Polish Journal of Microbiology
2008, Vol. 57, No 3, 205–212

ORIGINAL PAPER

Effects of *Propionibacterium* on the Growth and Mycotoxin Production by some Species of *Fusarium* and *Alternaria*

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Received 7 September 2007, revised 12 December 2007, accepted 5 May 2008

Abstract

The aim of this research was to study the antifungal properties of propionibacteria. Three fractions from cultures of *Propionibacterium freudenreichii* ssp. *shermanii* 41 and ssp. *freudenreichii* 111 (*i.e*. culture containing viable bacteria, cell-free supernatant and bacteriocin preparation) were tested for their ability to inhibit the growth and mycotoxin production of *Alternaria alternata, Fusarium culmorum*, *Fusarium graminearum* and *Fusarium verticillioides*. The growth of the fungi was monitored during cultivation using a plating method. The concentration of toxins produced was measured by HPLC on the 14th day of culture. Altenuene and tenuazonic acid were determined in cultures of *A. alternata* whilst concentration of nivalenol, deoxynivalenol, fumonisin B_1 and zearalenone was measured in *Fusarium* cultures. The strongest inhibition of growth and toxin production was observed in the presence of cultures containing viable cells and supernatants obtained from propionibacteria cultures. The bacteriocin extracts generally had a weak fungistatic effect on the growth of *A. alternata*, *F. culmorum* and *F. graminearum*. Despite the fact that growth was slower in the presence of bacteriocin extracts than in control trials, none of the preparations prepared from the propionibacteria significantly reduced the level of mycotoxin production. The ability of *P. freudenreichii* ssp. *freudenreichii* 111 to remove zearalenone from liquid medium was also evaluated. It was shown that both viable and non-viable cells caused a decrease in zearalenone concentration in the medium.

Key words: adsorption, fungi, inhibition, mycotoxin, propionibacteria

Introduction

Fungi represent a diverse group of eukaryotic organisms responsible for the majority of plant diseases and also certain infectious human diseases. Fungi are also significant spoilage microorganisms occurring in food; additionally they may be harmful to human due to the production of mycotoxins. These metabolites may have mutagenic, teratogenic or carcinogenic effects on humans as well as on animals. Mycotoxins may occur in different agricultural products, entering the food chain during cultivation, harvest, transportation and storage. Kuiper-Goodman *et al.* (1996) have stated that mycotoxins are the most important chronic dietary risk factor. It is estimated that worldwide about 5–10% of total food production may be spoiled by the above-mentioned organisms (Pitt and Hocking, 1999).

The genera *Fusarium* and *Alternaria* are commonly occurring in nature, and are also frequently found in various commodities, especially those of agricultural origin. Many species of *Fusarium* produce mycotoxins such as trichothecenes, fumonisins and zearalenone. The first group, *i.e*. trichothecenes includes nivalenol, deoxynivalenol, fusarenon-x, T-2 toxin, neosolaniol and diacetoxyscirpenol, which are commonly found in cereals, especially in countries with a moderate climate (Côté *et al.*, 1986). The fumonisins are the second group of related mycotoxins produced mainly by *Fusarium verticillioides*. They are often found in the corn, which is used mainly for animal feed and also in some corn-based foods intended for human consumption (Marasas, 2001; Kuiper-Goodman *et al.*, 1996). The most frequently detected, but not the most toxic mycotoxins produced by *Fusarium*, are fumonisins

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and deoxynivalenol. The last of the above-mentioned *Fusarium* toxins is zearalenone which is a nonsteroidal estrogenic mycotoxin produced by different species of *Fusarium*, such as *F. culmorum* or *F. graminearum*, which are regular contaminants of cereal crops worldwide (Haggler *et al.*, 2001). The genus *Alternaria* may be responsible for spoilage of commercially important commodities such as fruit and vegetables (Stinson *et al.*, 1980). *Alternaria* species produce numerous toxic secondary metabolites, such as tenuazonic acid, alternariol, altenuene, alternariol methyl ether, and altertoxin I, II and III (King and Schade, 1984). *Alternaria* toxins exhibit toxic effects on cultures of bacterial, plant, animal and also human cells (Stack and Prival, 1986; Yekeler *et al.,* 2001).

Bacteria can protect food from fungal spoilage by competitive growth or production of antimicrobial metabolites. Some reports indicate that different bacteria, such as some probiotic species of *Lactobacillus* (El-Nezami *et al*., 2002a, b) or yeast (Böswald *et al*., 1995) may cause degradation or removal of mycotoxins from the growth medium. Some authors have reported the possibility of degradation or biotransformation of mycotoxins by microorganisms. El-Sharkawy *et al*. (1991) described the conversion of zearalenone by few fungal species selected from group of 23 microorganisms belonging to 7 genera. Böswald *et al*. (1995) observed the transformation of zearalenone to α- and β-zearalenol by the yeasts *Candida tropicalis*, *Torulaspora delbrueckii*, *Zygosaccharomyces rouxii* and some strains of *Saccharomyces*. El-Nezami *et al*. (2002a) described a significant reduction in the concentration of zearalenone in liquid medium by *Lactobacillus rhamnosus*. The authors suggested that zearalenone was bound to the surface of cells because the removal of toxin was observed by both viable and nonviable cells. The same authors described the ability of *L. rhamnosus* GG and LC705 and *Propionibacterium shermanii* JS, which have been characterized as probiotics, to bind trichothecenes and aflatoxins (El-Nezami *et al*., 1998, 2000, 2002b; Peltonen *et al.*, 2000).

The aim of the work presented here is the evaluation of the antifungal activity of *P. freudenreichii* ssp. *shermanii* 41 and ssp. *freudenreichii* 111 against *A. alternata* and three species of *Fusarium* (*F. culmorum*, *F. graminearum* and *F. verticillioides*). In the majority of articles the antifungal activity of propionibacteria was investigated by the plating method, *i.e.* firstly pre-growth of bacteria on agar, then overlaid with fungi in soft agar (Lind *et al*., 2005; Miescher *et al*., 2000). In the present paper we have examined the effect of different fractions obtained from propionibacteria cultures on both the growth of the fungi in liquid medium and on mycotoxin production.

Because some authors have shown that propionic acid bacteria are able to remove mycotoxin from medium (El-Nezami *et al*., 2000, 2002b), we also investigated the ability of one strain of *P. freudenreichii* to remove zearalenone from PBS (phosphate buffered saline).

Experimental

Materials and Methods

Microorganisms and media. *Propionibacterium freudenreichii* ssp. *shermanii* 41 was obtained from the Agricultural University of Poznań (Poland) collection and *P. freudenreichii* ssp. *freudenreichii* 111 was obtained from the University of Warmia and Mazury (Olsztyn, Poland). Accessions of fungi: *Alternaria alternata* 684, *Fusarium culmorum* F724, *Fusarium graminearum* F722 and *Fusarium verticillioides* F728 were obtained from the Collection of Pathogen Microorganisms (BPR) Institute of Plant Protection in Poznań, Poland. Casein medium was used for bacterial cultivation. The composition of this medium has been described previously (Gwiazdowska and Trojanowska, 2006). Potato Dextrose Broth (PDB; BTL, Łódź, Poland) was used for fungal cultures.

Preparation of different fractions of propionibacteria cultures. The test strains of propionibacteria (10% v/v inoculum) were grown in casein medium in Erlenmeyer flasks at 30°C for 10 days. The pH of these cultures was adjusted to 7.0 ± 0.1 every day using 25% ammonia. Three fractions were then prepared from each culture. One fraction consisted of a sample of the culture. The cell-free supernatant fraction was obtained by centrifugation at $3800 \times g$ for 15 min and filtration through a 0.45 μm pore size low protein-binding membrane (Durapore Low Protein Binding, Millipore Bedford MA, USA). The bacteriocin extract was prepared as described earlier (Gwiazdowska and Trojanowska, 2006).

Cultivation of fungi with different fractions from propionibacteria cultures. Cultivation of fungi with propionibacteria cultures, supernatants and bacteriocin extracts was carried out according to the method described by Czaczyk *et al*. (2002) with some modifications. Fungal cultures were cultivated on agar slants at 24°C for 7–10 days. Well developed cultures were rinsed with 10 ml of sterile water and suspensions containing 10^4 CFU/ml were prepared. Of the liquid medium (PDB; Potato Dextrose Broth) 60 ml was mixed with 30 ml of an appropriate fraction from the propionibacteria culture and then 10 ml of fungal suspension was added. Fungal cultures with the addition of sterile casein medium were used as controls. All flasks were incubated at 24°C, with shaking (100 rpm) for 14 days. At the beginning and after 7 and 14 day of growth, the growth of the fungi was determined as

CFU/ml by the dilution plating method using PDA (Potato Dextrose Agar; BTL, Łódź, Poland).

Sample preparation for HPLC analysis of mycotoxins. The samples were prepared on the 14th day of culture according to the method described earlier by Perkowski (1993). A total of 50 ml of culture was homogenized and blended with 100 ml acetonitrile. After 24 h the mixture was filtered and to 25 ml of filtrate 25 ml of hexane was added, the mixture was shaken and the hexane removed after 5 minutes of extraction. The residue was evaporated, dissolved in 5 ml of methanol and purified on a HPLC column. The column was packed with 7.5 g anhydrous sodium sulfate, 12 g Florisil (Sigma-Aldrich, St.Louis, USA) suspended in hexane and followed 10 g anhydrous sodium sulfate. The methanol extract was applied to the column and washed twice with 25 ml of hexane. The mycotoxins were eluted with 150 ml of a mixture of chloroform and methanol (9:1 v/v). The eluate was evaporated to dryness and dissolved in 3 ml of the eluent used for HPLC analysis.

Adsorption assays. The experiment was based on the method described by El-Nezami *et al.* (2002b) with some modifications. Propionibacteria were cultivated in casein medium for 72 hours at 30°C. The culture was then centrifuged (7000×g for 15 min) and the biomass was suspended in PBS (phosphate buffered saline) pH 7.0 until a bacterial density of 10^{11} CFU/ml was achieved. The suspension was divided into 2-ml fractions and the methanolic solution of zearalenone was added to achieve a final concentration of 10 and 20 µg/ml. The samples were incubated at 4, 24 and 30°C for 1, 5 and 24 h. An identical experiment was carried out with a heat-inactivated biomass, which had been prepared by boiling a sample of the culture for 20 minutes at 100°C to kill the cells. The positive control in both cases was the biomass suspended in PBS and the negative control consisted of PBS containing an appropriate concentration of toxin. After the incubation, the samples were centrifuged (7000×g for 5 min), the supernatants were filtered through Millex GS 0.22 µm membranes (Millipore Bedford MA, USA) and finally the concentration of mycotoxins was determined by HPLC.

HPLC analysis of organic acids. The concentration of propionic and acetic acids was determined by liquid chromatography (HPLC) using a Merck-Hitachi (Merck, Darmstadt, Germany) chromatographic system. HPLC analysis was carried out on an Animex HPX-87H 300×7.8 mm column (Bio-Rad, Philadelphia USA). The column was eluted with 0.005 M H_2SO_4 at a flow rate 0.8 ml/min and a temperature of 30°C. The samples were filtered through Millex-GP 0.22 µm membranes (Millipore Bedford MA USA). The concentrations of the standard solutions were 4 g/l (propionic acid) and 2 g/l (acetic acid). The levels of acid were quantified by computer integration of the peak areas.

HPLC analysis of mycotoxins. Determination of mycotoxins was carried out using a Merck-Hitachi system (Merck, Darmstadt, Germany) consisting of an autosampler (L-7250), pump (L-7100), fluorescence detector (L-7480) or diode array detector – (DAD; L-7455) and one of three different columns: Lichrospher RP-18, 250×4.6 mm (Merck, Darmstadt), ODS Hypersil, 5 µm, 200×4.6 mm (Hewlett Packard) or Adsorbosphere C18, 150×4.6 mm, 5 µm (Alltech, Lexington KY USA). Nivalenol, deoxynivalenol and zearalenone were determined using the Lichrospher RP-18 column and the DAD detector at 222 nm (NIV and DON) and 230 nm (ZON). The same column was also used to separate fumonisin B_1 but with a fluorescence detector operating at 460 nm (excitation wavelength) and 500 nm (emission wavelength). HPLC analysis of altenuene was carried out using an ODS Hypersil column and a DAD detector to record the absorption at 240 nm whereas tenuazonic acid was determined using an Adsorbosphere C18 column and an absorption wavelength of 280 nm. The mobile phase for the separation of fumonisin B_1 was methanol:0.1 M sodium dihydrogen phosphate (80:20 v/v), adjusted to pH 3.4 with *o*-phosphoric acid. For the analysis of all other mycotoxins the mobile phases consisted of mixtures of methanol-water of variable ratios. Analysis was performed isocratically at a flow rate of 1 ml/min and at a temperature of 25°C (NIV, DON, ZON) or 30°C (ALT, TA) (Jiménez and Mateo, 1997; Muller and Lepom, 1992; Motta and Soares, 2000). Standard calibration solutions for all mycotoxins (Sigma-Aldrich, St.Louis USA) were prepared within the range 0.1–0.01 mg/ml. The detection limits for the pure standards taken as 3 times the baseline standard deviation, were 30 ng/ml for altenuene, 50 ng/ml for tenuazonic acid, 20 ng/ml for deoxynivalenol and nivalenol and 15 ng/ml for zearalenone, and 10 ng/ml for fumonisin B_1. Analytical recovery studies were carried out by adding a known amount of toxin (0.1 mg/ml) to noncontaminated samples. Mean recovery rates were determined based on 10th replicate analyses. The average recoveries for the mycotoxins under study: altenuene, tenuazonic acid, deoxynivalenol, nivalenol, zearalenone and fumonisin B_1 were 90, 88, 89, 85, 78 and 86%, respectively.

Protein determination. The method of Bradford (1976) was used to determine the protein concentration.

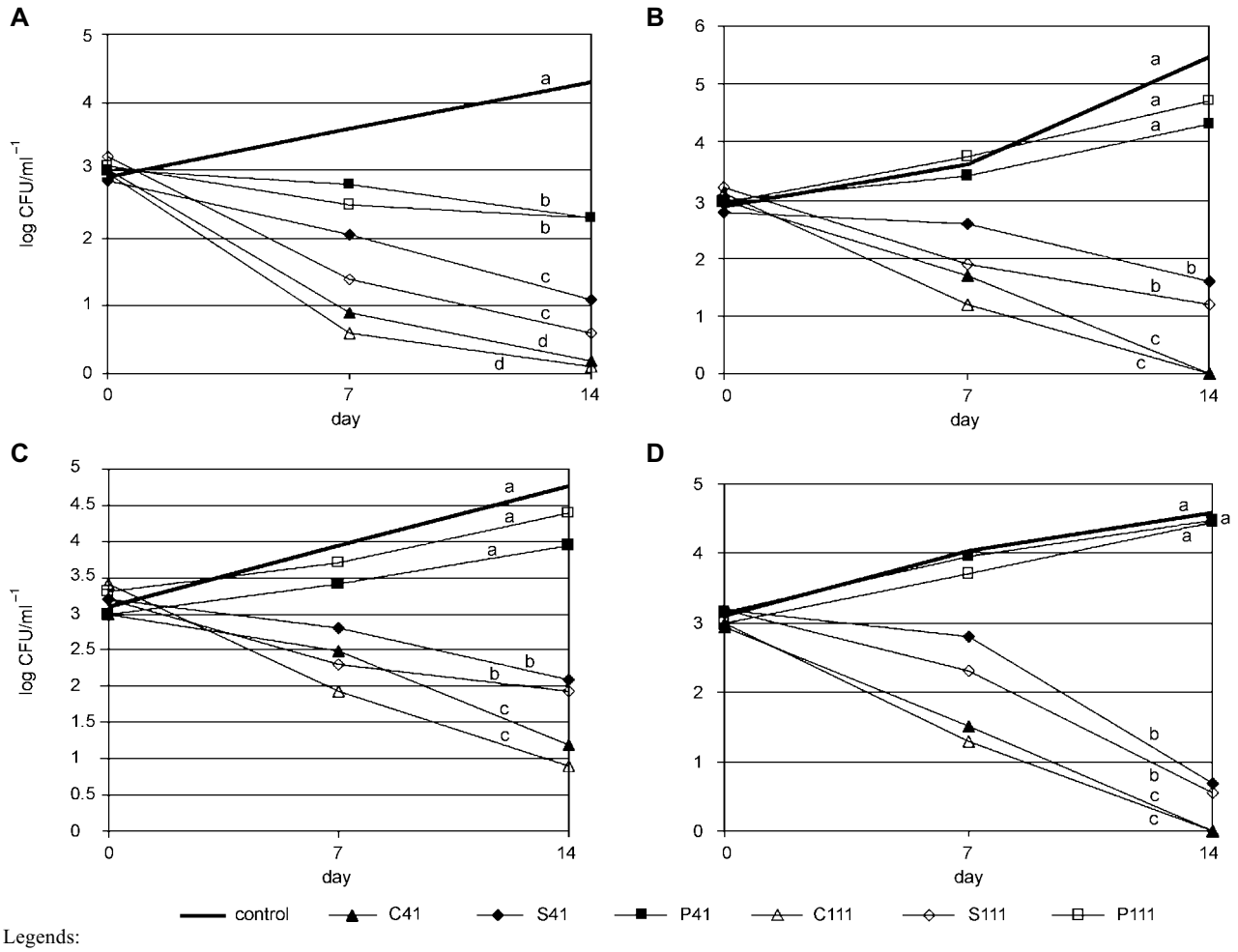
Statistical analysis. The data presented are the mean (± standard deviation) of three replicate trials. The results of growth inhibition and mycotoxin production were subjected to Student's t-test to determine the significance of the differences ($p<0.05$) between the controls and the experimental samples to which various fractions of the propionibacteria cultures had

been added. The influence of different factors on the adsorption of toxins was determined using ANOVA at a 95% confidence level. Analyses were carried out using STATISTICA for Windows version 5.1.

Results and Discussion

The research presented here mainly concerns the inhibition of fungal growth and mycotoxin production in liquid medium by two strains of *Propionibacterium*. Three fractions: culture, supernatant and bacteriocin preparation were added to fungal cultures. During culture (0, 7 and 14 days) the growth of the fungi was evaluated (CFU/ml) and the results are presented in Figure 1. The strongest inhibition of fungal growth was achieved by the addition of cultures of propionibacteria. After 14 days, the growth of *F. culmorum* and *F. verticillioides* was completely inhibited and that of *A. alternaria* almost completely inhibited, and the CFU/ml of *F. graminearum* was significantly reduced. The supernatants from the propionibacteria cultures were also shown to have a strong inhibitory effect on the growth of all the fungi under study. The supernatant from the *P. freudenreichii* ssp. *freudenreichii* 111 culture was often slightly more effective at inhibiting growth of fungi than that from the *P. freudenreichii* ssp. *shermanii* 41 culture (Fig. 1). The bacteriocin preparations were only weakly fungistatic against *A. alternata* (Fig. 1A). In the presence of bacteriocin preparations the growth of *F. culmorum* and *F. graminearum* was slower than that under control conditions. In turn, *F. verticillioides* was insensitive to this particular fraction. No differences were observed in the effects of the same fractions from *P. freudenreichii* ssp. *shermanii* 41 and *P. freudenreichii* ssp. *freudenreichii* 111 cultures (Fig. 1A-D).

The concentration of some mycotoxins was measured after 14 days of fungal growth. Altenuene (ALT) and tenuazonic acid (TA) were determined in cultures



Legends:
C41 – culture of *P. freudenreichii* ssp. *shermanii* 41
C111 – culture of *P. freudenreichii* ssp. *freudenreichii* 111
S41 – supernatant from *P. freudenreichii* ssp. *shermanii* 41 culture
S111 – supernatant from *P. freudenreichii* ssp. *freudenreichii* 111 culture
P41 – bacteriocin preparation from *P. freudenreichii* ssp. *shermanii* 41 culture
P111 – bacteriocin preparation from *P. freudenreichii* ssp. *freudenreichii* 111 culture
a, b, c – significant differences at $p<0.05$

Fig. 1. The growth of fungi in the presence of different fractions of propionibacteria cultures: A) *Alternaria alternata*, B) *Fusarium culmorum*, C) *Fusarium graminearum* D) *Fusarium verticillioides*

Table I
The effect of culture fractions of *Propionibacterium freudenreichii* ssp. *shermanii* 41 and *freudenreichii* 111 on the production of mycotoxins by *Alternaria alternata* determined on the 14th day of culture

Fraction of propionibacteria culture	Mycotoxins concentration (μg/ml)	
	Altenuene	Tenuazonic acid
Control	0.775 ± 0.16[a]	0.242 ± 0.07[a]
Culture 41	0.000 ± 0.00[b]	0.000 ± 0.00[a]
Supernatant 41	0.057 ± 0.05[b]	0.056 ± 0.04[a]
Bacteriocin preparation 41	0.671 ± 0.10[a]	0.129 ± 0.18[a]
Culture 111	0.000 ± 0.00[b]	0.000 ± 0.00[a]
Supernatant 111	0.073 ± 0.07[b]	0.072 ± 0.04[a]
Bacteriocin preparation 111	0.701 ± 0.08[a]	0.179 ± 0.13[a]

a, b – Significantly different at $p<0.05$

of *A. alternata* (Table I), while nivalenol (NIV), deoxynivalenol (DON) and zearalenone (ZEN) were determined in cultures of *F. culmorum* and *F. graminearum* and the concentration of fumonisin B_1 was measured in the case of *F. verticillioides* (Table II). The propionibacteria cultures and supernatants caused a decrease in the concentrations of all the mycotoxins examined. In cultures of *A. alternata* ALT and TA were not detected or were significantly reduced in the presence of propionibacteria cultures and supernatants, respectively. As far as *F. culmorum* is concerned, a similar situation was observed. All the toxins examined were either undetectable or were present at very low levels in samples treated with propionibacteria cultures or supernatants. In the case of *F. graminearum* ZEN could not be detected in either the control trial or in samples treated with propionibacteria cultures or supernatants. The concentration of the remaining toxins, NIV and DON, was only significantly reduced in the presence of *Propionibacterium* cultures. Fumonisin B_1 was significantly reduced when cultures and supernatants of propionibacteria were added to *F. verticillioides* cultures. In the presence of bacteriocin preparations the level of all the mycotoxins examined was a little lower than that in the control trial but the differences were not significant.

The results revealed that extracellular metabolites of propionibacteria can effectively inhibit fungal

Table II
The effect of culture fractions of *Propionibacterium freudenreichii* ssp. *shermanii* 41 and ssp. *freudenreichii* 111 on the production of mycotoxins by *Fusarium* species determined on the 14th day of culture

Fraction of propionibacteria culture	Mycotoxins concentration (μg/ml)			
	Fumonisin	Nivalenol	Deoxynivalenol	Zearalenone
Fusarium culmorum				
Control	nd	5.084 ± 0.20[a]	1.085 ± 0.28[a]	32.313 ± 6.36[a]
Culture 41	nd	0.000 ± 0.00[b]	0.000 ± 0.00[b]	0.487 ± 0.08[b]
Supernatant 41	nd	0.000 ± 0.00[b]	0.000 ± 0.00[b]	0.000 ± 0.00[b]
Bacteriocin preparation 41	nd	4.939 ± 0.31[a]	0.881 ± 0.09[a]	25.99 ± 3.21[a]
Culture 111	nd	0.000 ± 0.00[b]	0.000 ± 0.00[b]	0.000 ± 0.00[b]
Supernatant 111	nd	0.000 ± 0.00[b]	0.000 ± 0.00[b]	0.000 ± 0.00[b]
Bacteriocin preparation 111	nd	5.001 ± 0,21[a]	1.012 ± 0.07[a]	30.89 ± 0.19[a]
Fusarium graminearum				
Control	nd	40.27 ± 0.54[a]	5.583 ± 0.67[a]	0.000 ± 0.0
Culture 41	nd	32.13 ± 3.49[b]	3.73 ± 0.72[a]	0.000 ± 0.0
Supernatant 41	nd	39.74 ± 4.52[a]	4.374 ± 0.91[a]	0.000 ± 0.0
Bacteriocin preparation 41	nd	40.12 ± 2.45[a]	5.22 ± 1.02[a]	0.000 ± 0.0
Culture 111	nd	0.000 ± 0.00[b]	0.000 ± 0.0[b]	0.000 ± 0.0
Supernatant 111	nd	26.32 ± 1.30[a]	3.490 ± 0.46[a]	0.000 ± 0.0
Bacteriocin preparation 111	nd	40.583 ± 0.98[a]	5.018 ± 0.65[a]	0.000 ± 0.0
Fusarium verticillioides				
Control	25.11 ± 0.59[a]	nd	nd	nd
Culture 41	5.618 ± 0.35[b]	nd	nd	nd
Supernatant 41	5.574 ± 0.93[b]	nd	nd	nd
Bacteriocin preparation 41	24.52 ± 2.50[a]	nd	nd	nd
Culture 111	2.512 ± 0.02[c]	nd	nd	nd
Supernatant 111	5.422 ± 0.01[b]	nd	nd	nd
Bacteriocin preparation 111	25.13 ± 3.12[a]	nd	nd	nd

a, b, c – Significantly different at $p<0.05$; nd – not determined

growth and mycotoxin production in liquid medium. Numerous investigations have reported the antimycotic and antimycotoxigenic activity of lactic acid bacteria (Gourama and Bullerman, 1995; Karunaratne *et al*., 1990). Kimura and Hirano (1988) reported that *Bacillus subtilis* isolated from soil inhibited both growth and aflatoxin production by *Aspergillus parasiticus* NRRL 2999 in media as well as in peanuts and corn. Osman (2004) observed the inhibition of *Aspergillus flavus* growth, sporulation and aflatoxin production by *Brevibacterium linens*.

The strongest effect of propionibacteria cultures containing viable cells was probably caused by the production of antimicrobial metabolites after adding that fractions to fungal cultures. It is also possible that some compounds were lost during the preparation of cell-free supernatants. In support of this contention, Osman (2004) observed a decrease in antifungal activity in the cell-free supernatant of *Brevibacterium linens* compared to the whole cell culture and affirmed that centrifugation and filtration might be the reason for this reduction in activity. But, as the author suggested, a possible alternative cause for the decline in activity may also be the volatility of antifungal compounds.

It is probable that the main products of fermentation, propionic and acetic acids, play the most important role in the inhibition of fungal growth. As shown in Table III, *P. freudenreichii* ssp. *freudenreichii* 111 produced more propionic and acetic acids than *P. freudenreichii* ssp. *shermanii* 41 which parallels the greater inhibitory effect of *P. freudenreichii* ssp. *freudenreichii* 111 on the growth of all the fungi examined. However, the differences between the antifungal properties of the two propionibacteria strains were not statistically significant. There are several reports in the literature which document the inhibitory effect of propionic acid on the growth of various fungi such as *Aspergillus*, *Fusarium* and *Penicillium* (Higgins and Brinkhaus, 1999; Paster *et al*., 1999). Lind *et al*. (2005) compared the antifungal effect of five species of propionibacteria and proved that propionic acid followed by acetic acid, was the most potent antifungal agent. However they also observed that inhibition of fungal growth can only be partly explained by the presence of organic acids, moreover, the synergistic effect between these two acids is crucial.

We have observed that the protein fractions obtained from cultures of the two strains of propionibacteria examined also posses a certain degree of antifungal activity (Fig. 1). Also our previous research has shown the inhibition of some fungi on solid medium by partially purified bacteriocins produced by *P. freudenreichii* ssp. *freudenreichii* 11 and 111 and *P. freudenreichii* ssp. *shermanii* 41 (Gwiazdowska and Trojanowska, 2006). Similarly, propionicin PLG-1 produced by *Propionibacterium thoenii* P126 showed antifungal activity against *Aspergillus ventii*, *Apiotrichum curvatum*, *Fusarium tricinctum*, *Phialophora gregata* and yeasts belonging to the genera *Candida*, *Saccharomyces* and *Scopularopsis* (Lyon and Glatz, 1991). Proteinaceous compounds with antifungal activity were found to be produced by lactic acid bacteria (Magnüsson and Schnürer, 2001; Ström *et al.*, 2002) and *Bacillus* species (Munimbazi and Bullerman, 1998). In recent years, several new antifungal compounds produced by lactic acid bacteria have been isolated, *e.g*. phenyllactic acid (Ström *et al*., 2002) and hydroxylated fatty acids (Sjögren *et al.*, 2003). Thus, it is very probable that compounds other than organic acids such as bacteriocins produced *via* secondary metabolic pathways, may influence the antifungal properties of propionic acid bacteria.

According to the literature data, bacteria can remove mycotoxins by binding them to the cell surface (El-Nezami *et al*., 2002a; 2002b). In our study we investigated whether any of the strains under study is able to adsorb zearalenone. *P. freudenreichii* ssp. *freudenreichii* 111 was chosen for this purpose as it possesses the strongest antifungal activity. The results are shown in Table IV. The data indicate that the concentration of zearalenone was significantly reduced by viable as well as by non-viable bacteria, so it is probable that this effect may be a result of surface binding. This is

Table III
Concentration of short chain fatty acids and protein in the various fractions

Strain	Fraction	Concentration of		
		Propionic acid (g/l)	Acetic acid (g/l)	Protein (mg/ml)
P. shermanii 41	Culture Supernatant	15.28	8.53	nd
	Bacteriocin extract	0.00	0.00	14.60
P. freudenreichii 111	Culture Supernatant	21.89	11.34	nd
	Bacteriocin extract	0.00	0.00	14.05

nd – not determined

Table IV
Removal of zearalenone by *P. freudenreichii* ssp. *freudenreichii* 111

Temperature	Time of incubation	Concentration (μg/ml) of remaining toxin			
		10 μg		20 μg	
		V	NV	V	NV
Control		10 ± 0.01		20 ± 0.01	
30°C	1 h	2.58 ± 0.44	6.41 ± 1.71	9.03 ± 1.75	5.01 ± 0.69
	5 h	6.48 ± 0.34	5.30 ± 0.01	17.9 ± 0.03	8.49 ± 0.45
	24 h	4.97 ± 0.54	4.77 ± 0.61	16.12 ± 0.30	7.44 ± 0.18
24°C	1 h	5.45 ± 0.08	8.96 ± 1.71	12.39 ± 4.11	4.07 ± 0.69
	5 h	5.51 ± 0.73	7.67 ± 1.25	16.49 ± 1.55	4.66 ± 1.20
	24 h	5.33 ± 0,19	5.76 ± 1.20	16.68 ± 0.62	7.44 ± 0.71
4°C	1 h	5.69 ± 1.60	6.01 ± 0.76	15.13 ± 0.76	7.64 ± 0.21
	5 h	6.68 ± 0.59	5.18 ± 0.21	17.65 ± 1.32	7.78 ± 0.08
	24 h	6.29 ± 0.01	5.18 ± 0.07	17.59 ± 0.19	8.24 ± 0.15

V – viable cells; NV – nonviable cells

consistent with reports indicating that toxins are adsorbed by bacteria rather than metabolized. Binding of aflatoxin B_1, aflatoxin M_1 and some *Fusarium* toxins by lactic acid bacteria and propionibacteria has also been reported in the literature (El-Nezami *et al*., 1998, 2002a, 2002b; Gratz *et al*., 2004).

We found in our study that the decline in the concentration of toxins was not dependent on temperature or incubation time. The greatest reduction ZEN concentration was observed during the first hour of incubation. After 5 hours a certain concentration of toxins was released, however, when the incubation was continued, the toxins were once again adsorbed. These results are similar to those of other reports which describe the ability of lactic acid bacteria to adsorb mycotoxins. El-Nezami *et al*. (2002a) showed that removal of zearalenone and its derivative α-zearalenol by *L. rhamnosus* GG and *L. rhamnosus* LC 705 was a rapid reaction. But when incubation was continued, the toxins were released into the media during the first 4 hours and then re-adsorbed by the bacteria. Similar results were reported with regard to the removal of aflatoxin B_1 by the same strains of lactic acid bacteria (El-Nezami, 1998).

In our investigations the efficiency of toxin reduction was dependent on the initial concentration of toxin. Viable bacteria reduced toxin levels more effectively at a lower concentration of ZEN (10 μg/ml). In the case of non-viable cells the process of reducing the concentration of ZEN was more effective when the initial concentration was high. Data reported in the literature also indicates that non-viable cells can adsorb a greater concentration of toxins than viable bacteria. El-Nezami *et al*. (2002b) observed a significant enhancement in the ability of two strains of lactic acid bacteria to remove zearalenone and α-zearalenol from liquid media after heat or acid treatment of the bacteria. The authors explained this observation by suggesting that perturbation of the cell wall would facilitate toxin binding to various constituents of both the cell wall and plasma membrane.

Summing up: we have found that propionibacteria can effectively inhibit the growth of certain fungal species belonging to the genera *Alternaria* and *Fusarium* and that they can also reduce the concentration of mycotoxins produced by these fungi. However, the inhibition of growth and toxin production was dependent on the fungal species and on which fraction of the propionic acid bacterial culture was used. As our results indicate, fungistatic activity is dependent on the presence and cooperation of various metabolites, including protein substances. No significant differences were observed in the fungistatic activity of the two strains of bacteria tested: *P. freudenreichii* ssp. *shermanii* 41 and *P. freudenreichii* ssp. *freudenreichii* 111. The results of our study show that *P. freudenreichii* ssp. *freudenreichii* 111 can reduce the concentration of zearalenone in solution and that adsorption is probably responsible for this process.

Acknowledgments

This work was supported by an interdisciplinary grant AE-AR 51103-514.

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