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Inhibition of Fungal Growth and *Fusarium* Toxins by Selected Cultures of Lactic Acid Bacteria

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Abstract

Two species of Lactobacilli (*Lactobacillus rhamnosus* and *Lactobacillus paracasie* subsp. paracasie) were tested for their ability to inhibit growth and mycotoxins production by three species of *Fusarium*, (*F. graminearum*, *F. culmorum* and *F. proliferation*) which are the main producers of mycotoxins deoxynivalenol, zearalenone and fumonisin B1, respectively. *L. paracasie* subsp. paracasie was found to be effective in reducing the amount of toxins produced, although fungal growth was not affected. The inhibition levels of Deoxynivalenol, Zearalenone and Fumonisin B1 production reached to 56.8, 73.0 and 76.5%, respectively. Meanwhile, *L. rhamnosus* showed the highest inhibitory activity against both fungal growth and mycotoxins production. It completely suppressed mycelium growth of all the studied *Fusarium* species and consequently, no toxin was produced in the presence of this bacterium. The obtained results confirm that, selected species of Lactic acid bacteria may be successfully used as a biological control agent of food contamination with molds and mycotoxins. This bio-preservation action has interesting technological possibilities for a variety of fermented food and dairy products.

Keywords: Inhibition; *Fusarium*; Lactic acid bacteria; Deoxynivalenol; Zearalenone; Fumonisin B1

Introduction

Fusarium is one of the most important genera of plant pathogenic fungi, with a record of devastating infections in various economically important plants [1]. Certain species of fusaria are capable of producing mycotoxins which can be accumulating in the infected plants and stored materials. The International Agency for Research on Cancer [2] has grouped the main *Fusarium* mycotoxins on the basis of the fungal producing them as it represents the best way for identify the real situation through which humans become exposed to these naturally occurring toxins. Therefore, the most important mycotoxins, in terms of natural occurrence and toxicology, have been grouped in: toxins derived from *F. sporotrichioides* (T-2 toxin and related trichothecenes), toxins derived from *F. graminearum*, *F. culmorum* and *F. crookwellense* (Deoxinivalenol, Nivalenol, Fusarenone and Zearalenone), and toxins derived from *F. moniliform* (Fumonisins and Fusarine C). Only the group of toxins deriving from *F. moniliform* was identified as group 2B, i.e. possible carcinogenic to humans and with sufficient evidence of carcinogenicity towards experimental animals, whereas the data relevant to the other groups of toxins or to individual toxins were not sufficient (or adequate) to make them classifiable as to their carcinogenicity to humans [2].

Deoxynivalenol (DON) is a mycotoxin produced by *F. graminearum* and *F. culmorum*, which are abundant in various cereal crops (wheat, corn, barley and oats) and processed grains (malt, beer and bread). Chemically, it belongs to trichothecenes and is a very stable compound, both during storage/milling and the processing/cooking of food and it does not degrade at high temperatures [3]. DON inhibits the synthesis of DNA, RNA and protein synthesis at the ribosomal level. The toxin has hemolytic effect on erythrocytes. An acute dose of DON can induce vomiting in pigs, whereas at lower concentrations in the diet it reduce growth and feed consumption [4].

Zearalenone is a secondary metabolite produced mainly by *F. culmorum*. It possesses strong estrogenic activity and can result in severe reproductive and infertility problems when they are fed to domestic animals in sufficient amounts. Swine appears to be the most sensitive of the domestic animals species, therefore the most frequently reported with problems caused by zearalenone, which include enlargement or swilling and reddening of the vulva in gilts and sows (vulvovaginitis), swelling of the mammary glands and atrophy of the ovaries, vaginal and rectal prolaspses. In young male it can cause swelling of the prepuce, testicular atrophy, enlargement of the mammary glands, while in boars it causes reduced libido and a marginal reduction in sperm quality. Effects in other species are much less pronounced. High concentrations of zearalenone have been associated with infertility and development of a typical secondary sexual characteristic in heifers [5].

Fumonisins are structurally related mycotoxins produced mainly by *F. verticilloides* and *F. proliferatum*, both of which are frequently found in corn worldwide [6,7]. Of the several fumonisins identified, fumonisin B1 (FB1) is considered to be the most abundant and the most toxic [7]. FB1 has been associated with several fatal diseases in animals, including Equine Leuko encephalomalacia [8]. FB1 has also been demonstrated experimentally to be hepatotoxic and hepato carcinogenic in rats [6]. Epidemiological data also indicate a possible correlation between the consumption of fumonisin/*F. verticillioides* contaminated corn and the high incidence of esophageal cancer in countries where corn is a dietary staple [9-11].

Although the prevention of mycotoxin contamination of grain is the main goal of food and agricultural industries throughout the world, under certain environmental conditions the contamination

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of various cereal grains with *Fusarium* fungi and mycotoxins are unavoidable for grain producers. Several strategies have been applied to eliminate mold growth and mycotoxins production or to remove/destroy the preformed toxins from grains and foodstuffs [12,13]. The ideal decontamination procedure should be easy to use, inexpensive and should not lead to the formation of compounds that are still toxic or can alter the nutritional and palatability properties of the food. Chemical antifungal agents have long been used to control fungi in foods [12-15]. In some situations, the prolonged use or overuse of these chemicals has led to the development of fungal resistance. Also, the levels of chemicals used in the food industry are fungistatic and do not kill or completely inhibit growth of an indefinite time [15]. However, increasing consumer concerns about chemicals in foods has led to interest in novel presentation methods. It has been reported that antagonistic microorganisms or their antimicrobial metabolites have some potential as natural bio preservatives to control undesirable fungi. Mold growth and mycotoxins production are inhibited by antifungal metabolites produced by lactic acid bacteria [16-18] and some *Bacillus sp.* [19-21]. Recently, Nanis [22] and Chelkowski [1] demonstrated that some lactic acid bacteria and yeast species have promising ability as natural food-grade bio control agents of mold growth and mycotoxin production.

The present study was aimed to investigate the ability of both *Lactobacillus rhamnosus* and *L. paracasie* subsp. Paracasie to: (a) inhibits growth of three toxigenic *Fusarium* species i.e. *F. graminearum* R6574, *F. proliferatum* M 5991 and *F. culmorum* R 5321 and (b) prevents the formation of the mycotoxins deoxynivalenol, zearalenone and fumonisin B1 by the over mentioned *Fusarium* species.

Materials and Methods

Microbial cultures

The lactic acid bacteria used in this study were *Lactobacillus rhamnosus* and *L. paracasie* subsp. paracasie obtained from the Culture Collection of the Department of Food Science and Technology at Nebraska University, Nebraska-Lincoln, USA.

Fungal cultures of *F. graminearum* R6574, *F. proliferatum* M 5991 and *F. culmorum* R 5321 were obtained from the *Fusarium* research center at Pennsylvania State University, University Park, PA., US.

Inoculum preparation

Frozen stock cultures of Lactic acid bacteria were prepared by thawing under optimum temperature, activated in litmus milk. Then, cultured in de Man Rogosa Sharpe (MRS) broth and incubated at 37°C for 24 h before use. Spore suspensions of molds, grown on carnation leaf agar slants for 14 days prior to the experiment, were prepared by washing the slants with 10 ml of sterilized solution of tween 80 (0.5% V/V), then the spores were loosened by gentle brushing with sterile spatula. Mycelium debris was removed by filtration through sterile cheese cloth. Spores count in the resulting suspension was determined by Petroff-Houser counting chamber [23]. Spores count was finally adjusted to 10^7 spores of each fungus per ml and stored at 4°C until use.

Antifungal activity assay

This assay was a modification of the overlay technique described by [24]. Three 5 µl drops from an active culture of each bacterial strains tested were spotted into agar plates and incubated until well- grown colonies could be observed (Ca. 24–48 h).The plates then were overlaid with 10 ml of Glucose Yeast Extract Agar (GYA) medium, on which 0.1 ml of a mold spore suspension (10^7 spores per ml) was finally spread out. After incubation for up to 5 days, the plates were examined for a halo zone formation around the bacterial colonies. These experiments were performed in triplicate.

Inhibition of mycotoxin production

The ability of *Lactobacillus rhamnosus* and *Lactobacillus paracasie* sub sp. paracasie strains to inhibit production of deoxynevalenol, fumonisin B1 and zearalenone by the investigated fungi was examined by the simultaneous antagonism assay as described by [21]. Hundred ml portions of glucose yeast extract broth medium were sterilized at 121°C for 15 min in 250 ml Erlenmeyer flasks. Each flask was inoculated with 1 ml of fungal spores' suspension containing 10^7 spores/ml, and 1 ml containing 10^7 Cells of bacterial culture grown at 30°C for 24 h in MRS medium without acetate. All flasks were incubated at 28°C and analyzed for toxin production after 10, 15 and 20 days of incubation. Mycelium growth inside flasks was visually determined as indication of growth rate or growth inhibition.

Determination of deoxynivalenol

At the end of the incubation period, the deoxynivalenol content was extracted by adding 50 ml of MeOH: H_2O (84:16) in Erlenmeyer flasks and shaken for 1.5 h at room temperature (25°C). Extracts (10 ml) were filtered and 6 ml of filtered extract were cleaned up using Mycosep columns (Coring System Diagnostix GmbH, Gernsheim/Rhein, Germany). Two ml of cleaned extract were transferred to round bottom flasks and evaporated to dryness). The dried extracts were re-dissolved in 500 µl aqueous 12.5% MeOH, transferred to 0.8 ml glass vials and stored at -70°C until analysis [15]. Deoxynivalenol contents of the extracts were determined by comparison with standards (Supelco, Bellefonte, PA, USA) using a High Performance Liquid Chromatography (HPLC) as described by Stiles [28] and Bullerman [15]. The system consisted of a model 510 HPLC pump and a U6K loop injector (Waters Associates, Milford, MA), a high speed reverse phase column (C18, 4.6 mm×33 mm, 3 µm particle size, perkin-Elmer Corp., Norwalk, CT), and a model 474 scanning fluorescence detector (Waters, Milford, MA). All chromatographic analysis were monitored with computer millennium software (version 2.15, Waters Associates, Milford, MA), connected to the HPLC instruments. Fumonisin B1 was quantified by correlating peak area of the extract to that of reference standard. The entire experiment was repeated three times in duplicate.

Fumonisin B1 and zearalenone assay

The contents of each flask were extracted with 50 ml of acetonitrile: water (50:50, V/V) for 60 min using a wrist–action shaker (Burrell Corp., Pittsburgh, PA). The extracts were filtered through Whatman No.1 filter paper and then 2 ml extracts were purified with a solid phase extraction column (C18, Sep-Pak cartridge, Waters Association, Milford, MA). Before loading the sample, the SPE column was connected to a 10 ml syringe barrel and conditioned with 2 ml of acetonitrile, and 2 ml of 1% KCI solution. The 2 ml of filtered extract was pipetted into the barrel of the syringe and diluted by adding 6 ml of 1% KCI. Diluted sample was then loaded onto the column at a flow rate of 1 ml/min, facilitated by a vacuum manifold (Visiprep, Supelco, Inc., Bellefonte, PA). The column was washed with 2 ml of 1% KCI followed by 2 ml of acetonitrile: water (15: 85, V/V), followed by air forced through the column. Fumonisin B1 was eluted with 2 ml of acetonitrile: water (70: 30, V/V). Fumonisin B1 and zearalenone levels were determined with competitive direct Enzyme-Linked Immunosorbent Assay (ELISA) test kits (Veratox, Neogen Corp., Lansing, Mich.) in accordance with the kit instructions [25,26].

Results and Discussion

The antifungal activities of both *L. paracasie* sub sp. paracasie and *Lactobacillus rhamnosus* against each of *F. graminearum* R6574, *F. proliferatum* M 5991 and *F. culmorum* R 5321 are shown in Tables 1-3. Control treatments in all experiments revealed that all the studied molds were extensively grown and produced high levels of their mycotoxins when cultivated individually in the absence of the antagonistic bacteria. Concentrations of all mycotoxins markedly increased with increasing the incubation period from10 to 20 days. Data in Table 1 showed that, when fungus *F. graminearum* was grown simultaneously with *L. paracasie* sub sp. Paracasie, mycelium growth was slightly affected butdeoxynevalenol production was reduced by about 45.2% and 56.8% after 10 and 20 days of incubation, respectively. At the same time, cultivation of *F. graminearum* in the presence of *L. rhamnosus* resulted in complete inhibition of bothmycelium growth and deoxynevalenol production.

Data presented in Tables 2 and 3 indicated that *L. paracasie* sub sp. paracasie partially inhibited production of zearalenone and fumonisin B1 by *F. culmorum* and *F. proliferatum* although mycelium growth of both fungi was not affected. Percentages of inhibition of, zearalenone production were 57.4 and 73.0% of that produced in the control treatment after incubation periods of 10 and 20 days, respectively (Table 2). Meanwhile, fumonisin B1 production was inhibited by about 61.5% and 76.5% after 10 and 20 days of incubation, respectively (Table 3). On the other hand, *Lactobacillus rhamnosus* showed the highest antifungal as well as, antimycotoxigenic effects, it completely suppressed growth of *F. culmorum* and *F. proliferatum*, and hence no zearalenone or fumonisin B1 was formed (Tables 2 and 3).

Results of the present study are in agreement with that recorded by other investigators [16,22,27-30] who described the antifungal properties of different lactic acid bacteria against fungal contamination of dairy and food products. El-Nezami and Ahokas, Peltonen et al. and Haskard et al. [18,32,33] reported that *L. rhamnosus* strains removed

Bacterial strain	Incubation period (days)	Mold growth (visual)	DON conc. (ppm)	DON inhibition (%)
Control	10	+++	69.2	-
	20	+++	481.6	-
L. paracasie subsp. *paracasie*	10	++	37.9	45.2
	20	++	208.1	56.8
L. rhamnosus	10	-	ND	100
	20	-	ND	100

Legend: (-) no mold growth, (+) weak growth, (++) moderate growth, (+++) strong growth, (ND) not detected.

Table 1: Effect of *L. paracasie* subsp. *Paracasie* and *Lactobacillus rhamnosus* on growth of *F. graminearum* R6574 and Deoxynivalenol production.

Bacterial strain	Incubation period (days)	Mold growth (visual)	Zearalenone conc. (ppm)	Zearalenone inhibition (%)
Control	10	+++	3300	-
	20	+++	4900	-
L. paracasie subsp. *paracasie*	10	+++	1405	57.4
	20	+++	1322	73
L. rhamnosus	10	-	ND	100
	20	-	ND	100

Legend: (-) no mold growth, (+) weak growth, (++) moderate growth, (+++) strong growth, (ND) not detected

Table 2: Effect of *L. paracasie* subsp. *Paracasie* and *Lactobacillus rhamnosus* on growth of *F. culmorum* R 5321and Zearalenone production.

Bacterial strain	Incubation period (days)	Mold growth (visual)	Fumonisin B1 conc. (ppm)	Fumonisin B1 inhibition (%)
Control	10	++	200	-
	20	+++	300	-
L. paracasie subsp. *paracasie*	10	++	77	61.5
	20	+++	70.5	76.5
L. rhamnosus	10	-	ND	100
	20	-	ND	100

Legend: (-) no mold growth, (+) weak growth, (++) moderate growth, (+++) strong growth, (ND) not detected.

Table 3: Effect of *L. paracasie* subsp. *Paracasie* and *Lactobacillus rhamnosus* on growth of *F. proliferatum* M 5991 and Fumonisin B_1 production.

54.6 and 80.0% of aflatoxin B1, while *L. lactis* and *L. casei* removed 59.0 and 21.8% of aflatoxin B1, respectively. El-Nezami et al. [34] demonstrated that strains of *L. rhamnosus* have the ability to remove 55.0% of zearalenone and its derivative α-zearalenol with a rapid reaction instantly after mixing with the bacteria.

Our results clearly showed that *L. rhamnosus* is completely suppressed mycelial growth of all the studied molds, consequently no toxin was formed in the presence of this bacterial strain. On the other hand, *L. paracasie* subsp. paracasie mostly did not affect mold growth but reduced mycotoxin concentrations by about 56.8, 73.0 and 76.5% for Deoxynivalenol, Zearalenone and Fumonisin B1, respectively. This reduction may be explained on the basis of some degradation or removal of the preformed toxins was occurred by the bacterial cells. This postulation is in accordance with that demonstrated by [35]. They found that some lactic acid bacteria have potential to bind aflatoxins, and this binding is a physical phenomenon that is associated with the bacterial cell wall. Morotomi and Mutai [36] suggested that the binding of mutagens to lactic acid bacteria cell wall may occur through cationic binding. Also, Tanabe et al. [37] suggested that such binding may take place through the cell wall peptidoglycan. Furthermore Kollarczik et al. [38] found that zearalenone and deoxynevalenol were degraded in vitro by the normal bacterial gut flora from the distal section of the gastrointestinal tract of pigs. Deoxynevalenol was deep oxidated and zearalenone was hydrolyzed to α-zearalenol and an unknown metabolite.

In conclusion, the use of antifungal lactic acid bacteria instead of chemical preservatives would enable to produce organic foods without addition of chemical substances. In addition to the already known excellent properties of lactic acid bacteria they could enhance the nutritional value and prolong conservation of foods and dairy products.

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