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Impact of *Pediococcus pentosaceus* strain L006 and its metabolites on fumonisin biosynthesis by *Fusarium verticillioides*

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ABSTRACT

The aim of this work was to study the effectiveness of a *Pediococcus pentosaceus* strain L006, isolated from maize leaf and previously characterised for its high antifungal efficiency, on fumonisin biosynthesis by *Fusarium verticillioides*. Studies performed in GYEP medium supplemented with amylopectin showed a significant increase in fumonisin production when the *F. verticillioides* strain was simultaneously co-inoculated with the *P. pentosaceus* strain or inoculated in a three-day-old culture of this lactic acid bacteria. Our studies also demonstrated that some extracellular metabolites produced in MRS medium by the *P. pentosaceus* strain L006 were able to significantly reduce fumonisin production in liquid medium as well as on maize kernels. Fumonisin yields by *F. verticillioides* inoculated on autoclaved maize kernels were reduced by a factor ranging from 75% to 80% after 20 days of incubation. Our results illustrate the potential risk linked to the use of an antagonistic bacterial agent to manage fumonisin contamination, while emphasizing the potential use of bacterial metabolites to counteract fumonisin accumulation in kernels.

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1. Introduction

Fumonisins, mainly produced by *Fusarium verticillioides* and *Fusarium proliferatum*, are common contaminants of maize and maize-based products worldwide (Shephard, Thiel, Stockenstrom, & Sydenham, 1996). Of the many structural forms of fumonisins, the most important ones found in naturally contaminated foods and feed are fumonisin B_1 (FB1) and B_2 (FB2) (Niderkorn, Morgavi, Aboab, Lemaire, & Boudra, 2009).

Considerable interest in fumonisins emerged after discovering their high degree of toxicity. Fumonisins have been shown to be directly responsible for several animal diseases, including equine leukoencephalomacia and porcine pulmonary oedema syndrome (Fandohan et al., 2005; JECFA, 2001, pp. 103–279). Moreover, these mycotoxins have been associated with nephrotoxic, hepatotoxic and immunosuppressing effects in various animal species (Merrill, Sullards, Wang, Voss, & Riley, 2001; Morgavi & Riley, 2007; Voss et al., 2002). In fact, because of a structural feature that is analogous to that of the co-substrates of ceramide synthase, fumonisins, and particularly FB1, are assumed to act as inhibitors of sphingolipid biosynthesis and, consequently, to interfere with several aspects of cell regulation (Marasas et al., 2004). Fumonisins are considered to be hazardous to the health of humans. These toxins have been reported to be associated with oesophageal cancer in South Africa (Sydenham et al., 1990) and in China (Chu & Li, 1994) and with liver cancer in China (Ueno et al., 1997). FB1 is considered to be a possible carcinogen to humans and classified as class 2B (IARC, 2002). Therefore, recommendations for maximum admissible levels of FB1 in foods and feed have been established in the United States by the Food and Drug Administration (Allaben, Bucher, & Howard, 2001). In Europe, legislation (EC Regulation No. 11/26/2007) dealing with maximum levels of fumonisins in maize and maize-derived products has recently been implemented. Surveys on the occurrence of FB1 in corn kernels have shown that it is a common contaminant of maize ears worldwide (Shephard et al., 1996). An overview of mycotoxins in food (Logrieco & Visconti, 2004) revealed the significant occurrence of FB1 in southern European countries such as Portugal, Spain, France, Italy and Croatia. Investigations carried out on commercial corn kernels in northern Italy from 1995 to 1999 reported a high incidence of FB1-positive samples for all of the years considered (Pietri, Bertuzzi, Pallaroni, & Piva, 2004). European legislation concerning FB1 has

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a strong impact on the European agricultural economy and, more particularly, on corn production in southern regions of Europe. Therefore, limiting FB1 occurrence in corn kernels is a crucial challenge.

In the past decade, tremendous progress has been made in identifying the fungal genes required for fumonisin biosynthesis. Since it commonly occurs in toxigenic fungi, these genes are organised in a cluster referred to as the *FUM* gene cluster (Proctor, Brown, Plattner, & Desjardins, 2003). Among the clustered genes, *FUM1* plays a crucial role in fumonisin production since it encodes the polyketide synthase that catalyses the first step of the biosynthetic pathway. However, despite this increasing knowledge about fumonisin production, there is still no obvious solution in sight with respect to fungal and mycotoxin contamination of maize.

Several strategies (physical and chemical) have been developed to prevent and/or to reduce mycotoxin accumulation in cereals. However, these methods require sophisticated equipment and expensive chemicals or reagents (Reddy, Nurdijati, & Sallech, 2010). Moreover, growing public concern about the use of pesticides and the development of new resistance to fungicides by several fungal pathogens has led to the search of alternative approaches (Montesinos, 2003).

The use of biological antagonism appears to be a promising alternative strategy. Among natural biological antagonists, lactic acid bacteria have been described as suitable antifungal agents (Dalié, Deschamps, & Richard-Forget, 2010; Gerez, Torino, Rollan, & de Valdez, 2009; Lavermicocca et al., 2000; Sathe, Nawani, Dhakephalkar, & Kapadnis, 2007; Sjögren, Magnusson, Broberg, Schnürer, & Kenne, 2003), and some authors have reported their capacity to inhibit mycotoxin biosynthesis, in addition to their ability to act as binders. These authors have focused on aflatoxins, and according to Gourama and Bullerman (1995), the capacity of lactic acid bacteria to modulate aflatoxin biosynthesis is probably linked to the production of specific bacterial metabolites that, up until now, have never been identified.

To our knowledge, even if Niderkorn, Boudra, and Morgavi (2006) investigated the ability of lactic acid bacteria to scavenge fumonisins, no published report has described their capacity to directly affect the production of fumonisins by *F. verticillioides* or *F. proliferatum*. In a previous study, we isolated lactic acid bacteria from maize leaf and tested their potential as bioprotective agents against *Fusarium* spp. (Dalié, Deschamps, Atanasova-Penichon, & Richard-Forget, 2010). The most effective antifungal isolate was identified as *Pediococcus pentosaceus* strain L006. The aim of the present work was to test the *in vitro* capacity of this strain and its metabolites to modulate fumonisin biosynthesis by *F. verticillioides*.

2. Materials and methods

2.1. Microorganisms and media

F. verticillioides strain INRA 62 and *P. pentosaceus* strain L006 were provided by the Mycology and Food Safety Research Unit, INRA, Bordeaux, France.

De Man, Rogosa and Sharpe broth (MRS) was used for the growth of *P. pentosaceus* strain L006. GYEAP media, corresponding to a GYEP medium (Ponts, Pinson-Gadais, Barreau, Richard-Forget, & Ouellet, 2007) supplemented with amylopectin (10 g/L), and autoclaved maize kernels were used for the culture of *F. verticillioides* INRA 62 in liquid and solid medium, respectively. Stock cultures were maintained at 4 °C on Potato Dextrose Agar (PDA) slants under mineral oil. When inoculum was required, the strain INRA 62 was grown on PDA slants at 25 °C in the dark for seven days, and spore suspensions were prepared by adding sterile water containing 0.05% Tween 80 to the PDA slant with gentle shaking. Spore count was determined with the aid of a Malassez cell and suspensions were adjusted to 10^7 spores per mL and stored at −20 °C until used.

2.2. Simultaneous co-inoculation

50 mL of GYEAP medium in 250-mL Erlenmeyer flasks were simultaneously inoculated with 0.1 mL of a suspension of *F. verticillioides* INRA 62 spores (10^6 spores mL^{-1}) and 0.5 ml of an overnight culture of *P. pentosaceus* strain L006. Cultures were incubated at 25 °C and 150 rpm in the dark in a Multitron incubator shaker (INFORS AG, Bottmingen, Switzerland). Cultures were then stopped at 4, 8, 12, 20 and 30-day intervals for analysis of fungal growth and fumonisin levels. pH values were monitored for each batch treated and the control. The experiment was performed in triplicate.

2.3. Differed co-inoculation

Erlenmeyer flasks with 50 mL of GYEAP were inoculated with 0.5 mL of an overnight culture of *P. pentosaceus* strain L006. Cultures were incubated at 30 °C for three days. After this incubation period, the cultures were inoculated with 0.1 mL of a suspension of spores of *F. verticillioides* strain INRA 62 (10^6 spores. mL^{-1}) and incubated under the same conditions as those described above. Cultures were then stopped at 4, 8, 12, 20 and 30-day intervals for analysis of fungal growth and fumonisin levels. Control treatment consisted in 50 mL of fresh GYEAP with 0.1 mL of a suspension of spores of the strain INRA 62. pH values were monitored for each batch treated and the control. The experiment was performed in triplicate.

2.4. Effect of the metabolites produced by P. pentosaceus strain L006 on fumonisin production in GYEAP medium

Erlenmeyer flasks with 600 mL of MRS broth (pH 5.5) were inoculated with 1% (v/v) of an overnight culture of *P. pentosaceus* strain L006, and fermentation was carried out at 30 °C for five days with shaking (200 rpm). Following the incubation period, the cells of strain L006 were removed from the culture by centrifugation at 10000 g for 10 min. The cell-free supernatant was concentrated 10-fold by lyophilisation and sterilised by passage through 0.22-μm filters (Sigma). Erlenmeyer flasks containing 50 mL of GYEAP medium were supplemented with 0.5, 1, and 2 mL of the 10-fold-concentrated cell-free supernatant and inoculated with 1 mL of a suspension of *F. verticillioides* strain INRA 62 spores (10^6 spores. mL^{-1}). The cultures were incubated at 25 °C and 150 rpm in the dark in a Multitron incubator shaker (INFORS AG, Bottmingen, Switzerland). The cultures were stopped after 20 days of incubation for analysis of fungal growth and fumonisin levels. Concentrated cell-free supernatant was replaced by sterile water in the control. The experiment was performed in triplicate.

2.5. Effects of P. pentosaceus L006 and its metabolites on fumonisin biosynthesis on maize kernels

Erlenmeyer flasks containing 50 g of sterile maize grains (sterilised at 105 °C for 30 min, aw = 1) were inoculated with 1 mL (10^6 spore mL^{-1}) of *F. verticillioides* strain INRA 62 and 1 mL (10^8 UFC/mL) of *P. pentosaceus* strain L006. Control treatment consisted in 50 g of maize kernels inoculated with 1 mL (10^6 spore mL^{-1}) of the strain INRA 62. The cultures were incubated for 20 days at 25 °C and three replicates per treatment were removed at 4, 8, 12 and 20-day intervals for analysis of fungal growth and fumonisin levels.

To determine the influence of the metabolites produced by the strain L006 on the growth and fumonisin accumulation of the *F. verticillioides* strain INRA 62, the same experiment described above was performed. In this experiment, however, maize kernels were treated with 2 mL of a 10-fold-concentrated cell-free supernatant (according to the procedure described above), instead of the L006 cells. In the controls, the cell-free supernatant was replaced by sterile water.

2.6. Determination of fungal growth

Fungal biomass production in GYEAP cultures was measured by weighing the mycelia after 48 h of freeze-drying. Ergosterol analysis was used to evaluate the fungal growth on inoculated maize kernels, following a protocol adapted from Marin, Ramos, and Sanchis (2005). Briefly, 2 mL of 10% KOH in pure methanol were added to 30 mg of lyophilised and ground maize in a screw cap tube and placed in a hot bath (80 °C) for 60 min. The tubes were then allowed to cool on ice. Forty µL of 7-dehydrocholesterol (500 mg/L), 480 µL of 6N HCL, 520 µL of MilliQ water and 2 mL of n-hexane were added to each tube, which was then agitated using a Vortex mixer for 1 min. Following agitation, the mixture was centrifuged at 3000 rpm for 3 min and the organic phase was removed and transferred to a 5-mL vial. A two-step hexane extraction was carried out. The extracts were combined and evaporated to dryness under a nitrogen stream. The dry extracts were dissolved in 1 mL of methanol and filtered through a 0.45-µm Millipore filter before HPLC analysis. Twenty µL of this solution were injected into a Zorbax SB-C18, 5µ, 250 mm × 46 mm column at a flow rate of 1 mL min^{-1}, using an HPLC device (LC 1100, Agilent Technologies, USA). The isocratic phase was methanol and eluate and was monitored at 282 nm. Quantification was performed by using standard solutions of ergosterol and 7-dehydrocholesterol purchased from Sigma (St. Louis, MO, USA).

2.7. Fumonisin extraction, clean up and analysis

Fumonisins were extracted from culture filtrates following a previously described protocol (Shephard, Sydenham, Thiel, & Gelderblom, 1990), which was adapted to our samples. Briefly, 5 mL of culture filtrates were adjusted at pH 6.5 with 1M NaOH, and fumonisins were purified by using Bond Elut Strong Anion Exchange (SAX) cartridges (Varian, Palo Alto, CA, USA). Fumonisins were eluted at acidic pH with 10 mL of methanol/acetic acid (99/1, v/v) and evaporated to dryness under a nitrogen stream. Dried samples were dissolved in 200 µL of methanol before high performance liquid chromatography analysis with fluorescence detection. Ten µL of the sample were derivatised with 90 µL of *o*-phtaldialdehyde reagent. Twenty µL of this solution were injected into the HPLC system within 1 min of derivatisation. HPLC-FLD analysis was performed following the protocol of Shephard et al. (1990). Quantification was performed by using external calibration with FB1, FB2 and FB3 standard solutions prepared from commercial pure powders (FB1 and FB2 were purchased from Sigma–Aldrich Co, St Louis, MO, USA; FB3 was purchased from MRC/PROMEC, Tygerberg, South Africa). Fig. 1a illustrates a typical chromatogram of fumonisins separation, extracted from liquid culture filtrates.

Fumonisins were extracted from inoculated maize kernels following a procedure adapted from Kedera, Plattner, and Desjardins (1999). A quantity of 12.5 g of ground maize was extracted with methanol-water (3:1 v/v). After 15 min agitation at 150 rpm and centrifugation (3 min, 3000 rpm), the supernatant was filtered through Whatman 2V filter paper. Fumonisins were then purified and analysed according to the protocol described above. A typical chromatogram of fumonisins production on maize kernels is provided in Fig. 1b.

2.8. Extraction of total RNA and preparation of cDNA

Total RNA was extracted from mycelium, using the RNeasy® Mini Kit according to the manufacturer's instructions (Qiagen). A quantity of 100 mg of mycelium was ground with the TissueLyser System® (Qiagen-Retsch), using one stainless steel bead in an Eppendorf tube containing 450 µL of RLC Buffer (Qiagen) for 1.5 min at 30 Hz. Total RNA was quantified using a Nanodrop® spectrophotometer (Nanodrop Technology®). The quality of the RNA was verified by agarose gel electrophoresis. Seven ng of total RNA were reverse-transcribed using the Superscript™ II First-Strand Synthesis System (Invitrogen Life Technologies).

2.9. Real-time PCR analysis

The abundance of the transcripts of the *FUM1* and *GAPDH* genes was evaluated by real-time PCR with a LightCycler® real-time detector (2.0 instrument; Roche Applied Science), using 1 µL of each cDNA diluted in 10 µL reaction mixture. Quantification of the transcripts of the gene *FUM1* was performed using the LightCycler® FastStart DNA Master SYBR® Green I (Roche Applied Science) and quantification of the gene *GADPH* using the QuantiTectTM SYBR_Green PCR kit (Qiagen). Analyses were performed in triplicate. Expression of *GAPDH* was used as an endogenous reference (Merhej, Boutigny, Pinson-Gadais, Richard-Forget, & Barreau, 2010). The primer pairs used to amplify these genes were FUM1-F(CTCGGGCACATATCGT)/FUM1-R(AGCATCTGGGGACAAA) (Tm: 58 °C) which were designed from *FUM1* gene (accession number AY577458) to amplify a 353 pb fragment, and GAPDH-F (CCTTTCATTGAGCCTCAC)/GAPDH-R (CGTACATGGGAGCGTC) (Tm: 59 °C), which were designed to amplify a 289 pb fragment. They were used at a final concentration of 500 nM.

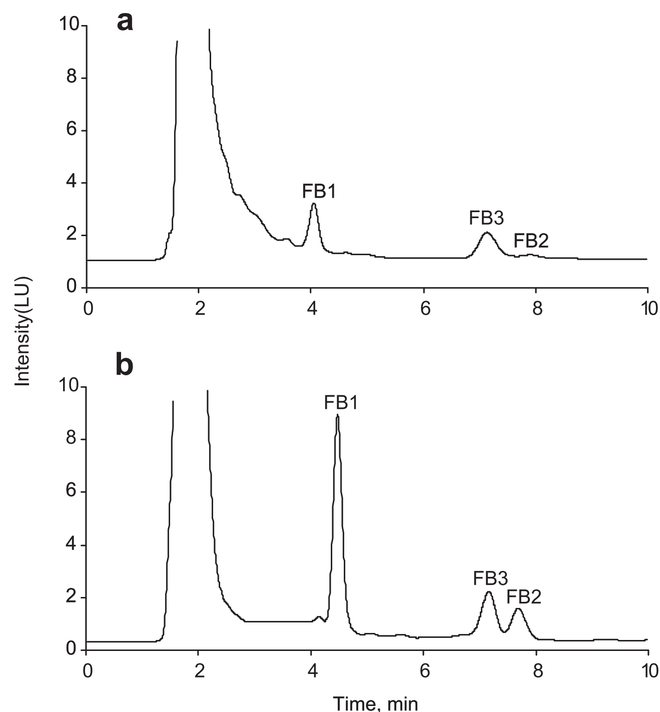

Fig. 1. Example chromatograms of fumonisin extracts from a 8-days-old liquid culture (a) and a 12 days-old kernel culture of *Fusarium verticillioides* strain INRA 62 (b). The peaks are identified at retention time: 4.1 min for FB1, 7.7 min for FB2 and 7.2 min for FB3.

All cDNA samples were tested for residual genomic DNA using *GAPDH* primers. The absence of genomic DNA was verified by running both melting curves and agarose gel analyses on the final PCR products. The same procedure was followed to check the absence of non-specific PCR amplification or primer dimer formation.

Five μL of each cDNA sample solution were mixed together to prepare a standard mixture to be used to determine PCR efficiencies (E) by external calibration, according to a previously described protocol (Ponts et al., 2007). E was calculated using LightCycler® Software, version 4.05 (Roche Applied Science). PCR efficiencies and standard errors obtained for reference and target genes were 1.95 for *FUM1* and 2.04 for *GAPDH*.

2.10. Real-time PCR data analysis

The crossing point values (Cp) obtained experimentally (mean value of three replicates) for *GAPDH* were compared in control and treated fungal liquid cultures to verify that the level of *GAPDH* expression was not affected by the presence of the bacterial strain. Cp values obtained experimentally for the target *FUM1* gene were compared under control and treated conditions and normalised relative to the Cp values obtained for the reference gene *GAPDH* using REST®-384 software (Relative Expression Software Tool). The mathematical model used accounts for differences in efficiencies for the reference gene and the target gene and for the mean crossing point deviation between control and treated conditions (Pfaffl, 2001; Pfaffl, Horgan, & Demppfle, 2002). The expression levels of the target genes, normalised to reference gene expression, are expressed as a regulation factor in the treated condition relative to the control, with the ratio $= (\text{Etarget})^{\Delta \text{CP target (control-treated)}}/(\text{Eref})^{\Delta \text{CP ref (control-treated)}}$. $P = 0.001$ was chosen as the statistical point throughout.

3. Results

3.1. The occurrence of P. pentosaceus strain L006 affects the growth of F. verticillioides and its fumonisin production in liquid cultures

The effect of *P. pentosaceus* strain L006 on *F. verticillioides* growth and fumonisin accumulation in liquid cultures inoculated with a mixture of *F. verticillioides* and *P. pentosaceus* is reported in Table 1. Growth of *F. verticillioides* and toxin production were monitored over time for up to 30 days. Data indicated that biomass accumulation kinetics slowed down in the presence of *P. pentosaceus*, supporting the previously reported antifungal efficiency of this lactic acid bacteria strain (Dalié, Deschamps, Atanasova-Penichon et al., 2010). Our data also clearly illustrated a significant increase in fumonisin production. Thus, in 30-day-old control cultures, the concentration of fumonisin was close to 42 μg mL^{-1} when it reached 120 μg mL^{-1} in treated cultures. This increase was observed for each day of culture analysed.

The effects of a differed inoculation procedure, *i.e.*, when the spores of *F. verticillioides* strain INRA 62 were inoculated in a three-day-old culture of *P. pentosaceus* strain L006, on biomass accumulation and toxin production are summarised in Table 2. The *P. pentosaceus* treatment led to a significant reduction in biomass yield that could most certainly be ascribed to the production of bacterial antifungal metabolites as previously described by Dalié, Deschamps, Atanasova-Penichon et al. (2010). Our results also showed that for each point of the kinetics, FB1 + FB2 accumulation was considerably enhanced in the treated cultures compared to the controls. The activation factors ranged between 3 and 7, depending on the day of culture. To investigate if this toxin increase was linked to a transcriptional control induced by the occurrence of *P. pentosaceus*, we decided to compare *FUM1* gene expression in supplemented and control cultures. *FUM1* encodes a polyketide synthase that catalyses the first step in fumonisin biosynthesis and therefore plays a critical role in the production of this toxin (Seo, Proctor, & Plattner, 2001). Since the presence of FB1 was detected as early as the fourth day of cultures when inoculated by *P. pentosaceus* strain L006 while it remained undetectable in the control cultures (Table 2), we decided to examine levels of expression of *FUM1* gene in four-day-old cultures of *F. verticillioides* INRA 62 grown in the presence of *P. pentosaceus* strain L006. Moreover, according to Brown, Butchko, Busman, and Proctor (2007), transcripts of most *FUM* genes reach their highest level between three and five days in liquid cultures. Analysis of the *GAPDH* Cp values measured in control and treated cultures showed that levels of *GAPDH* mRNA in total RNA were not significantly affected by the treatment, supporting the use of *GAPDH* as a reference gene. The expression values of the *FUM1* gene compared to the reference gene were expressed as up- or down-regulation factors in the treated conditions relative to the control. The *P. pentosaceus* treatment was shown to induce a significant up-regulation of *FUM1* genes with an up-regulation factor close to 46 ± 8 in accordance with the significant enhancement of the FB1+FB2 accumulation that was reported above.

In conclusion, for the two previously described inoculation procedures, our results supported the antagonistic behaviour of *P. pentosaceus* strain L006 and clearly illustrated a significant activation of fumonisin production by *F. verticillioides* in GYEAP medium inoculated with the bacterial strain. pH measurements in the control and treated cultures (data not shown) indicated that the increased fumonisin production could not be ascribed to an acidification of the broths. In our opinion, there could be two rationales for this enhancement. First, activation of the biosynthesis of fumonisins might be the direct result of stressful conditions perceived by *F. verticillioides* in competition with *P. pentosaceus*. Second, bacterial metabolites might be released in the liquid media, inducing a perturbation in the environmental conditions of *F. verticillioides* growth. These changes might be perceived by *F. verticillioides* and might, in turn, interfere with fumonisin biosynthesis.

Table 1
Influence of *Pediococcus pentosaceus* strain L006 on growth and fumonisin (FB1 + FB2) production by *Fusarium verticillioides* strain INRA 62 when both strains were simultaneously co-inoculated in GYEP medium.

Days	Dry biomass (mg/mL)		FB1 + FB2 (μg/mL)	
	INRA 62	INRA 62 + L006	INRA 62	INRA 62 + L006
4	2.22 ± 0.19	1.68 ± 0.71	1.12 ± 0.13	3.06 ± 0.21
8	5.91 ± 0.60	4.70 ± 0.49	13.57 ± 2.05	44.18 ± 1.73
12	8.98 ± 0.46	7.50 ± 0.46	20.70 ± 4.21	52.53 ± 1.25
20	10.25 ± *0.26*	8.62 ± 0.97	35.78 ± 4.18	101.50 ± 4.08
30	12.05 ± 0.59	9.18 ± 0.82	42.40 ± 0.76	120.35 ± 8.84

Table 2
Growth and fumonisin (B1 + B2) production by *Fusarium verticillioides* strain INRA 62, inoculated in a three-day-old culture of *Pediococcus pentosaceus* strain L006 (cultivated in GYEP medium).

Days	Dry biomass (mg/mL)		FBl + FB2 (μg/mL)	
	INRA 62	INRA 62 + L006	INRA 62	INRA 62 + L006
4	2.17 ± 0.42	0.61 ± 0.06	0.00 ± 0.00	1.59 ± 0.32
8	6.14 ± 0.96	4.60 ± 0.09	6.78 ± 0.80	46.23 ± 8.86
12	8.94 ± 0.07	6.94 ± 0.43	12.51 ± 1.41	82.14 ± 9.76
20	10.30 ± 0.17	7.75 ± 0.46	27.48 ± 3.43	98.30 ± 12.08
30	11.94 ± 0.84	8.54 ± 1.00	35.17 ± 3.45	110.01 ± 9.38

3.2. P. pentosaceus strain L006 produces some metabolites capable of affecting fumonisin production by F. verticillioides in liquid cultures

To study the impact of the metabolites produced by *P. pentosaceus* strain L006 on fumonisin production by *F. verticillioides* strain INRA 62, we carried out the following experiment. After five days of culture of the bacterial strain in MRS medium, three volumes (0.5 mL, 1 and 2 mL) of concentrated cell-free supernatant were added to the 50-mL GYEAP media. Fungal biomass and fumonisin accumulation were compared in 30-day-old cultures for control and treated conditions. Our data indicated a reduction of the fungal development in supplemented cultures that was only significant when a 2-mL volume was used (data not shown). This result supported the production of extracellular antifungal metabolites previously reported for this *P. pentosaceus* strain (Dalié, Deschamps, Atanasova-Penichon et al., 2010). Regarding fumonisin accumulation (Fig. 2), a significant reduction was induced by GYEAP supplementation with 0.5 and 1 mL of bacterial concentrated supernatant, and no fumonisin was quantified in the cultures treated with 2 mL of concentrated supernatant. These data suggested that in MRS medium, the *P. pentosaceus* strain L006 was able to produce some metabolites that interfere with fumonisin production. Since no reduction of fungal growth was observed in 0.5 and 1 mL supplemented media, while fumonisin accumulation was significantly reduced, we could reasonably suppose that the “antimycotoxin” metabolites are different from the antifungal ones.

3.3. On maize kernels, P. pentosaceus strain L006 affects F. verticillioides growth and fumonisin production due to its production of extracellular metabolites

When co-inoculated with *P. pentosaceus* L006 on autoclaved maize kernels, growth and fumonisin production of *F. verticillioides* were not significantly affected (data not shown). These results suggested a weak adaptation of the strain L006 (initially isolated from maize leaves) on maize grains, or that, although well adapted to this substrate, the lactic acid bacteria was unable to produce its antifungal metabolites. When maize kernels were mixed with 2 mL of concentrated supernatant of *P. pentosaceus* strain L006 before inoculation by *F. verticillioides*, a significant modulation of fungal development and fumonisin production was observed (Figs. 3 and 4). As can be clearly seen in Fig. 3, *F. verticillioides* growth was significantly reduced in the presence of the cell-free supernatant of *P. pentosaceus* strain L006. The reduction in ergosterol content was close to 50% after 12 days of culture and remained close to 40% at the end of the culture. In addition, *P. pentosaceus* cell-free extract supplementation led to a large decrease (close to 75% after 15 or 30 days of culture) in FB1 production by *F. verticillioides* (Fig. 4). According to our results, the extracellular metabolites produced by *P. pentosaceus* strain L006, whose efficiency to repress fungal growth and FB1 production in liquid media was previously demonstrated, also appeared to be potent antifungal and “antimycotoxin” agents when tested on maize kernels.

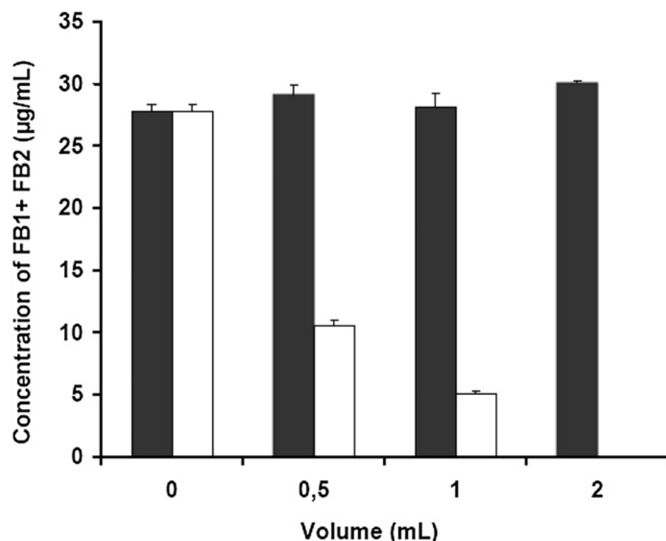

Fig. 2. Production of fumonisins (FB1 + FB2) by *Fusarium verticillioides* strain INRA 62 in GYEAP medium in the absence (■) or presence (□) of metabolites produced by *Pediococcus pentosaceus* strain L006 in MRS medium. In the control cultures (■), metabolites were replaced by sterile water.

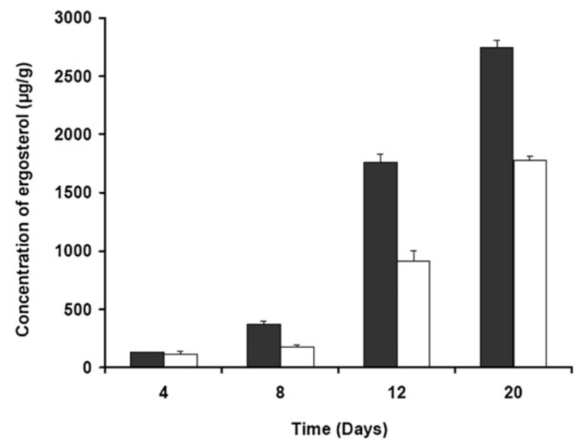

Fig. 3. Growth of *Fusarium verticillioides* strain INRA 62 on maize kernels in the absence (■) or presence (□) of metabolites produced by *Pediococcus pentosaceus* strain L006 in MRS medium. In the control cultures (■), metabolites were replaced by sterile water.

4. Discussion

Mycotoxin contamination of feed often begins in the field and may continue throughout harvest, transportation and storage, depending on the prevailing environmental conditions. Thus, the most effective way to prevent its occurrence is to limit the

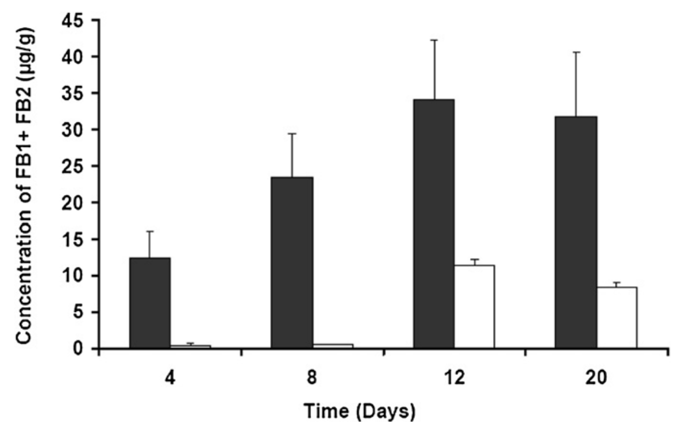

Fig. 4. Production of fumonisins (FB1 + FB2) by *Fusarium verticillioides* strain INRA 62 on maize kernels in the absence (■) or presence (□) of metabolites produced by *Pediococcus pentosaceus* strain L006 in MRS medium. In the control cultures (■), metabolites were replaced by sterile water.

biosynthesis by the fungus when the crop is cultivated. Although management practices have been defined in order to substantially decrease mycotoxin contamination, no management strategy is effective enough to avoid mycotoxin contamination in years favourable to disease development. Physical and chemical methods have also been developed to reduce the occurrence of toxigenic moulds and mycotoxins in contaminated food and feed, but no effective treatment has yet been proposed. The use of biopreservation, *i.e.*, the control of one organism by another, could be an effective alternative to prevent "mycotoxigenic" fungal contamination. Various studies have clearly demonstrated that diverse microorganisms could act as natural antagonists of plant pathogens. Among these microorganisms, lactic acid bacteria that have a long history of use in food and feed and are generally considered as safe organisms have been reported to effectively reduce mould growth and to interact with mycotoxins (El-Nezami, Polychronaki, Salminen, & Mykkanen, 2002; Niderkorn et al., 2006, 2007, 2009). However, little is known regarding the impact of these microorganisms on mycotoxin production and especially *Fusarium* toxins. In this work, we demonstrated that the efficiency of a bacterial strain used as a biocontrol agent to prevent fumonisin contamination is strongly dependent on the inoculation procedure and that, surprisingly, this treatment can under certain conditions lead to an unexpected result, *i.e.* higher levels of fumonisins. Using *P. pentosaceus* strain L006, which was previously recognized as a potent antifungal agent (Dalié, Deschamps, Atanasova-Penichon et al., 2010), we never observed a reduction of fumonisin accumulation by *F. verticillioides* but demonstrated a significant increase in two cases: simultaneous inoculation of *F. verticillioides* with the bacterial strain and inoculation of the fungal strain in broths previously inoculated by the *P. pentosaceus* strain L006. Fumonisin production has been shown to be significantly affected by pH conditions, optimal within a pH range of 2–4.5 in liquid cultures (Keller, Nesbitt, Sarr, Phillips, & Burow, 1997) and repressed under alkaline conditions (Flaherty, Pirttila, Bluhm, & Woloshuk, 2003). In our work, pH measurements indicated that the occurrence of the *P. pentosaceus* bacterial strain, which has the potential to produce high levels of organic acids, did not modify the pH in *F. verticillioides* liquid cultures compared to control cultures. Therefore, the enhancement of fumonisin yields was not the result of a strong acidification. Fumonisin production has been shown to be significantly affected by pH conditions, optimal within a pH range of 2–4.5 in liquid cultures (Keller et al., 1997) and repressed under alkaline conditions (Flaherty et al., 2003). Fumonisin production has also been viewed as a response to stressful environmental conditions (Picot et al., 2010). We could therefore suppose that the occurrence of an antagonistic agent such as *P. pentosaceus* L006 induces stressful conditions perceived by *F. verticillioides*. Then, the question of the benefit for the fungus to produce higher levels of fumonisin must be addressed. One hypothesis, that still remains to be demonstrated, is that fumonisin production helps *F. verticillioides* to compete with other microorganisms. Our data support the results of Hassan and Bullerman (2008) who indicated an activation of fumonisin production by *F. proliferatum* when it was cultivated simultaneously with *Lactobacillus paracasei* subp. *tolerans*. In another study, Stiles and Bullerman (2002) investigated the effect of *Lactobacillus rhamnosus* on growth and mycotoxin production by *Fusarium* species, including *F. proliferatum* and *F. verticillioides* and in contrast to our data, their results showed a significant reduction of FB1 accumulation (up to 63.2%). However, according to Stiles and Bullerman (2002), the mechanism leading to this reduction was linked to the ability of *L. rhamnosus* to trap fumonisin rather than to its capacity to inhibit FB1 biosynthesis. Besides, according to Luchese and Harrigan (1990) who studied the ability of some *Lactobacillus* strains to affect aflatoxin biosynthesis, the impact a bacterial strain could have on toxin biosynthesis is strongly dependent on the bacterial strain used.

Activation of fumonisin production could also be linked to the yield of extracellular metabolites by *P. pentosaceus* strain L006. Indeed, lactic acid bacteria are known for their high production of hydrogen peroxide. For several toxinogenic moulds, it has been shown that higher levels of oxidative stress result in enhanced mycotoxin production (Ponts et al., 2007; Reverberi, Ricelli, Zjalic, Fabbri, & Fanelli, 2008). To our knowledge, this has not been demonstrated for fumonisin as of this time. However, since the fumonisin biosynthetic pathway contains many oxidation steps, it is tempting to speculate that fumonisin biosynthesis may also be enhanced by increasing ROS levels.

Studies regarding the influence of the metabolites produced by *P. pentosaceus* strain L006 in MRS medium on growth and fumonisin production in GYEAP medium and on maize kernels revealed a significant inhibition of fungal growth and fumonisin accumulation. This indicates that in MRS medium, *P. pentosaceus* strain L006 produced some metabolites that were able to significantly repress fumonisin production. Our data suggested that these "antimycotoxin" metabolites are different from those affecting fungal growth. These results support the studies of Karunaratne, Wezenberg, and Bullerman (1990) and Gourama (1991) who also reported the occurrence of a production of bacterial metabolites that were effective in inhibiting the biosynthesis pathway of some mycotoxins. However no "anti-mycotoxigenic" metabolites have been characterised, up to now.

In conclusion, according to our results, *P. pentosaceus* strain L006 could be a promising agent for reducing the fumonisin contamination of food and feed due to its production of antifungal and "antimycotoxin" metabolites. The direct prospect of this study is the purification and the characterisation of these biochemical entities, as well as the investigation of their *in planta* efficiency during the development of maize kernels. Our data also clearly illustrated the potential risk induced by the use of antagonistic agents to control mycotoxin contamination of food and feed. Under certain conditions, the presence of the bacterial strain could be perceived as a stressful signal and induce an enhancement of mycotoxins production. Therefore, it is strongly recommended to carefully check the lack of unexpected effect before using a new microorganism as a biocontrol agent for mycotoxin contamination.

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