns with the results of other research groups (**Del Prete *et al.*, 2007; Hernandez-Mendoza *et al.*, 2009; Niderkorn *et al.*, 2006**). The actual binding sites of different mycotoxins were recently investigated by **Niderkorn *et al.* (2009**). In that study, factors that led to the degradation of peptidoglycan polymers of bacterial cell walls resulted in decreased binding capacities, while conditions that increased the tricarballylic acid (TCA) component of bacterial cell walls were responsible for higher binding affinities.

CONCLUSION

In conclusion, this isolate of *Lactobacillus paracasei* subsp. *tolerans* showed the ability to bind DON in liquid cultures. Products that contain the heat-inactivated cells (such as baked products or pasteurized yoghurt) may have some advantages in this regard; however the practical use of such mycotoxin-binding strains need to be further investigated using *in vivo* models.

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