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Tracking new insights into antifungal and anti-mycotoxigenic properties of a biofilm forming *Pediococcus pentosaceus* strain isolated from grain silage

Carlos M.N. Mendonça [a,b], Rodrigo C. Oliveira [a], Lucas J.L. Pizauro [c], Wellison A. Pereira [a], Kahlile Abboud [c], Sonia Almeida [d], Ii-Sei Watanabe [d], Alessandro M. Varani [c], José M. Domínguez [e], Benedito Correa [f], Koen Venema [c], Pamela O.S. Azevedo [a,g], Ricardo P. S. Oliveira [a,*]

[a] *Laboratory of Microbial Biomolecules, Department of Biochemical and Pharmaceutical Technology, University of São Paulo, 05508-000 São Paulo, Brazil*
[b] *Centre for Healthy Eating and Food Innovation (HEFI), Faculty of Science and Engineering, Maastricht University – campus Venlo, Villaflorapark 1, 5928 SZ Venlo, the Netherlands*
[c] *Department of Agricultural and Environmental Biotechnology, School of Agricultural and Veterinary Sciences (FCAV), UNESP, Jaboticabal, Brazil*
[d] *Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, 05508-000 São Paulo, Brazil*
[e] *Industrial Biotechnology and Environmental Engineering Group "BiotecnIA", Chemical Engineering Department, University of Vigo (Campus Ourense), As Lagoas s/n, 32004 Ourense, Spain*
[f] *Laboratory of Mycotoxins and Toxigenic Fungi, Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, 05508-900 São Paulo, Brazil*
[g] *SAZ Animal Nutrition, São Paulo, Brazil*

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ABSTRACT

The present study offers detailed insights into the antifungal and anti-mycotoxigenic potential of a biofilm forming lactic acid bacterium (*Pediococcus pentosaceus*) against one atoxigenic (*Aspergillus flavus*) and two toxigenic (*Aspergillus nomius* and *Fusarium verticillioides*) fungal strains. The antifungal effect of *P. pentosaceus* LBM18 strain was initially investigated through comparative analysis of fungi physiology by macroscopic visual evaluations and scanning electron microscopy examinations. The effects over fungal growth rate and asexual sporulation were additionally accessed. Furthermore, analytical evaluations of mycotoxin production were carried out by HPLC-MS/MS to provide insights on the bacterial anti-mycotoxigenic activity over fungal production of the aflatoxins B1, B2, G1 and G2 as well as fumonisins B1 and B2. Finally, reverse transcription quantitative real-time PCR (RT-qPCR) analysis was employed at the most effective bacterial inoculant concentration to evaluate, at the molecular level, the down-regulation of genes *aflR*, *aflQ* and *aflD*, related to the biosynthesis of aflatoxins by the strain of *Aspergillus nomius*. The effects over mycotoxin contamination were thought to be result of a combination of several biotic and abiotic factors, such as interaction between living beings and physical-chemical aspects of the environment, respectively. Several possible mechanisms of action were addressed along with potentially deleterious effects ascribing from *P. pentosaceus* misuse as biopesticide, emphasizing the importance of evaluating lactic acid bacteria safety in new applications, concentrations, and exposure scenarios.

1. Introduction

Mycotoxins are fungal secondary metabolites occurring under natural conditions in almost all agricultural commodities worldwide (as much as 50 % including feed grains), and known for being toxic for humans and animals even at low concentrations (Quetta Balochistan et al., 2022). Produced by toxigenic fungi, mycotoxins onset has been associated with different environmental factors or stress-related signals (*e.g.* temperature, water activity, nutrient stress, oxidative stress, and pH) (Reverberi et al., 2010).

The ability of different microorganisms to degrade or reduce mycotoxins contamination levels through different biodetoxification mechanisms was previously reported (Assaf et al., 2018). Several studies evaluating the association mechanism of Gram-positive bacteria with different mycotoxins have been described. For instance, the association mechanism may occur through the ability of interaction and adsorption

* Corresponding author at: Department of Biochemical and Pharmaceutical Technology, University of São Paulo, 05508-000 São Paulo, Brazil.
E-mail address: rpsolive@usp.br (R.P.S. Oliveira).

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by the bacterial cell wall polysaccharides (Assaf et al., 2018). Due to LAB's GRAS status, based on their safe use in traditional food fermentation, processes have been proposed to reduce mycotoxin contamination in food and feed matrices (Bata and Lásztity, 1999). Fungal growth retardation or inhibition is considered one of the most conventional approaches to prevent fungal food spoilage and mycotoxin production.

Numerous bacterial strains isolated from a vast variety of sources have shown the ability to slow down or inhibit fungal growth, affect its morphological features, and modulate the production of secondary metabolites such mycotoxins. Due to their recognized ability to produce a wide range of antifungal metabolites, their acknowledgement as biopesticides for fungi biocontrol has been postulated in a growing number of scientific studies under a wide range of *in vitro* approaches. LAB biofilms are known for providing a set of protective effects against different biotic and abiotic stress inducing conditions (Kubota et al., 2008). The successful use of biofilm forming LAB as bio-preservatives against different pathogens and spoilage bacteria has been widely explored (Tatsaporn and Kornkanok, 2020). Moreover, such ability has been proposed as a preponderant feature during the selection of bacterial biological control agents against plant pathogenic fungi (Khezri et al., 2011). Considered to act as a natural absorbent with multi-mycotoxin binding capacity, biofilms have been capable of either inactivate or remove mycotoxins from food and feed products (Kavitake et al., 2020). By affecting mycotoxins diffusion, LAB biofilms are believed to promote limitations over fungal antimicrobial compounds mass transfer, decreasing its concentration across cell aggregates to sub-lethal levels and increasing the resistance of microbial biofilm communities to high mycotoxin contamination levels (Flemming et al., 2016). Together with their enhanced ability to setup such physical and chemical protection barrier, we suggest that the LAB capacity to produce antifungal metabolites might provide advantageous improvements over bacterial modulation of fungal growth environment.

Under such scenario, the bacterial competitiveness against fungal defense mechanisms is expected to increase comparatively to planktonic cells. Although recently described to be correlated with the decrease of fungal growth and genetic down-regulation of genes related to mycotoxins biosynthesis, the understanding about the potentialities of biofilm forming bacteria against filamentous toxigenic fungi remain unclear. Vastly used on farms around the world, corn silage is considered one of the most important conserved forages in modern protein production systems (Yuan et al., 2022). The contamination of this high-quality forage crop by mycotoxigenic fungi before and during silage storage is a well-known phenomenon (Ogunade et al., 2018). Owning to that, tests evaluating a biofilm forming *Pediococcus pentosaceus* strain antifungal and anti-mycotoxigenic potential against one atoxigenic (*Aspergillus flavus*) and two toxigenic (*Aspergillus nomius* and *Fusarium verticillioides*) fungal strains were performed using a corn-based medium. Given that the LAB's GRAS status does not automatically apply to every application or unlimited quantities, it is crucial to conduct specific evaluations and obtain regulatory approvals for different concentrations, usage scenarios, and exposure conditions. Therefore, in this study, the safety and suitability of the *Pediococcus pentosaceus* strain was thoroughly assessed within the specific intended context and conditions of use.

2. Materials and methods

2.1. Microbial strains

The *Pediococcus pentosaceus* strain LBM18, previously isolated from corn grains silage (Azevedo et al., 2020), was used as a biocontrol agent against fungal growth and mycotoxin production. The strain was characterized by 16S rRNA gene sequencing after partial amplification by PCR, using the following primers: F5′GAGAGTTTGATCCTGGCTCAG3′ and R5′CGGTGTGTACAAGGCCCGGGAAC G3′. DNA amplification was carried out in a volume of 25 μL, and the temperature profile of the reaction was as follows: after the denaturation step at 94 °C for 30 s, the annealing temperature was set at 60 °C for 30 s, and 35 additional cycles were carried out. Extension was performed at 72 °C for 10 min, and the final cycles were followed by an additional step at 72 °C for 10 min. Sequencing of the PCR product was performed at USP Genome Center using the following primers: F5′GAGAGTTTGATCCTGGCTCAG3′ and R5′CGGTGTGTACAAGGCCCGGGAAC G3′; F5′AACGCGAAGAACCTTA C3′ and R5′CCGTCAATTCCTTTRAGTTT3′. The resulting sequences were compared using the nucleotide blast (BLASTn) network service at the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The *P. pentosaceus* LBM18 was cultivated in De Man, Rogosa, and Sharpe (MRS) medium (Difco Laboratories, Detroit, MI, (USA)) at 37 °C in static mode and stored at −80 °C in MRS medium supplemented with 20 % (v/v) glycerol. The fungal strains *Aspergillus flavus* (Zorzete et al., 2013), *Aspergillus nomius* (Yunes et al., 2020) and *Fusarium verticillioides* (Reis et al., 2020), previously isolated from Brazil nuts and fresh maize samples respectively, were used in the present work as environmental fungal contaminants. All strains were previously identified by partial sequencing of β-tubulin gene (*tub2*) (Zorzete et al., 2016). The fungal strains were grown in potato dextrose agar (PDA) and were maintained at −80 °C in PDA medium supplemented with 20 % (v/v) glycerol.

2.2. Bacterial growth conditions and cell harvest procedure

P. pentosaceus LBM18 was cultivated at static mode at 37 °C for 10 h in 500 mL Erlenmeyer flasks containing 300 mL of MRS medium. The bacteria cell harvest experiments were performed using a tangential flow filtration (TFF) system. The used TFF system was a Sartorius Sartoflow Slice 200 Benchtop Cross-flow (Goettingen, Germany), consisting of a SciLog Peristaltic Pump Tandem 1082 with a coupled cylindrical vessel (total volume equal to 400 mL), a Sartocon Slice 200 stainless steel holder (160 mm length, 120 mm width and 275 mm height), and a Sartorius TE4101 precision compact balance. The ultrafiltration membrane used was a Sartocon Slice Cassette (Goettingen, Germany), made of Hydrosart® (stabilized cellulose), with a pore size of 0.2 μm and an effective filtration area of 0.1 m^2. The assembled system was sterilized according to supplier protocol. The obtained cell suspension was fractioned into three sterile falcon tubes (50 mL), frozen with liquid nitrogen, further stored at −80 °C for 24 h, and finally, lyophilized using a freeze dryer (L101, Liobras, São Carlos, SP, Brazil).

2.2.1. Bacterial viability

P. pentosaceus LBM18 viability was assessed by plate count method before harvest, after cell concentration by the TFF system, after lyophilization and along stocking under different conditions. Samples obtained before harvest and after cell concentration were serially 10-fold diluted in sterile saline solution (0.8 % w/v NaCl) and poured into MRS medium supplemented with 1.0 % (w/v) agar. The grown colonies were counted after aerobic incubation of plates at 37 °C for 48 h. Results were expressed as log of CFU/mL. For the lyophilized samples, approximately 15 mg of lyophilized biomass was rehydrated in 1 mL of sterile saline solution (0.8 % w/v NaCl) at room temperature and processed as previously stated. Results were expressed as log of CFU/g of lyophilized biomass. All analyses were performed in triplicate.

2.3. Spore suspension preparation

The conidia from each fungus strain used to inoculate the corn-based culture medium were removed from 7-day-old fungi cultures grown in PDA culture medium incubated at 26 °C. The mycelium surface of each fungi culture was vigorously rubbed with a sterile L-shaped spreader after addition of a 5 mL sterile water solution containing 0.1 % Tween 20 (*v*/v). The conidia suspension was transferred to a 15 mL sterile Falcon tube, centrifuged, and adjusted to a final spore concentration of 10^6 spores/mL using a hemocytometer slide. The spore suspensions were prepared prior to the inoculation and held at 4 °C until used.

2.4. Corn-based culture medium preparation

The corn-based culture medium used in the present work was prepared by mixing a blended corn grains aqueous suspension 2× concentrated (50 % of the final culture volume) with an equal volume of a *P. pentosaceus* LBM18 aqueous suspensions at the concentrations required to attain a final bacterial inoculant concentration of 0.2, 2, and 20 mg/mL. The corn-based medium was prepared by initially mixing 8 % (w/v) blended corn grains with deionized water supplemented with 2 % (w/v) agar. The medium was sterilized by autoclave (121 °C, 1 bar for 30 min) and led to cool down to the temperature of 45 °C on a heating laboratory incubator. Different *P. pentosaceus* LBM18 suspensions were prepared by resuspending the required lyophilized bacteria masses in sterile deionized water. Both, 2× concentrated corn-based culture medium and inoculant suspensions were vigorously mixed, distributed in aliquot of 5 mL on disposable plastic petri dishes (30 mm × 15 mm) and allowed to solidify at room temperature. A corn-based medium without bacterial inoculant (control group) was additionally prepared at a final concentration of 4 % (w/v) blended corn grains and 1 % (w/v) agar.

2.5. Fungal growth rate analysis

Fungal growth rate analysis was performed on solid corn-based culture medium supplemented with a final *P. pentosaceus* LBM18 concentration of 0.2, 2, and 20 mg/mL. Each treatment was centrally inoculated with 10 μL of a freshly made spore suspension (10^6 spores/mL). The plates were incubated for 7 days at 26 °C. A corn-based medium without bacterial inoculant (control group) was also inoculated with each fungus and monitored along incubation time. The mycelium growth rate was evaluated as previously described by Patriarca et al. (2001).

2.6. Spore production analysis

Total spore production was analyzed for each fungi strain at the seventh day of incubation with the aid of a hemocytometer. The conidia were removed and collected after vigorously rubbing of whole petri plate surface with 15 mL of sterile deionized water (wash 5 mL each for three times). The spore concentration has been counted and expressed as number of conidia/mm^2. All analyses were performed in duplicate.

2.7. Fungal physiology analysis

Hyphae and fungal reproductive structures were analyzed by Scanning Electron Microscopy (SEM) after growth for 7 days either in the absence and presence of *P. pentosaceus* LBM18 (0.2 mg/mL). Mycelial agar plugs of 5 mm diameter were scoped out from the culture with a sterile cork borer, added to a 2 mL Eppendorf tube and then fixed at 4 °C for 24 h with a 2.5 % solution of glutaraldehyde in 0.1 M cacodylate buffer (TC). After fixation, the samples were washed twice with TC for 10 min and a post-fixation step was carried out for 2 h at room temperature using 1 % osmium tetra oxide (Sigma Aldrich). The samples were subsequently washed twice with TC and further submitted to serial dehydration steps with different ethanol-water mixtures (v/v) in the following sequence: 50 % for 10 min, 70 % for 10 min, 95 % for 5 min and 100 % for 1 min. After dehydration, the samples were led to dry at room temperature and further dehydrated in liquid CO_2. Finally, the samples were sputter coated with gold under cathodic pulverization in an argon-ion atmosphere (EDWARDs, model S150 Sputter Coater) and examined in a Scanning Electron Microscope, model Neoscope JCM-5000 (JEOL, Peabody, MA, USA).

2.8. Mycotoxin analysis

The corn-based culture medium either with and without *P. pentosaceus* LBM18 was analyzed for mycotoxin production after fungal contamination and incubation for 14 days. Regarding the tests performed with *A. flavus* and *A. nomius*, analysis aiming the detection and quantification of aflatoxin B1/B2 (AFB1/AFB2), as well as aflatoxin G1/G2 (AFG1/AFG2) were performed. To the test evaluating the contamination with *F. verticillioides*, analysis aiming the detection and quantification of fumonisin B1/B2 (FB1/FB2) were executed. After incubation, 5 g of corn-based culture medium of each treatment were separately collected to a 50 mL Falcon tube, smashed and further mixed with 20 mL of: acetonitrile:water solution in a proportion of 84:16 (v/v) for the analysis of aflatoxin B and G, as well as acetonitrile:methanol: water solution (25:25:50, v/v/v) for the analysis of fumonisins and shaked at room temperature for 90 min. The samples were subsequently centrifuged at 1029 *g* for 5 min, and an aliquot of 100 μL of supernatant was transferred to an amber vessel and dried at a heating block under a nitrogen stream. All samples were analyzed using an API 5000 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an Ion Electrospray Ionization (ESI) source in the positive ionization mode. Chromatographic separation was performed at 30 °C with an Eclipse XDB-C8 column (4.6 × 150 mm, 5 μm particle diameter) (Agilent, Palo Alto, USA). For aflatoxins a mobile phase gradient made of a methanol:water:ammonium acetate mixture (95:4:1, *v/v/v*) (solution A) and water:ammonium acetate solution (99:1, v/v) (solution B) was used. For fumonisins, a mobile phase made of acetronitrile:water:acetic acid (49:51:1, v/v/v) was used under isocratic mode at a flow rate of 1.4 mL/min. The recovery, linearity, limits of detection (LOD), and limits of quantification (LOQ) of the applied method were previously validated. A calibration curve of each mycotoxin was prepared using different standard concentration and validated based on its correlation coefficients.

2.9. Aflatoxin gene expression

2.9.1. RNA extraction and cDNA synthesis

Total RNA was extracted from mycelium of *A. nomius* grown in the absence (control group) and presence of *P. pentosaceus* (0.2 mg/mL) using the RNeasy PlantMini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNA synthesis was performed in a Veriti thermal cycler (Applied Biosystems) using the following conditions: hybridization step of 10 min at 25 °C, reverse transcriptase (RT) step of 120 min at 37 °C and 5 min at 85 °C. The cDNA samples were stored at −20 °C.

2.9.2. Quantitative real-time PCR (RT-qPCR)

The transcription profiles of genes related to the biosynthesis of aflatoxins (*aflR*, *aflQ* and *aflD*) were analyzed by using qRT-PCR. β-tubulin (TUB2) gene was used as a reference gene. The primers used in this study were previously designed by Yunes et al. (2020). The qRT-PCR assays were carried out using 2X Power SYBR Green PCR master-mix (Invitrogen, USA), according to the manufacturer recommendations. Primer optimization was performed following the manufacturer guidelines. The comparative ΔΔCt method was used for the analysis of the qRT-PCR including the melting curve (Step One Plus Real time PCR system, Applied Biosystems). The PCR efficiencies for the genes were performed using the protocol suggested by the manufacturer.

2.10. Statistical analysis

Statistically significant differences of the several assays were evaluated by a one-way ANOVA. A significant difference was considered if $p < 0.05$ applying the Tukey multiple-comparisons test. Statistical analyses were performed using Statgraphics Centurion XVI software (The Plains, Virginia, USA).

3. Results and discussion

3.1. Bacterial inoculant

The bacterial inoculant used in the present work was formed by the LAB *Pediococcus pentosaceus* LBM18 in the form of biofilm. The bacterial strain ability to form a non-dispersible, non-pelletable, unattached polymeric biofilm of glycosidic nature (Fig. 1), was detected during the exponential growth at submerged cultivation. Strain self-aggregation capacity was confirmed at its solid state by SEM analysis (Fig. 2-A).

Based on the SEM photomicrographs, a structured community of clustered *P. pentosaceus* LBM18 cells arranged in the form of sheets or amorphous aggregates enclosed by an extracellular polymeric substance (EPS) was observed. These aggregate morphotypes differed from the typical pairs or tetrads cluster shapes known to occur at the planktonic form of most *Pediococcus* strains. Moreover, its unadherent profile seemed to diverge from the behavior previously described for other *P. pentosaceus* strains formers of surface attached biofilms. To properly evaluate bacterial-fungal interactions, culture medium contaminants and biofilm non-associated products formed during biomass production were eliminated using a tangential flow filtration (TFF) system. Furthermore, considering the numerous advantages of freeze-drying in the production and distribution of commercial bacterial inoculant products (such as standardized and consistent cell counts, accurate dosing, extended shelf life, and easy reconstitution through rehydration), the washed biomass was subjected to lyophilization. The bacterial viability at the end of the fermentation (3.5×10^9 CFU/mL), after cell harvest by TFF (1.4×10^{10} CFU/mL) and after freeze-drying process (1.1×10^9 CFU/g), were evaluated to guarantee cell viability and bioactivity before corn-based medium supplementation.

3.2. Fungal morphology, growth, and conidia production

Fig. 2-B shows fungal cultures visualization at the seventh day of incubation in the absence (control) and presence of different concentrations of *P. pentosaceus* LBM18. Changes on fungal physiology were macroscopically visible amongst all tested conditions in comparison to control.

Fungal growth was affected on a bacterial dose-dependent manner. Moreover, the effects were dependent of fungal genera. The highest growth-inhibitory effects occurred for both *Aspergillus* strains at 0.2 mg/mL of *P. pentosaceus* LBM18. Under such supplementation level, the growth of the *Aspergillus* strains was less vigorous, and total biomass production was substantially reduced. *P. pentosaceus* LBM18 strongly influenced *A. flavus* mycelia physiology and conidiogenesis. A substantial reduction on mycelial growth and number of conidiophores were microscopically visible (Fig. 2-C). In addition, the diameter of conidiophore vesicles seemed to be smaller. Similar changes on *A. nomius* mycelial growth were shown to occur (Fig. 2-D). Strain ability to differentiate towards both asexual spores or sclerotia during fungal development was shown to be influenced. The restriction of conidiophores to the center of the fungal colonies (observed at the control condition) was expanded for a wider radial area, suggesting that an earlier conidial heads maturation occurred in the presence of low bacterial inoculant concentrations (Miller, 2011). The production of sclerotia, known to remain in dormant state at unfavorable environment conditions until it germinates into mycelia and produces new conidiophores (Li et al., 2022), seemed to be substantially inhibited. Given its role as fungal survival structures (Li et al., 2017), it was concluded that under such co-culturing conditions, *A. nomius* pathogenicity was capable of being substantiated decreased. Increasing the concentrations of *P. pentosaceus* LBM18 in corn-based medium resulted in a growth biostimulation and a rise on asexual sporulation. Alongside with the increase of both strains' mycelial growth, a strong influence on conidiogenesis itself seemed to occur. These deleterious effects were evidenced by the increase of total fungal biomass production and delay of *A. flavus* conidial heads maturation, that similarly to the control had its conidiophores restricted to the center of the colony. Such effects disagreed with our previous findings on *P. pentosaceus* LBM18 growth inhibitory activity against *A. alternata* co-cultured in corn-based medium (continuous increase of growth inhibition with the increase of bacterial inoculant) (Oliveira et al., 2022). Yet, it may be plausible to postulate that such outcome might be associated to different biotic or abiotic effects related either to a substantial increase on bacterial-fungal competition for nutrients and space or to nutritional changes driven from corn-based medium supplementation with high bacterial concentrations. Contrastingly to the findings made for the *Aspergillus* strains, it was concluded that *P. pentosaceus* LBM18 had no antifungal activity against *F. verticillioides*. Total biomass production by *F. verticillioides* was substantially stimulated in all tested bacterial concentrations in a similar effect to that described for *Aspergillus* strains co-cultured with high bacterial inoculant concentrations. Fig. 2-E shows the morphology of *F. verticillioides* mycelium at the bacterial concentration of 0.2 mg/mL. Identically to the control, a plumpish homogeneous mycelium of smooth surface was observed. Besides the increase of biomass density and aerial hyphae network occurred at higher bacterial concentrations, any

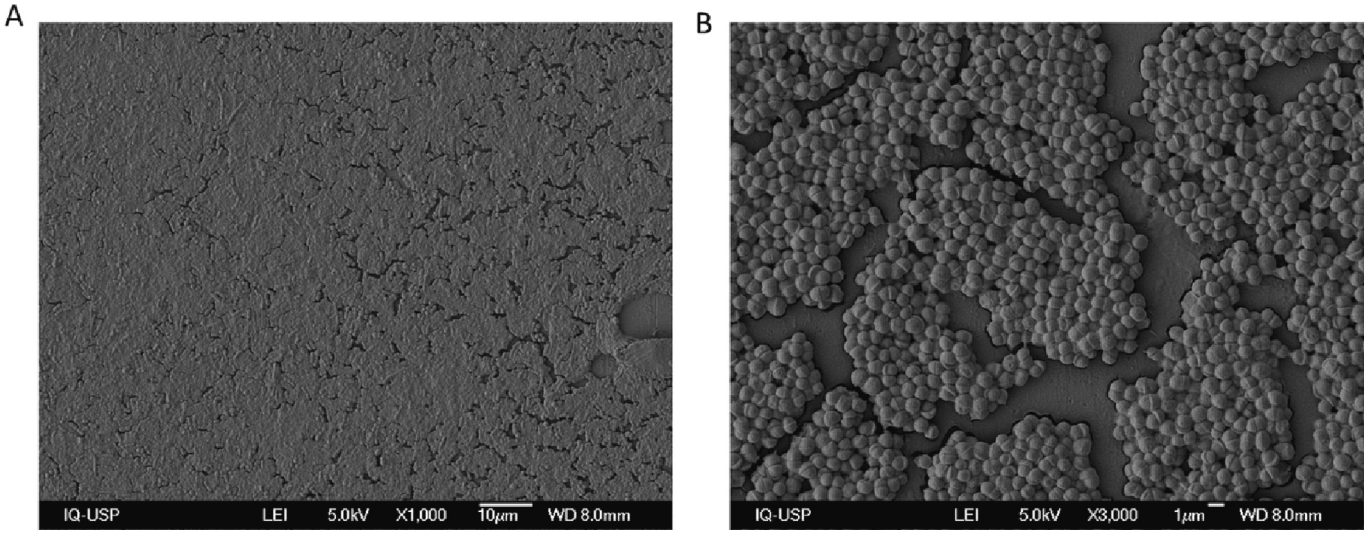

Fig. 1. Biofilm of glycosidic nature produced by *Pediococcus pentosaceus* LBM18. A) Scanning electron microscopy (×1000); B) Scanning electron microscopy (×3000).

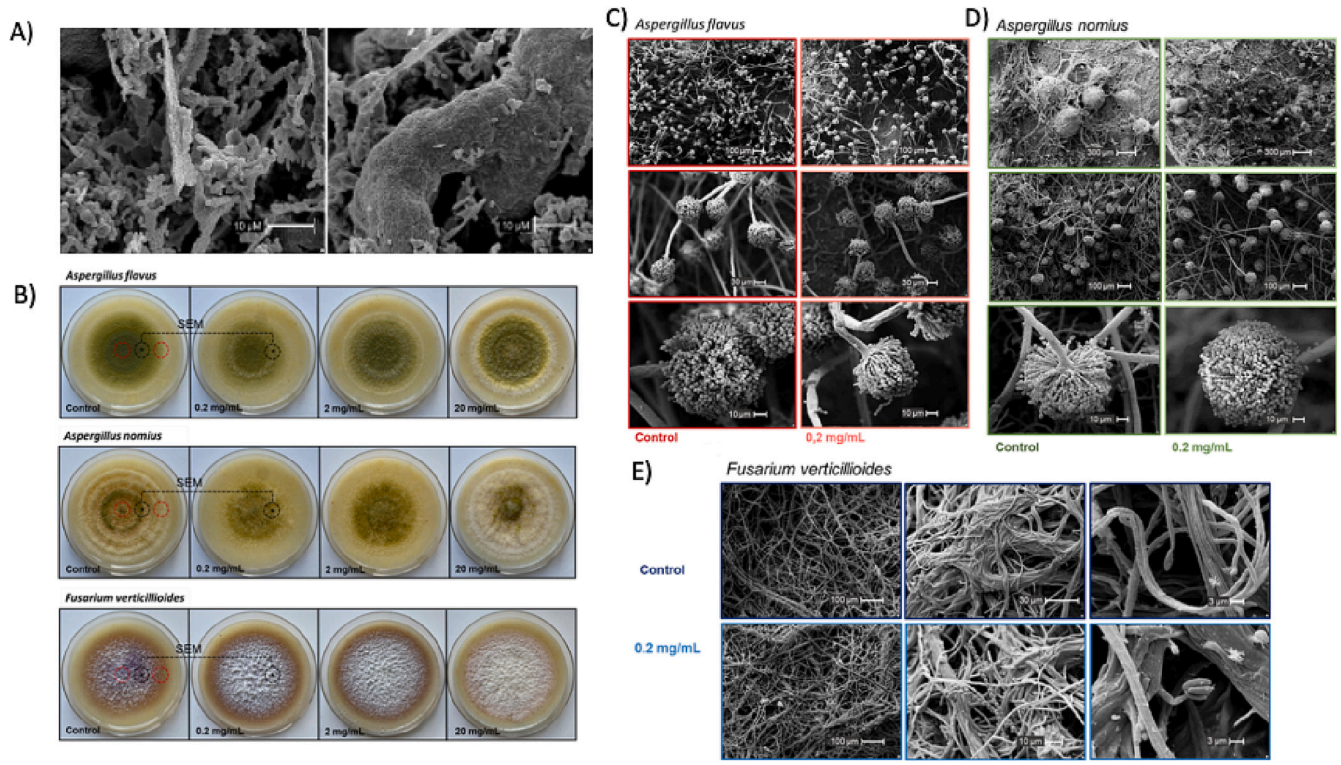

Fig. 2. A) SEM photomicrographs of freeze-dried *P. pentosaceus* LBM18 after purification; B) representative visual images of *A. flavus, A. nomius* and *F. verticillioides* inoculated at a solid corn-based medium in the absence (control) and presence of *P. pentosaceus* LBM18 at the concentrations of, 0.2, 2 and 20 mg/mL at the end of the seventh day of incubation; and SEM photomicrographs of mycelia, hyphae and reproductive structures after growth in the absence (control) and presence of *P. pentosaceus* LBM18 in a concentration of 0.2 mg/mL for C) *A. flavus,* D) *A. nomius* and E) *F. verticillioides.*

noticeable morphological changes were considered to occur.

Aiming to better understand *P. pentosaceus* LBM18 inhibitory effects, the growth of each fungus aerial mycelium radius was daily monitored for up to 7 days after co-cultured. The detailed growth rates data for each fungus in mm/day are presented in Fig. 3-A.

As previously shown on fungal physiology analysis, *P. pentosaceus* LBM18 was unable of totally inhibit the growth of any studied fungi. However, except for the tests performed to *F. verticillioides* at 0.2 and 2 mg/mL of bacterial inoculant where there were no significant differences regarding the control, all tested concentrations induced a mycelium growth rate deceleration in a dose-dependent manner. Significant growth-deceleration effects resulted from the supplementation of both *Aspergillus* strains with *P. pentosaceus* LBM18. Therefore, a significant decrease of approximately 14 % of mycelial growth rate was determined for *A. flavus* (8.12 mm/day at a bacterial inoculant concentration of 2 mg/mL compared to 9.46 mm/day at the control), and significant was the reduction with *A. nomius* (8.93 mm/day at both 0.2 and 2 mg/mL compared to 10.38 mm/day at the control). These growth

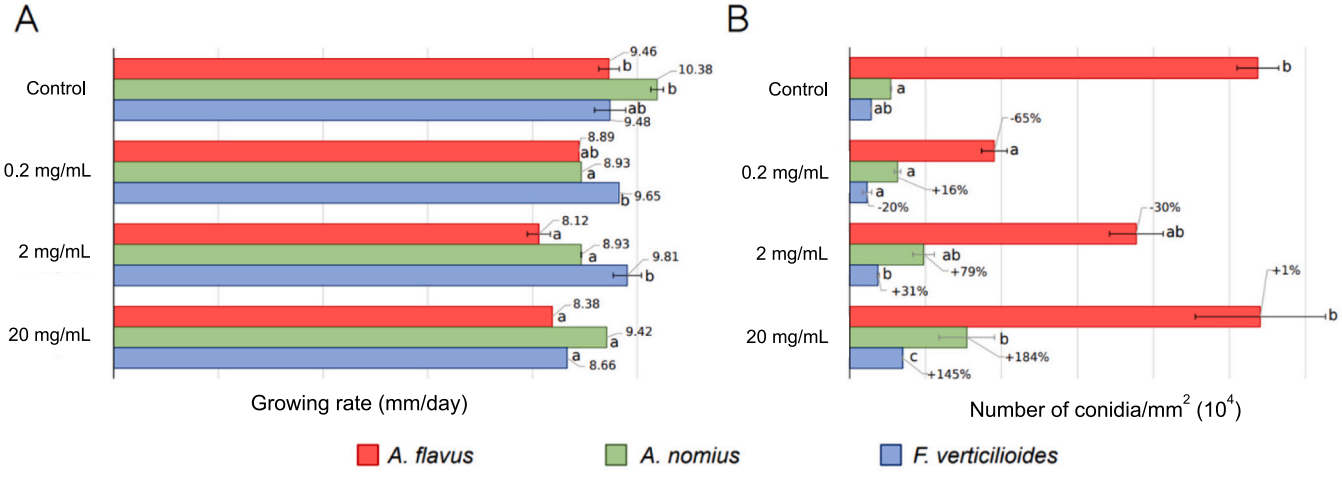

Fig. 3. Comparison of A) the radial mycelium growth rate (mm/day) and B) analysis of conidial production (number of conidia/mm^2) of the non-toxigenic *A. flavus* strain and two toxigenic *A. nomius* and *F. verticillioides* strains grown on solid corn-based medium in the absence (control) and presence of *P. pentosaceus* LBM18. Tests were performed using a bacterial inoculant concentration of 0.2, 2 and 20 mg/mL. Mycelium radius was daily monitored for up to 7 days after co-culture at 26 °C. Bars indicate Average ± Standard Error. The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($p < 0.05$); values with shared letters in the same strain are not significantly different.

deceleration effects were slightly lower than the previously reported for the *A. alternata* strain co-cultured with *P. pentosaceus* LBM18 on corn-based medium (28, 32 and 31 % for 0.2, 2 and 20 mg/mL of bacterial inoculant, respectively) (Oliveira et al., 2022). Yet, the observed trends were in line with the overall fungal growth inhibition effects previously discussed in the physiology analysis section. Once considering the results obtained at the highest tested bacterial inoculant concentration compared to that of 2 mg/mL, a trend shifts over *Aspergillus* strains growth rate deceleration seemed to occur (a decrease of 2.8 and 4.8 % for *A. flavus* and *A. nomius*, respectively). These results agreed with the growth biostimulation occurred at the highest bacterial inoculant concentrations. Regarding the effects of *P. pentosaceus* over mycelium growth of *F. verticillioides*, it was possible to observe that at the bacterial inoculant concentrations of 0.2 and 2 mg/mL rates were kept like the control. However, a decrease of approximately 9 % was noticed to occur at 20 mg/mL of *P. pentosaceus* LBM18. Such low deceleration of fungal growth rate agrees with the lack of antagonistic effects previously described by the bacterial strain against *F. verticillioides*.

To address *P. pentosaceus* LBM18 effects over fungal asexual reproduction, fungal conidiogenesis was assessed after the seventh day of incubation. The quantitative analysis of conidial production presented in Fig. 3-B shows that, similarly to the growth rate, the bacteria effects over fungal sporulation acted under a bacterial dose-dependent manner. At a bacterial concentration of 0.2 and 2 mg/mL, *A. flavus* presented a significant sporulation decrease of approximate 65 and 30 %, respectively. However, no significant differences were observed at 20 mg/mL.

A. nomius showed an increase of spore production under all tested conditions. The highest increase on fungal sporulation occurred at a concentration of 20 mg/mL with a significant enhancement of approximately 184 % compared to the control. However, a reduction of approximate 20 % was observed for *F. verticillioides* at the lowest bacterial concentration tested, which was unexpected given the effects previously described for total biomass and hyphae growth rate, followed by an increment under the remaining conditions. The highest augment on fungal sporulation was observed at the highest concentration of 20 mg/mL with a significant increase of 145 % compared to the control.

Such effects were in an opposite direction than those previously reported for *P. pentosaceus* LBM18 over the inhibition of *A. alternata* asexual reproduction (sporulation reduction of 52,71 and 81 % for 0.2, 2 and 20 mg/mL of bacterial inoculant, respectively) (Oliveira et al., 2022). Additionally, it disagrees with the great majority of scientific findings attesting the action of different LAB metabolites against the inhibition of spore production by different fungal species (Svanström et al., 2013). Despite that, the registered trends agreed with the observation made under fungal culture physiology analysis. Given the relationship between asexual sporulation rate and crops airborne infection (Svanström et al., 2013), the trends registered for *A. nomius* and *F. verticillioides* at high bacterial inoculant concentrations were considered deleterious, specially under the scope of fungal dispersal and contamination management in crops.

3.3. Mycotoxin production

The anti-mycotoxigenic effect of *P. pentosaceus* LBM18 was evaluated as a function of mycotoxin production after 14 days of fungal growth on corn-based medium. According to the performed mycotoxins detection and quantification analysis it was possible to confirm that *A. flavus* strain was incapable of producing aflatoxins either in the absence or presence of *P. pentosaceus* LBM18. Therefore, its previous assignment as non-aflatoxigenic fungi was confirmed. Different atoxigenic *A. flavus* strains are already being used at crops fields as biopesticides applied on the prevention of mycotoxins contamination (Shabeer et al., 2022).

Considering *P. pentosaceus* LBM18 effect over the atoxigenic *A. flavus* mycelium growth rate and sporulation, it may be plausible to consider that some bacterial biopesticides may not be selective enough to act only against fungal toxigenic strains present at grains microbiota. In such scenario, bacterial biopesticides could cause an imbalance in the crops microflora when used in the field, which may disrupt grains microbiota homeostasis and weaken natural competitive exclusion phenomenon (Paasch and He, 2021) fundamental for grains natural biopreservation before and after harvest (Mousa et al., 2015). The ability of *A. nomius* strain to produce aflatoxins (AFB1, AFB2, AFG1 and AFG2) on corn-based medium was validated (Fig. 4). Thus, its previous established toxigenic profile was confirmed (Yunes et al., 2020).

According to the quantified levels of total mycotoxins production, it was concluded that the effects induced by *P. pentosaceus* LBM18 against *A. nomius* occurred in a significant bacterial dose-dependent manner. Although not capable of completely inhibiting *A. nomius* from producing mycotoxins, *P. pentosaceus* LBM18 could induce a significant reduction of approximately 55 % of the total aflatoxin's biosynthesis at a concentration of 0.2 mg/mL. Such decrease of production was in agreement with the reduction of *A. nomius* total biomass production and growth rate deceleration, and is in line with the literature evidences describing the existence of a direct relationship between the fungal growth inhibition and the reduction of mycotoxin production (Sadiq et al., 2019). In addition, such decay was in conformity with the many studies showing LAB ability to arrest fungal growth and decrease mycotoxin production through the combination of bacterial competition for space and nutrients as well as the production of different antifungal metabolites (*e.g.* reuterin, cyclic dipeptides, carboxylic acids, fatty acids, organic acids and many others) (Guimarães et al., 2018). By ten-fold increasing the bacterial concentration, the decrease of mycotoxin production was incapable of reaching the extents detected at 0.2 mg/mL of *P. pentosaceus* LBM18. Yet, a significant decrease of 39 % of the total aflatoxin contamination occurred. Despite still lower than control, a fall of 16 % of the inhibitory effect was noticed comparatively to the tests using the lowest bacterial supplementation. These findings agree with the slight inhibition decay of fungal growth and sporulation occurred by rising ten-fold the *P. pentosaceus* LBM18 concentration. By the hundredfold increase of bacterial inoculant, fungal pathogenicity and fitness was enhanced up to a level at which *P. pentosaceus* LBM18 no longer succeeded to decrease mycotoxins contamination. Under such co-culturing conditions, a significant increase of approximately 95 % of total aflatoxin contamination was detected. Such result supports the existence of a relationship between mycotoxin production and the improvement of toxigenic fungal fitness and pathogenicity, previously evidenced in literature reports (López-Díaz et al., 2018). Studies have demonstrated that different stress-related conditions (*e.g.* temperature, water activity, light, oxidative stress, pH and nutritional conditions), occurring during mycelial growth could increase fungal virulence and strongly modulate the production of mycotoxins at the end of toxigenic fungal growth stage (Reverberi et al., 2010).

Although further studies are needed to fully understand the microbial synergetic interactions leading to the up-regulation of mycotoxin production by LAB strains, it is possible to postulate that scarcer environmental nutritional conditions imposed by high initial bacterial concentration may be perceived by *A. nomius* as a threat to its survival (Rangel et al., 2008). Thus, responses regarding viable cells dissemination could be triggered due to nutritional-stress in order to promote the colonization of new niches with a higher nutritional composition (Rangel et al., 2008). Under such scenario, *A. nomius* would promote a substantially increase of mycelial, conidia and sclerotial production, enhancing its chances of persisting at new less unfavorable environment. Despite unknowing the reliability of these considerations, it is highly probable that the increase of mycotoxin production by *A. nomius* ultimately targeted the elimination of the bacteria surrounding fungi microhabitat. Either based on competition for space, nutrients, or other specific stress-related phenomenon, it should be mentioned that the exact mechanisms of action through which mycotoxins may inhibit the growth of LAB present in fungal surroundings is still uncertain. Taking into account the distinct production pattern of each aflatoxin type by different fungi strains at varying environmental conditions (Frisvad

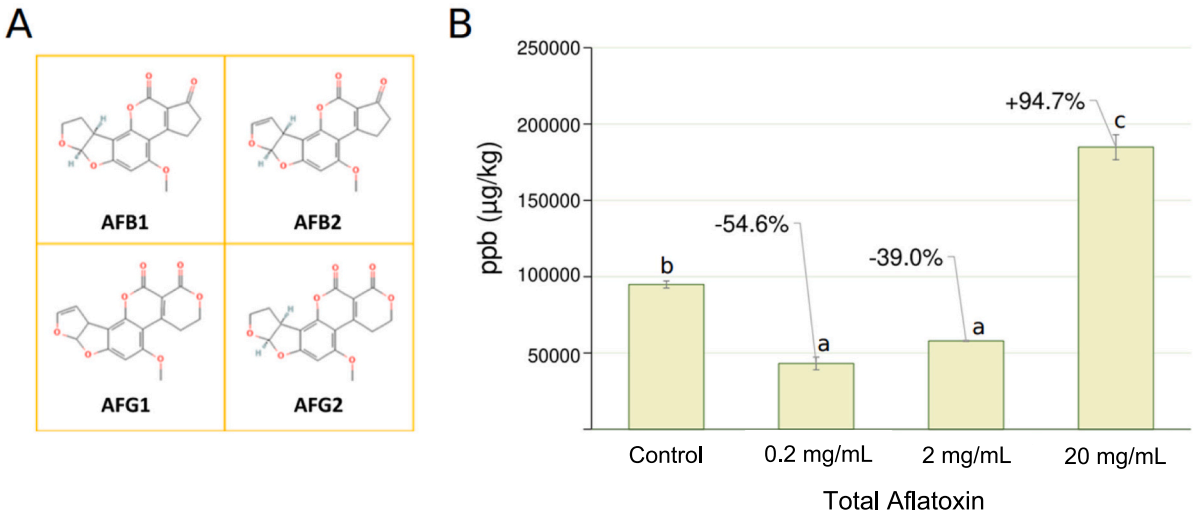

Fig. 4. Total aflatoxin contamination levels detected after 14 days of *A. nomius* growth at the corn-based medium in the absence (control) and presence of *P. pentosaceus* LBM18 over the concentrations of 0.2, 2 and 20 mg/mL at 26 °C. A) aflatoxin B1, B2, G1 and G2 chemical structure. B) *A. nomius* total aflatoxin levels. The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($p < 0.05$); values with shared letters in the same graph are not significantly different.

et al., 2005) and the differences on the lethality features and permissible limits within each of these forms (Battilani et al., 2012), the careful analysis of its specific quantification levels are of extreme importance. According to the specific aflatoxins quantification levels presented in Fig. 5, *P. pentosaceus* LBM18 was unable of completely inhibit the production of any mycotoxin under evaluation.

Within the four studied aflatoxin types, AFG1 presented the maximum decrease of contamination at a concentration of 0.2 mg/mL, with a significant reduction from 68,250 to 25,250 µg/kg. Contrastingly, the aflatoxin with minimum reduction of contamination was the AFB1, with a no significant reduction from 26,225 to 17,600 µg/kg. At such bacterial inoculant concentration, the decrease of contamination in descending order of percentage proportion described by each mycotoxin was listed as AFG1 < AFB2 < AFG2 < AFB1, under a production inhibition effect of approximately 63, 50, 40 and 33 %, respectively. At the concentration of 2 mg/mL of bacterial inoculant, AFB1 and AFG2 had their contamination levels still decreased. Even though smaller than at the lowest tested bacterial inoculum, these mycotoxins presented, respectively, no significant decrease of approximately 49 and 15 % compared to the control. Regarding aflatoxins AFB2 and AFG2, a no significant increment of 75 and 20 % was respectively determined. The fact that the contamination by these last two mycotoxins was hundredfold lower than the levels detected for AFB1 and AFG1, such substantial rise of contamination was not capable of fully affect *P. pentosaceus* LBM18 overall decrease effect at 2 mg/mL. Yet, deep, and careful considerations should always be made in such cases, so that by favoring the total mycotoxin decrease we will not neglect the increase of any mycotoxin over its permissible or lethal limits. At the concentration of 20 mg/mL of *P. pentosaceus* LBM18 all aflatoxins suffered an increase of production.

Interestingly, the aflatoxins produced in lower concentration at the control test (AFB2 and AFG2), were those registering the highest contamination rise (from 200/250 to 1550/1800 µg/kg, respectively). Such effect was described in descending order of percentage proportion by AFB2 < AFG2 < AFB1 < AFG1, which was equivalent to a significant increase of 675, 620, 142 and 73 %, respectively. According to the

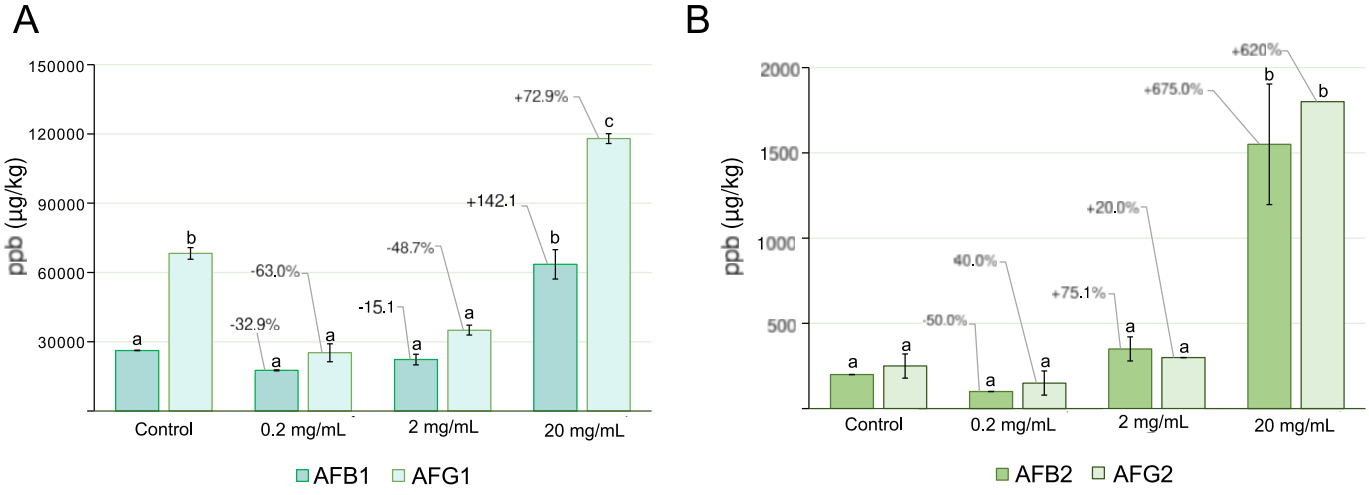

Fig. 5. Specific detection and quantification of aflatoxins produced by *A. nomius* after 14 days of growth at a solid corn-based medium in the absence (control) and presence of *P. pentosaceus* LBM18 over the concentrations of 0.2, 2 and 20 mg/mL at 26 °C. The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($p < 0.05$); values with shared letters in the same aflatoxin are not significantly different.

obtained dose-response results the tested bacterial concentration with highest effectiveness over the inhibition of total and specific aflatoxin production was that of 0.2 mg/mL. Concentrations of *P. pentosaceus* LBM18 equal or higher than 2 mg/mL was considered detainers of high safety risk if used on the management aflatoxin contamination. Fig. 6 shows total and specific levels of fumonisins FB1 and FB2 produced by *F. verticillioides* strain after 14 days of growth.

Based on the quantified mycotoxin contamination levels it was possible to conclude that *F. verticillioides* was incapable of producing the studied mycotoxins on corn-based medium. These results were contrary to the strain mycotoxigenic profile previous assigned during fungal isolation and characterization (Reis et al., 2020). Therefore, the alteration of strain post-isolation toxigenic status might have taken place due to changes on growth medium nutrient composition and incubation conditions (Kokkonen et al., 2010). Being so, it supports the evidences that mycotoxins are ecological metabolites and that fumonisin production by toxigenic fungi may vary according to certain environmental and nutritional factors (Milani, 2013). Even though, at corn-based medium supplementation with 0.2 and 2 mg/mL of *P. pentosaceus* LBM18, *F. verticillioides* retaken its ability to produce both fumonisin FB1 and FB2. These observations were in line with the substantial increase of fungus total biomass previously discussed in Section 3.1.

Therefore, it is possible to consider that the presence of *P. pentosaceus* LBM18 at fungi microhabitat surrounding might have activated, in a similar fashion than discussed for *A. nomius* behavior, a stress-induced response that triggered the reactivation of *F. verticillioides* mycotoxin production. Such considerations are supported by the findings of Dalié et al. (2012), which reported a considerably rise of production of these mycotoxins by a strain of *F. verticillioides* grown in the presence of *P. pentosaceus* L006 strain (Dalié et al., 2012). Although difficult to describe at the physiological and molecular level the exact set of conditions or triggers responsible for stimulate either the activation or deactivation of fumonisin production, it is expected that similar triggering effects would occur in the field. Thus, changes on transient atoxigenic status of different toxigenic strains naturally present at grains fungal flora could occur. Such consideration may further support the importance of ecological assessments regarding bacterial biopesticides effects prior its use in the fields.

By carefully analyzing the specific quantification levels of the two fumonisins under study, it was observed that FB1 described the highest increase of contamination, reaching the amounts of 1415 and 1135 μg/kg for 0.2 and 2 mg/mL of bacterial inoculant, respectively. Regarding FB2, the extent of the induced contamination level reached the amounts of 202 and 161 μg/kg, respectively. Interestingly, a fall of approximately 20 % of both mycotoxins occurred at the ten-fold increase of *P. pentosaceus* LBM18 concentration, comparatively to the lowest bacterial concentration tested. In spite of that, FB1 and FB2 concentrations remained conforming to the acceptable upper permissible limits of contamination at cereal based products by the US and the European Union (800–4000 and 2000–4000 μg/kg FB1 and FB2, respectively) (Anfossi et al., 2016). Nonetheless, at 20 mg/mL of bacterial inoculant no detection of FB1 and FB2 was observed, meaning that at high concentrations of *P. pentosaceus* LBM18 fumonisin contamination was totally suppressed. Given the substantial increase of fungus biomass density at hundredfold bacterial inoculant concentration, it was concluded that *P. pentosaceus* LBM18 effects over suppression of fumonisins contamination did not include a dependence on fungal growth decrease, even though a reduction of mycelial growth rate by 9 % was observed.

Therefore, the suppression of fumonisin contamination was thought to be result of a combination of several biotic and abiotic factors. Although the available information regarding alternative mechanisms behind the suppression of such toxic metabolites, biosynthesis excluding the arrestment of fungal growth are scarce in literature, few studies have described the ability of organic acids to modulate mycotoxin production without substantial affecting fungal growth (*e.g.* reduction of aflatoxin

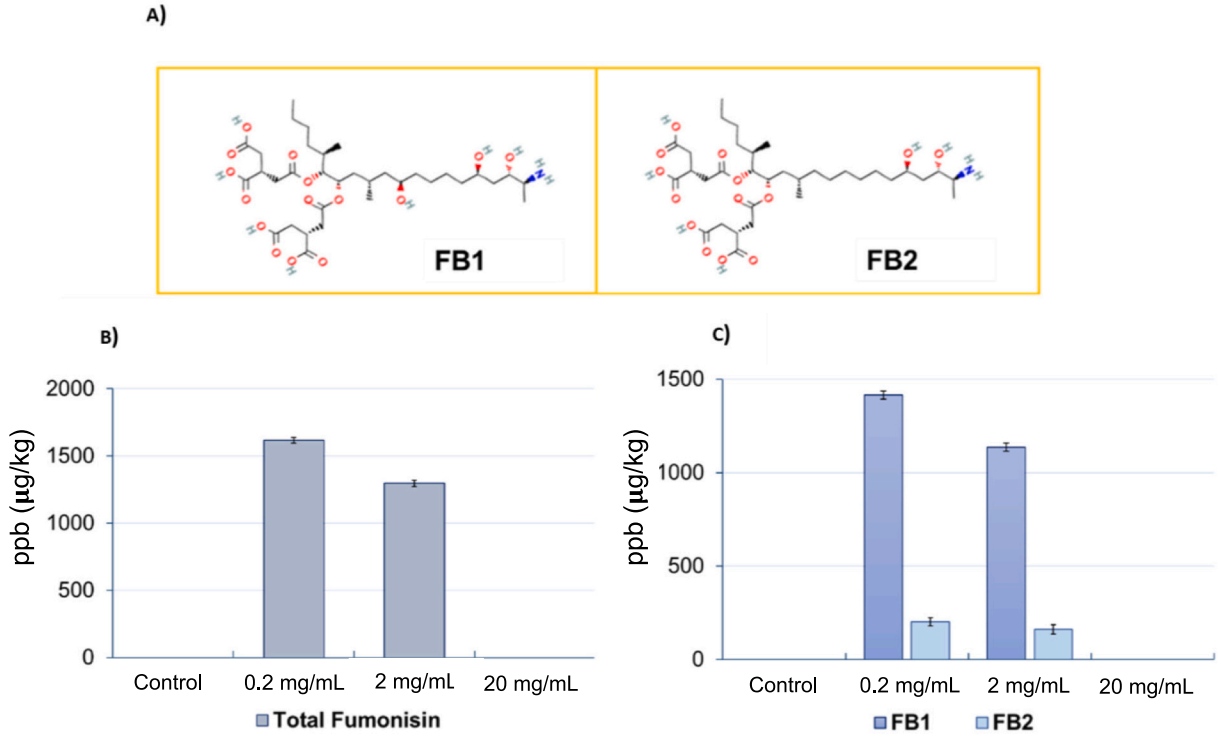

Fig. 6. Production of fumonisins (FB1 + FB2) by *Fusarium verticillioides* after 14 days of growth at a solid corn-based medium in the absence (control) and presence of *P. pentosaceus* LBM18 over the concentrations of 0.2, 2 and 20 mg/mL at 26 °C. A) Fumonisin B1 and B2 chemical structure. B) *F. verticillioides* total aflatoxin production. C) fumonisin B1 and B2 after *F. verticillioides* growth.

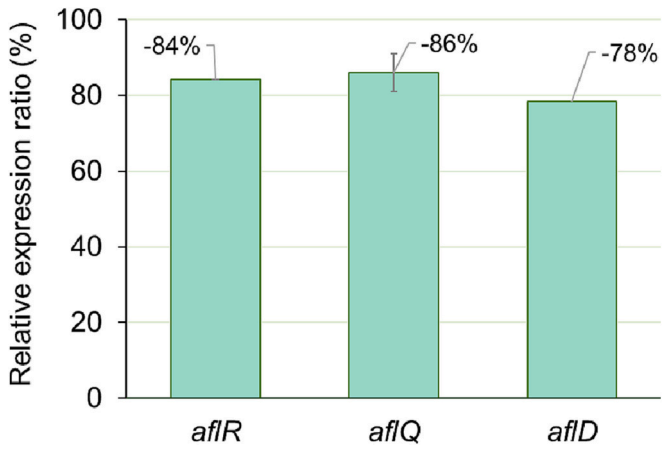

Fig. 7. Effect of *P. pentosaceus* LBM18 on transcriptional level of genes related to aflatoxin biosynthesis by *A. nomius*.

production by 99 % while reducing by only 15 % the fungal growth). Yet, more studies are needed to fully clarify the exact microbial interactions and molecular mechanisms contributing to the registered outcomes.

3.4. Analysis of mycotoxin gene expression

Despite the substantial focus given to the modulation of *A. flavus* mycotoxin biosynthesis and gene expression by different abiotic factors (Al-Jaza et al., 2022), there is a deficit of studies focused on the modulation of mycotoxin producing genes in *A. nomius*. Once being regulated majorly at transcriptional level (Wang et al., 2022), transcriptional genes down-regulation studies involving aflatoxin biosynthesis were performed in order to provide deeper insights over the mechanisms underlying *P. pentosaceus* LBM18 action over downregulation of aflatoxin production by the *A. nomius* strain. Such evaluation was performed at the concentration of 0.2 mg/mL of bacterial inoculant, previously proven to promote the highest inhibition of total and specific aflatoxin production in corn-based medium.

To accomplish that, RT-qPCR assays were performed aiming to access at the molecular level the relative expression of a transcriptional gene regulator, *aflR*, and of two structural genes, *aflD* and *aflQ*, involved in aflatoxin production. All validation parameters tested (*e.g.* primer efficiencies, compatibility between the tested genes, and melt-curve analysis) showed acceptable results, allowing the application of the ΔΔCt method. As shown in Fig. 7, all tested genes under evaluation were down regulated in the presence of *P. pentosaceus* LBM18 comparatively to the control group.

The expression of *aflR*, *aflD* and *aflQ* was reduced in approximately 84, 78 and 86 %, respectively. These results demonstrated that the anti-mycotoxigenic properties of *P. pentosaceus* LBM18 could be related to the modulation of important genes intrinsically associated to aflatoxin production, which may have directly impact on reduction of these mycotoxins contamination. Moreover, it supports the increasing evidence that bacterial-fungal interactions can affect transcriptional gene regulation that in turn can modulate mycotoxin production.

4. Conclusion

Continuous efforts to develop new management and preventive practices for the reduction or avoidance of mycotoxin contamination levels in food commodities are critical for future global food safety. Despite acknowledging the limited number of tested fugal strains and the lack of similarities between the performed *in vitro* tests and real ecological systems (known for their many occurring arbitrary variables), it seems plausible to consider that bacterial biopesticides misuse might present several deleterious effects over the so-aimed decrease of mycotoxin contamination in food commodities. Either during the development of future commercial bacterial biopesticides or during their use, all biocontrol approaches may take into consideration the complex net of intrinsic interactions occurring within the biological control agents and the specific ecological niches where they are meant to be applied. Moreover, ecological evaluations regarding the effects of these bacterial biopesticides on atoxigenic fungal competitors present in grain microbiota should be accessed to avoid weakening the competitive exclusion phenomenon fundamental for grain natural biopreservation before and after harvest. Furthermore, changes in the transient atoxigenic status of different toxigenic strains naturally present in grain fungal flora might occur in specific cases due to the onset of mycotoxin production triggered by bacterial biopesticide stress-related induced effects. Although challenging, the understanding of the synergic effects occurring between bacteria-fungi interactions is considered essential for both guarantying product efficacy and ecological safety. The performed assessment has revealed that the safety and suitability of *Pediococcus pentosaceus* LBM18 are specifically dependent on its concentrations, usage scenarios, and exposure conditions. Therefore, further comprehensive evaluation of its potential efficacy and safety under biocontrol strategies in real-world settings would be required in order to obtain regulatory approvals for its future commercial use.

CRediT authorship contribution statement

Carlos M.N. Mendonça: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Rodrigo C. Oliveira:** Investigation, Methodology. **Lucas J.L. Pizauro:** Methodology, Formal analysis. **Wellison A. Pereira:** Methodology, Data curation. **Kahlile Abboud:** Data curation, Writing – review & editing. **Sonia Almeida:** Formal analysis, Methodology. **Ii-Sei Watanabe:** Formal analysis, Methodology, Resources. **Alessandro M. Varani:** Resources, Methodology, Writing – review & editing. **José M. Domínguez:** Writing – review & editing. **Benedito Correa:** Resources, Writing – review & editing. **Koen Venema:** Resources, Writing – review & editing. **Pamela O.S. Azevedo:** Conceptualization, Methodology, Resources, Writing – review & editing. **Ricardo P.S. Oliveira:** Conceptualization, Supervision, Project administration, Funding acquisition, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The research described in the article used no data.

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References

Al-Jaza, D., Medina, A., Magan, N., 2022. Abiotic factors affect growth and aflatoxin B1 production by *aspergillus flavus* strains on chilli powder and red chillies. World Mycotoxin J. 15 (3), 251–260. https://doi.org/10.3920/WMJ2021.2715.

Anfossi, L., Giovannoli, C., Baggiani, C., 2016. Mycotoxin detection. Curr. Opin. Biotechnol. 37, 120–126. https://doi.org/10.1016/J.COPBIO.2015.11.005.

Assaf, J.C., Atoui, A., Khoury, A. El, Chokr, A., Louka, N., 2018. A comparative study of procedures for binding of aflatoxin M1 to *Lactobacillus rhamnosus* GG. Braz. J. Microbiol. 49 (1), 120–127. https://doi.org/10.1016/J.BJM.2017.05.003.

Azevedo, P.O.S., Mendonça, C.M.N., Moreno, A.C.R., Bueno, A.V.I., Almeida, S.R.Y., Seibert, L., Converti, A., Watanabe, I.-S., Gierus, M., Oliveira, R.P.S., 2020. Antibacterial and antifungal activity of crude and freeze-dried bacteriocin-like inhibitory substance produced by *Pediococcus pentosaceus*. Sci. Rep. 10 (1), 12291. https://doi.org/10.1038/s41598-020-68922-2.

Bata, Á., Lásztity, R., 1999. Detoxification of mycotoxin-contaminated food and feed by microorganisms. Trends Food Sci. Technol. 10 (6–7), 223–228. https://doi.org/10.1016/S0924-2244(99)00050-3.

Battilani, P., Rossi, V., Giorni, P., Pietri, A., Gualla, A., van der Fels-Klerx, H.J., Booij, C.J. H., Moretti, A., Logrieco, A., Miglietta, F., Toscano, P., Miraglia, M., Santis, B. De, Brera, C., 2012. Modelling, predicting and mapping the emergence of aflatoxins in cereals in the EU due to climate change. EFSA Support. Publ. 9 (1), 223E. https://doi.org/10.2903/SP.EFSA.2012.EN-223.

Dalié, D., Pinson-Gadais, L., Atanasova-Penichon, V., Marchegay, G., Barreau, C., Deschamps, A., Richard-Forget, F., 2012. Impact of *Pediococcus pentosaceus* strain L006 and its metabolites on fumonisin biosynthesis by Fusarium verticillioides. Food Control 23 (2), 405–411. https://doi.org/10.1016/J.FOODCONT.2011.08.008.

Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. Nat. Rev. Microbiol. 14 (9), 563–575. https://doi.org/10.1038/nrmicro.2016.94.

Frisvad, J.C., Skouboe, P., Samson, R.A., 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. Syst. Appl. Microbiol. 28 (5), 442–453. https://doi.org/10.1016/J.SYAPM.2005.02.012.

Guimarães, A., Santiago, A., Teixeira, J.A., Venâncio, A., Abrunhosa, L., 2018. Anti-aflatoxigenic effect of organic acids produced by *Lactobacillus plantarum*. Int. J. Food Microbiol. 264, 31–38. https://doi.org/10.1016/J.IJFOODMICRO.2017.10.025.

Kavitake, D., Singh, S.P., Kandasamy, S., Devi, P.B., Shetty, P.H., 2020. Report on aflatoxin-binding activity of galactan exopolysaccharide produced by *Weissella confusa* KR780676. 3. Biotech 10 (4), 1–4. https://doi.org/10.1007/S13205-020-02173-W/FIGURES/3.

Khezri, M., Ahmadzadeh, M., Jouzani, G.S., Behboudi, K., Ahangaran, A., Mousivand, M., Rahimian, H., 2011. Characterization of some biofilm-forming *Bacillus subtilis* strains and evaluation of their biocontrol potential against *Fusarium culmorum*. J. Plant Pathol. 93 (2), 373–382. https://doi.org/10.4454/jpp.v93i2.1192.

Kokkonen, M., Ojala, L., Parikka, P., Jestoi, M., 2010. Mycotoxin production of selected *Fusarium* species at different culture conditions. Int. J. Food Microbiol. 143 (1–2), 17–25. https://doi.org/10.1016/J.IJFOODMICRO.2010.07.015.

Kubota, H., Senda, S., Nomura, N., Tokuda, H., Uchiyama, H., 2008. Biofilm formation by lactic acid bacteria and resistance to environmental stress. J. Biosci. Bioeng. 106 (4), 381–386. https://doi.org/10.1263/JBB.106.381.

Li, B., Tian, X., Wang, C., Zeng, X., Xing, Y., Ling, H., Yin, W., Tian, L., Meng, Z., Zhang, J., Guo, S., 2017. SWATH label-free proteomics analyses revealed the roles of oxidative stress and antioxidant defensing system in sclerotia formation of Polyporus umbellatus. Sci. Rep. 7 (1), 1–13. https://doi.org/10.1038/srep41283.

Li, B., Liu, L., Shan, T., Xing, Y., Guo, S., 2022. Acetylome analysis of acetylation providing new insight into sclerotial generation in medicinal fungus *Polyporus umbellatus*. Sci. Rep. 12 (1), 1–11. https://doi.org/10.1038/s41598-022-11798-1.

López-Díaz, C., Rahjoo, V., Sulyok, M., Ghionna, V., Martín-Vicente, A., Capilla, J., Di Pietro, A., López-Berges, M.S., 2018. Fusaric acid contributes to virulence of *Fusarium oxysporum* on plant and mammalian hosts. Mol. Plant Pathol. 19 (2), 440–453. https://doi.org/10.1111/MPP.12536.

Milani, J.M., 2013. Ecological conditions affecting mycotoxin production in cereals: a review. Vet. Med. 58 (8), 405–411. https://doi.org/10.17221/6979-VETMED.

Miller, J.J., 2011. The metabolism of yeast sporulation: v. Stimulation and inhibition of sporulation and growth by nitrogen compounds. Can. J. Microbiol. 9 (2), 259–277. https://doi.org/10.1139/M63-032.

Mousa, W.K., Shearer, C.R., Limay-Rios, V., Zhou, T., Raizada, M.N., 2015. Bacterial endophytes from wild maize suppress *fusarium graminearum* in modern maize and inhibit mycotoxin accumulation. Front. Plant Sci. 6, 805. https://doi.org/10.3389/FPLS.2015.00805/BIBTEX.

Ogunade, I.M., Martinez-Tuppia, C., Queiroz, O.C.M., Jiang, Y., Drouin, P., Wu, F., Vyas, D., Adesogan, A.T., 2018. Silage review: mycotoxins in silage: occurrence, effects, prevention, and mitigation. J. Dairy Sci. 101 (5), 4034–4059. https://doi.org/10.3168/JDS.2017-13788.

Oliveira, R.C., Mendonça, C.M.N., Veríssimo, N.V., de Almeida, S.R.Y., Correa, B., Watanabe, I.-S., De Azevedo, P.O.S., Oliveira, R.P.S., 2022. Evaluating the potential of as a biocontrol agent against tenuazonic acid-producing on livestock feeds. J. Appl. Microbiol. https://doi.org/10.1111/jam.15746.

Paasch, B.C., He, S.Y., 2021. Toward understanding microbiota homeostasis in the plant kingdom. PLoS Pathog. 17 (4), e1009472 https://doi.org/10.1371/JOURNAL.PPAT.1009472.

Quetta Balochistan, D., Kakar, N., Kakar, A., Shahzad Sheikh, I., Din, M., Ahmed Khan, M., ur Rehman, F., Khan, A., Ahmed, R., Rehman, N., Shakeel, M., Mohammad, B., Hameed Shakir, A., Khan Kakar, D., 2022. Mycotoxins in dairy feed and its harmful impact on animal health: diagnostic aids and treatment: a big animal health challenge. Res. J. Chem. Pharm. 02 (01), 001–009. https://doi.org/10.53022/oarjcp.2022.2.1.0032.

Rangel, D.E.N., Alston, D.G., Roberts, D.W., 2008. Effects of physical and nutritional stress conditions during mycelial growth on conidial germination speed, adhesion to host cuticle, and virulence of *Metarhizium anisopliae*, an entomopathogenic fungus. Mycol. Res. 112 (11), 1355–1361. https://doi.org/10.1016/J.MYCRES.2008.04.011.

Reis, T.A., Oliveira, T.D., Zorzete, P., Faria, P., Correa, B., 2020. A non-toxigenic *Aspergillus flavus* strain prevents the spreading of *Fusarium verticillioides* and fumonisinas in maize. Toxicon 181, 6–8. https://doi.org/10.1016/j.toxicon.2020.04.091.

Reverberi, M., Ricelli, A., Zjalic, S., Fabbri, A.A., Fanelli, C., 2010. Natural functions of mycotoxins and control of their biosynthesis in fungi. Appl. Microbiol. Biotechnol. 87 (3), 899–911. https://doi.org/10.1007/S00253-010-2657-5/FIGURES/2.

Sadiq, F.A., Yan, B., Tian, F., Zhao, J., Zhang, H., Chen, W., 2019. Lactic acid bacteria as antifungal and anti-mycotoxigenic agents: a comprehensive review. Compr. Rev. Food Sci. 18 (5), 1403–1436. https://doi.org/10.1111/1541-4337.12481.

Shabeer, S., Asad, S., Jamal, A., Ali, A., 2022. Aflatoxin contamination, its impact and management strategies: an updated review. Toxins 14 (5), 307. https://doi.org/10.3390/TOXINS14050307.

Svanström, Å., Boveri, S., Boström, E., Melin, P., 2013. The lactic acid bacteria metabolite phenyllactic acid inhibits both radial growth and sporulation of filamentous fungi. BMC Res. Notes 6 (1), 1–9. https://doi.org/10.1186/1756-0500-6-464/FIGURES/6.

Tatsaporn, T., Kornkanok, K., 2020. Using potential lactic acid bacteria biofilms and their compounds to control biofilms of foodborne pathogens. Biotechnol. Rep. 26, e00477 https://doi.org/10.1016/J.BTRE.2020.E00477.

Wang, P., Xu, J., Chang, P.-K., Liu, Z., Kong, Q., 2022. New insights of transcriptional regulator AflR in aspergillus flavus physiology. Microbiol. Spectr. 10 (1), e0079121 https://doi.org/10.1128/spectrum.00791-21.

Yuan, X., Yang, X., Wang, W., Li, J., Dong, Z., Zhao, J., Shao, T., 2022. The effects of natamycin and hexanoic acid on the bacterial community, mycotoxins concentrations, fermentation profiles, and aerobic stability of high moisture whole-crop corn silage. Anim. Feed Sci. Technol. 286, 115250 https://doi.org/10.1016/J.ANIFEEDSCI.2022.115250.

Yunes, N.B.S., Oliveira, R.C., Reis, T.A., Baquião, A.C., Rocha, L.O., Correa, B., 2020. Effect of temperature on growth, gene expression, and aflatoxin production by *Aspergillus nomius* isolated from Brazil nuts. Mycotoxin Res. 36, 173–180. https://doi.org/10.1007/s12550-019-00380-w.

Zorzete, P., Baquiao, A.C., Atayde, D.D., Reis, T.A., Gonçalvez, E., Correa, B., 2013. Mycobiota, aflatoxins and cyclopiazonic acid in stored peanut cultivars. Food Res. Int. 52 (1), 380–386. https://doi.org/10.1016/j.foodres.2013.03.029.

Zorzete, P., Oliveira, R.C., Correa, B., 2016. Effects of conventional and high-oleic peanut medium on extracellular enzyme production and mycelial growth of *aspergillus flavus*. J. Food Saf. 36 (4) https://doi.org/10.1111/jfs.12262.