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Interaction of *Aspergillus flavus* and *A. parasiticus* with *Salmonella* spp. isolated from peanuts

Aline Morgan von Hertwig[a], Beatriz Thie Iamanaka[b], Dionísio Pedro Amorim Neto[a], Josiane Bueno de Rezende[b], Ligia Manoel Martins[b], Marta Hiromi Taniwaki[b], Maristela Silva Nascimento[a,*]

[a] Faculty of Food Engineering, University of Campinas, Campinas, SP, Brazil
[b] Institute of Food Technology - ITAL, Campinas, SP, Brazil

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ABSTRACT

Although *Aspergillus flavus* and *Aspergillus parasiticus* are the main microorganisms of concern in peanuts, due to aflatoxin contamination, several *Salmonella* outbreaks from this product have been reported over the last ten decades. Thus, it is important to understand the relationship between microorganisms to predict, manage and estimate the diversity in the peanut supply chain. The purpose of this study was to evaluate aflatoxin production during the co-cultivation of *Aspergillus* section *Flavi* and *Salmonella* both isolated from peanuts. Three strains of *A.* section *Flavi*: *A. flavus* producing aflatoxin B, *A. flavus* non-producing aflatoxin and *A. parasiticus* producing aflatoxin B and G were co-cultivated with seven serotypes of *Salmonella* of which six were isolated from the peanut supply chain (S. Muenster, S. Miami, S. Glostrup, S. Javiana, S. Oranienburg and S. Yoruba) and one was S. Typhimurium ATCC 14028. First of all, each *Salmonella* strain was inoculated by pour plate (ca. 5 log cfu/mL) in PDA (potato dextrose agar). Then, each pre-cultured fungus was inoculated in the center of the petri dish. The plates were incubated at 30 °C and the fungal colony diameter was measured once a day for 7 days. As a control each *Aspergillus* strain was cultivated in the absence of *Salmonella* culture. All three strains of *Aspergillus* with absence of *Salmonella* (control) reached the maximum colony diameter and their growth rate was influenced when co-cultivated ($p < 0.05$) with all *Salmonella* serotypes tested. The maximum inhibition in the colony diameter was 20% for *A. flavus* aflatoxin B producer and *A. parasiticus*, and 18% for *A. flavus* non- aflatoxin producer when cultivated with *Salmonella*. However, no significant difference ($p < 0.05$) in reduction of colony diameter was observed among the *Salmonella* serotypes. Aflatoxin production was determined previously, by using the agar plug technique on thin layer chromatography (TLC). The production of aflatoxin G by *A. parasiticus* in co-cultivation with *Salmonella* was not observed. On the other hand, *A. flavus* preserved their characteristics of aflatoxin B production. The quantification of aflatoxin reduction by *Salmonella* interaction was evaluated using HPLC method. There was a maximum reduction of aflatoxin production of 88.7% and 72.9% in *A. flavus* and *A. parasiticus,* respectively, when cultivated with *Salmonella*. These results indicate that some serotypes of *Salmonella* may interfere with aflatoxin production and fungal growth of *A. flavus* and *A. parasiticus* in the peanut supply chain.

1. Introduction

Aflatoxigenic fungi contamination in peanuts have been reported by several studies around the world (Gonçalez et al., 2008; Kamika et al., 2014; Martins et al., 2017; Xue et al., 2003), *Aspergillus flavus* and *Aspergillus parasiticus* usually infect peanuts when they are still on the ground, since these species are commensals in the environment (Pitt et al., 2013). Aflatoxins are fungi secondary metabolites, have immunosuppressive properties and are classified as the most potent natural carcinogens known, according to the International Agency for Research on Cancer (IARC), as group 1 (IARC, 2002). *Salmonella* outbreaks linked to peanut products have also been reported in the last few decades (CDC, 2007, 2009, 2013, 2014; Isaacs et al., 2005; Kirk et al., 2004) with thousands of cases and several deaths. Both contaminations may occur at any step of the peanut supply chain (ICMSF, 2011).

Mixed populations of bacteria and fungi coexist in an extensive

* Corresponding author at: Monteiro Lobato n° 80 - Barão Geraldo — C. P. 6121- CEP: 13.083-862, Campinas, SP, Brazil.
E-mail address: mnasci@unicamp.br (M.S. Nascimento).

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variety of ecological niches, giving rise to complex microbial communities. Microorganisms can sense the presence of other microorganisms and directly or indirectly influence each other in numerous aspects (Bertrand et al., 2014). Bacterial secreted factors can influence fungal growth, adaptation patterns, morphology and developmental patterns (Sandland et al., 2007), while fungi can trigger bacterial behavior and survival (Peleg et al., 2010).

Antagonistic growth-inhibiting interactions between microorganisms have been studied in soil communities (An et al., 2013; Sullivan et al., 2013) or specific bacterial–fungal interactions related to the synthesis of antibiotics (Park et al., 2009). Further, it has already been elucidated that many lactic acid bacteria are capable of inhibiting mould growth and can interact with mycotoxins (Dalié et al., 2010). Studies of co-cultivation are already consolidated as a potent tool to discover new molecules and applications with industrial, medical, environmental approaches and can be used for the purpose of controlling or reducing specific contaminations in food (Bader et al., 2010; Brakhage, 2013; Netzker et al., 2015; Serrano et al., 2017).

Yadav et al. (2005) studied the antifungal activity of 12 bacterial (*Pseudomonas aeruginosa, Bacillus cereus, Streptomyces thermonitrificans, Streptococcus pneumoniae, Enterobacter aerogenes, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella* Typhi and *Escherichia coli*) strains against *A. flavus*, *A. fumigatus* and *A. niger*, with the inhibition activity of bacterial components being analyzed by microbroth dilution, disc diffusion and spore germination inhibition assays. They conclude that the products of *Salmonella* Typhi demonstrated significant activity against *Aspergillus* species but reported the scarcity of research in this field. Efforts have been made to quantify and classify the populations of *Salmonella* and *Aspergillus* in peanuts, whereas little has been reported on interactions between these microorganisms. Thus, it is important to understand the interaction around the co-cultivation between *Aspergillus* section *Flavi* and *Salmonella* in order to establish a strategy to control these microorganisms in the peanut supply chain. Therefore, the main objective of this work was to evaluate the influence of microbial interaction of *Salmonella* on the development and production of aflatoxin by *A. flavus/A. parasiticus* both isolated from different stages of the peanut supply chain.

2. Materials and methods

2.1. Bacterial strains

Seven serotypes of *Salmonella* were used in this study of which six were previously isolated and identified from the peanut supply chain in Brazil: *S.* Muenster (P03.2 FEA), *S.* Miami (P10.5 FEA), *S.* Glostrup (P02.1 FEA), *S.* Javiana (P06.1 FEA), *S.* Oranienburg (P07.1 FEA) and *S.* Yoruba (P08.1 FEA) (Nascimento et al., 2018; von Hertwig et al., 2019), and one is a reference strain: *S.* Typhimurium ATCC 14028. The strains were stored in a biofreezer (−80 °C). Each *Salmonella* strain was cultivated in tryptic soy broth (TSB, Difco, Detroit, MI, USA) followed by tryptic soy agar (TSA, Difco, Detroit, MI, USA) at 37 °C for 18–24 h and maintained on slants at 4 °C for use.

2.2. Fungal strains

Three strains of *A.* section *Flavi* isolated from the peanut supply chain in Brazil in a previous study (Martins et al., 2017) were used: *A. flavus* producing aflatoxin B (11340); *A. flavus* non-producing aflatoxin (11219) and *A. parasiticus* producing aflatoxin B and G (8964F).

2.3. Co-cultivation of Salmonella and A. section Flavi

The *Salmonella* strains were individually cultivated twice in brain heart infusion broth (BHI, Difco, USA). Then each strain was inoculated separately by pour plate (ca. 5 log cfu/mL PDA) in potato dextrose agar (PDA, Difco, USA) and Czapek Yeast Autolysate (CYA) agar. After the agar solidification, each pre-cultured fungus (previously cultivated in PDA for 5 days) was one point inoculated, using a needle, in the center of the petri dishes (90 mm). The plates were incubated at 30 °C and the fungal colony morphological variations were observed and the diameter was measured once a day for 7 days. In addition, each *Aspergillus* strain was cultivated in the absence of *Salmonella* culture as control. The experiments were repeated three times.

Preliminary tests using PDA and CYA were conducted to determine the appropriate agar media for the growth of both target microorganisms (*Salmonella* and *Aspergillus*). *Salmonella* strains did not grow well in CYA agar when compared to PDA agar, probably because of the high concentration of glucose (data not shown). Furthermore, the results indicated that there was no significant difference ($p > 0.05$) among the *Aspergillus* colony diameters after 7 days at 25, 30 or 37 °C. Peanut growth occurs in temperatures ranging from 25 to 30 °C and temperatures above 33 °C harm pod yield (Akram et al., 2018). Therefore, 30 °C was adopted as the incubation temperature in the subsequent experiments.

2.4. Qualitative analysis of Aflatoxin production

This test was carried out as a screening to verify if the fungal strains were able to produce aflatoxins when co-cultivated with *Salmonella*. After 7 days of co-cultivation in PDA, the aflatoxin production was qualitatively determined using the agar plug technique on thin layer chromatography (TLC). According to the methodology described by Filtenborg et al. (1983), a small piece (approximately 3 mm diameter) of the agar plate were taken from the center of the colony and aflatoxins extracted with chloroform:methanol (1:1) solution. A plug was placed on thin layer chromatography (TLC) plate with aflatoxin B1, B2, G1 and G2 standards (Sigma Aldrich, St. Louis, MO, USA) and developed in a toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, v/v/v/v) mobile phase. The aflatoxins were visualized under UV light at 254 and 365 nm. The experiments were repeated three times. The test was also performed for the strains of *Aspergillus* cultivated in the absence of *Salmonella* (control).

2.5. Quantitative analysis of Aflatoxin production

Positive samples from qualitative aflatoxin analysis were submitted to a quantification test. Three small pieces of mycelium were removed (plugs) from the central portion of the colony of each aflatoxin producing strains after 7 days co-cultivation with *Salmonella* serotypes. The toxin was extracted with 2 mL of methanol and homogenized for 2 min manually. The extract was twice filtered in a 0.22 μm Millex membrane. The same procedure was carried out with control. The inoculation and aflatoxin quantification were repeated three times.

The HPLC system used was an Agilent 1260 Infinity model system (Santa Clara, CA, USA) with a fluorescence detector set at 362 nm excitation and 455 nm emission for aflatoxins G1 and G2 and 425 nm emission for aflatoxins B1 and B2. An ODS (1.8 μm, 40 × 15 mm; Agilent, Santa Clara, CA, USA) guard column and a Zorbax Eclipse Plus C18 column (5 μm, 4.6 × 150 mm; Agilent, Santa Clara, CA, USA) were used. The mobile phase was water: acetonitrile: methanol (6:2:3, v/ v/ v), containing KBr (119 mg) and nitric acid (4 M, 350 μL/L) at a flow rate of 1 mL/min with injection volume of 20 μL. A post-column derivatization of aflatoxins B1 and G1 was performed with bromine using a KobraCell (R-Biopharm Rhône Ltd., Darmstadt, Germany).

The concentration of aflatoxins in the sample was determined by interpolation of the resulting peak area of aflatoxin B1, B2, G1 and G2 standard curves (Sigma, St Louis, MO, USA). Recovery values were calculated spiking aflatoxin standards in the culture medium and detection and quantification limits were determined according to Magnusson et al. (2015).

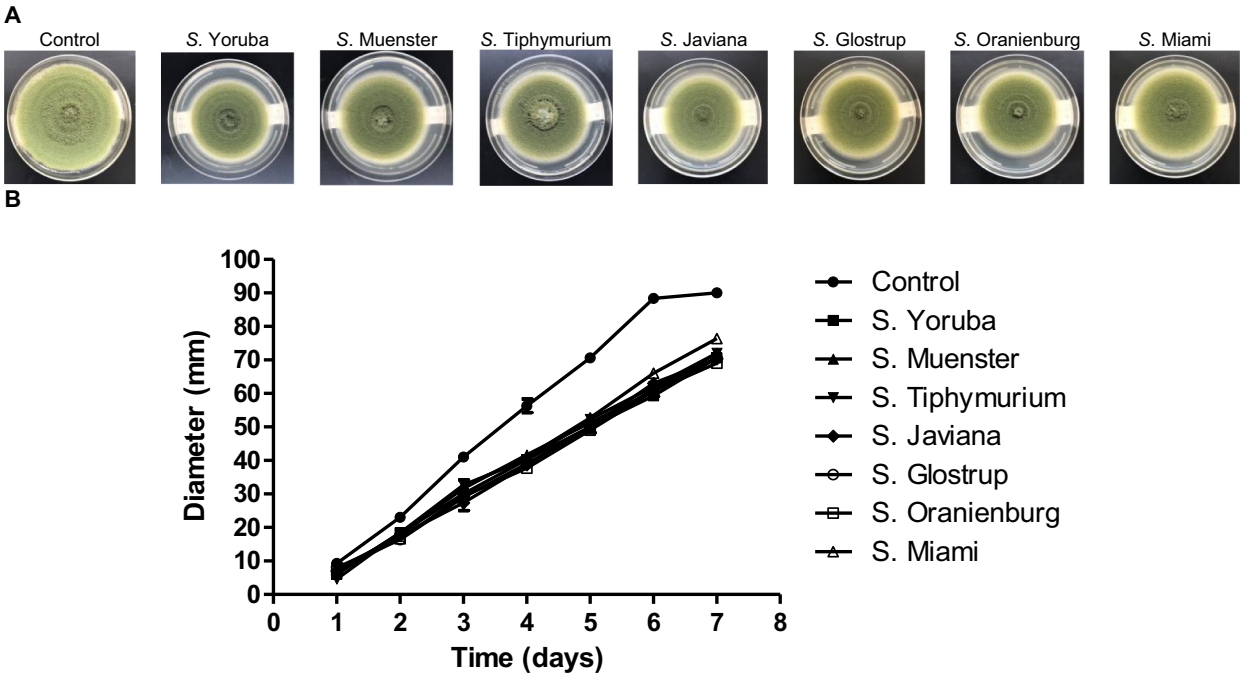

Fig. 1. Monitoring the morphology of *A. flavus* producing aflatoxin B for 7 days at 30 °C in PDA medium co-inoculated with *Salmonella* strains. (A) Figures representative of the colonies after 7 days of incubation. (B) Diameter of colonies over time.

2.6. Statistical analyses

The statistical analyses were carried out using Statistica software (version 10.0, StatSoft, CA, USA). The analysis of variance (ANOVA) and the Tukey test at the 5% level of significance were performed to verify differences among the fungal colony diameters and aflatoxin quantification.

3. Results

Maximum fungal growth was observed in the control sample, with colony diameter of 90 mm for both *A. flavus* strains (Figs. 1 and 2) and 88 mm for *A. parasiticus* (Fig. 3). The maximum inhibition was observed in co-cultivation with *S.* Oranienburg. The colony diameter was 69 mm (20% of reduction) for *A. flavus* aflatoxin B producer; 70 mm (18% of reduction) for *A. flavus* non-producer; and 66 mm (20% of reduction) for *A. parasiticus* (Figs. 1, 2 and 3). However, no significant difference ($p > 0.05$) was observed among *S.* Oranienburg and the other *Salmonella* serotypes tested.

Related to aflatoxin production, in the screening test (TLC) it was confirmed that both *A. flavus* strains preserved their characteristics of toxin production after co-cultivation with the seven *Salmonella* serotypes and aflatoxins group B were detected in all cases. On the other hand, for *A. parasiticus,* aflatoxin group G was not detected using qualitative method, in the presence of all *Salmonella* strains tested. According to HPLC results, aflatoxin production by the strains cultivated in the absence of *Salmonella* reached the total amount of 67,068.5 μg/kg for *A. flavus* and 92,458.7 μg/kg for *A. parasiticus* (Tables 1 and 2) and both *A. flavus* and *A. parasiticus* strains had the production of mycotoxin affected by the co-cultivation with all *Salmonella* serotypes tested (Tables 1 and 2). In *A. flavus* the reduction of aflatoxin B1 was above 70% for all *Salmonella* tested in co-cultivation, while for aflatoxin B2 the minimal reduction (80.5%) was observed in co-cultivation with *S.* Muenster (Table 1). For *A. parasiticus* the reductions ranged from 66.1% to 72.6% for aflatoxin B1, 74.0% to 79.1% for aflatoxin B2, 68% to 84.8% for aflatoxin G1 and 82.3% to 89.8% for aflatoxin G2 (Table 2).

4. Discussion

This is the first study on co-cultivation of *Salmonella* on *A.* section *Flavi* isolated from the peanut supply chain in a solid medium. Most reports have studied the occurrence of these microorganisms separately. However, to the best of the authors' knowledge, it is essential to understand the possible interactions between them to contribute to the establishment of control measures and assist in risk analysis and management for peanut and nut supply chains for all stakeholders.

A decrease in the fungal colony diameter and sporulation was verified. All *Salmonella* serotypes tested affected the growth of *Aspergillus* strains. Fungi are well adapted to develop on solid media because they use hyphal growth to colonize unexplored regions containing nutrients (Prosser and Tough, 1991). The growing fungal tip is the center of intense metabolic activity, mainly to ensure hyphal extension. Co-cultivated microorganisms might suffer modifications from genome to metabolome, exhibiting a differentiated phenotype driven by the various morphological interaction patterns (Bertrand et al., 2014).

Yadav et al. (2005) evaluated the antifungal activity of *Salmonella* Typhi (MTCCB 733) on clinical isolates of *Aspergillus* using the activity of supernatant and lysates. The lysate of *Salmonella* Typhi showed the highest antimycotic activity against *A. fumigatus*. In our study, *Salmonella* demonstrated significant ($p < 0.05$) activity against *Aspergillus* section *Flavi* species corroborating with Yadav et al. (2005). Probably the scarcity of data on co-cultivation with *Salmonella* may be linked to the fact that pathogenic bacteria produce toxic bioactive molecules limiting their usefulness (Lehrer et al., 1993).

The study of Yadav et al. (2005), showed information about proteins of *Salmonella* having an antifungal activity. Nevertheless, there is evidence that in certain cases the production of secondary metabolites requires the physical presence of a second microbe (cell–cell interaction) (Bertrand et al., 2014; König et al., 2013). Also, secreted molecules often involved in quorum sensing with single-species communities are capable of mediate interactions between bacteria and fungi, suggesting that the population density is linked to the effects of one microorganism on another (Goers et al., 2014). Inhibition of fungal growth has also been extensively reported for lactic acid bacteria (LAB)

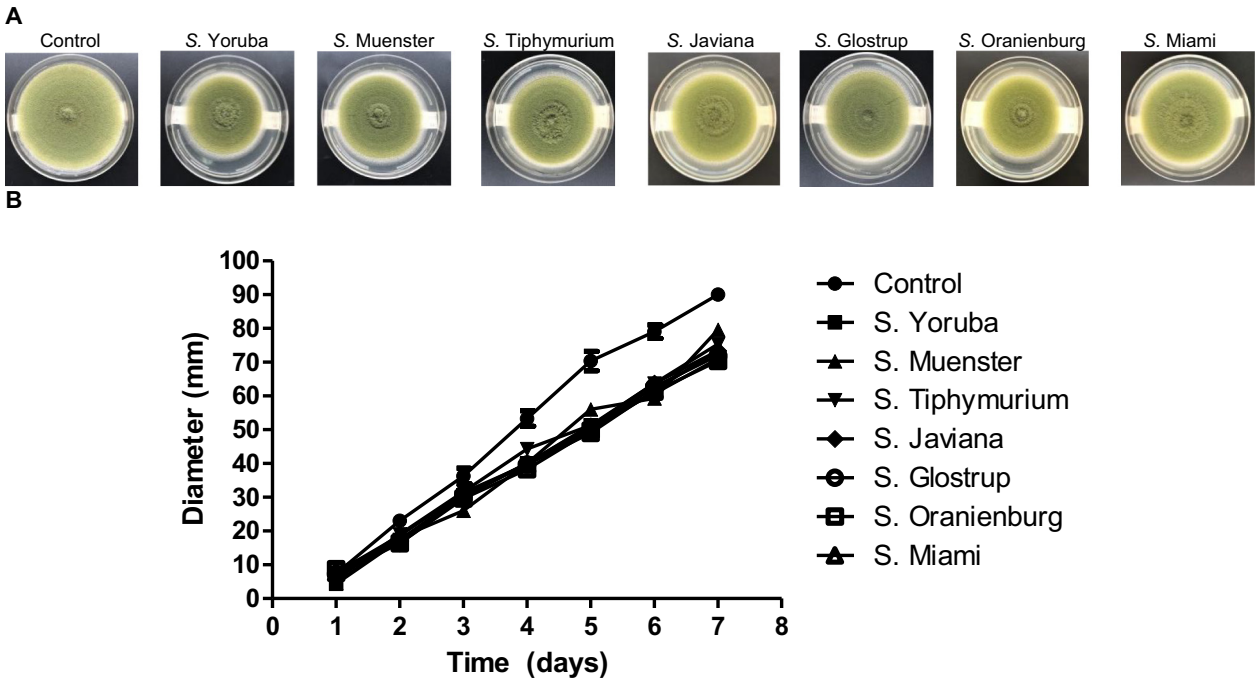

Fig. 2. Monitoring the morphology of *A. flavus* non-producing aflatoxin B for 7 days at 30 °C in PDA medium co-inoculated with *Salmonella* strains. (A) Figures representative of the colonies after 7 days of incubation. (B) Diameter of colonies over time.

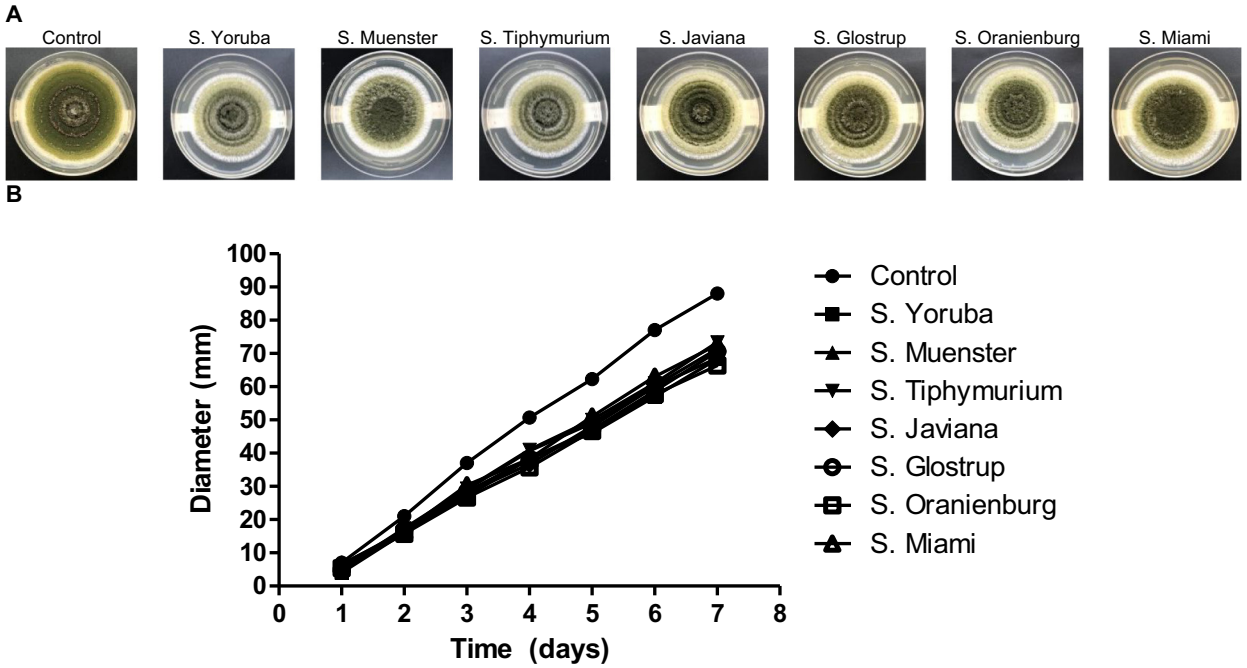

Fig. 3. Monitoring the morphology of *A. parasiticus* producing aflatoxin B and G for 7 days at 30 °C in PDA medium co-inoculated with *Salmonella* strains. (A) Figures representative of the colonies after 7 days of incubation. (B) Diameter of colonies over time.

(Dalié et al., 2010).

The production of secondary metabolites by microorganisms is strongly dependent on environmental factors, such as growth conditions and biotic and abiotic stresses, including sexual phases, growth inhibition, and defense or nutrient competition (Bertrand et al., 2014; Bode et al., 2002; Iwai and Omura, 1982). In fact, it is already known that microbes can produce compounds that function as transcriptional regulators and epigenetic modifiers (Charusanti et al., 2012). Also, co-cultivation of microbes can result in gene mutation and activate silent gene clusters, which could act reducing the aflatoxin production. Nevertheless, the exact molecular mechanisms by which this is achieved remain unknown (Bertrand et al., 2014).

S. Glostrup brought about the biggest reduction in the total of aflatoxin production by *A. flavus* (88.7%) whereas in *A. parasiticus* it was caused by *S.* Miami (72.9%). Ghazvini et al. (2016) had similar rates of aflatoxin reductions when cultivated acid lactic bacteria with *A. parasitcius*. Our results suggest the reduction of aflatoxin production might be serotype dependent when cultivated with *A. flavus*.

Table 1
Production (μg/kg) of aflatoxins in *A. flavus* strains.

	Control	*S.* Muenster	*S.* Yoruba	*S.* Javiana	*S.* Typhimurium	*S.* Oranienburg	*S.* Miami	*S.* Glostrup
Aflatoxin B_1	66,088.6 ± 8830.7	19,770.1 ± 964.7 (70.1%)	12,906.0 ± 1278.5 (80.5%)	12,816.9 ± 4076.0 (80.6%)	10,852.4 (± 1716.0) (83.6%)	8947.4 ± 1759.4 (86.5%)	8181.5 ± 91.6 (87.6%)	7508.4 ± 2993.6 (88.6%)
Aflatoxin B_2	979.9 ± 224.3	191.4 ± 19.0 (80.5%)	114.7 ± 11.8 (88.3%)	103.2 ± 33.8 (89.5%)	96.8 ± 22.9 (90.1%)	77.9 ± 11.9 (92.0%)	78.3 ± 4.1 (92.1%)	62.0 ± 16.2 (93.7%)
Total[a]	67,068.5[a] ± 8615.8	19,961.4b ± 970.0 (70.2%)	13,020.7bc ± 1290.2 (80.6%)	12,920.0bc ± 4109.8 (80.7%)	10,949.2bc ± 1738.7 (83.7%)	9025.3c ± 1771.2 (86.7%)	8259.8c ± 95.7 (87.7%)	7570.4c ± 3009.7 (88.7%)

Values between brackets represent the aflatoxin reduction in percentage.
LOD (limit of detection of total aflatoxins) = 1.7 μg/kg and LOQ (limit of quantification of total aflatoxins) = 5.7 μg/kg.
Recovery percentage of total aflatoxins = 81.3%.
[a] Values obtained from three independent trials with standard deviation. Means with different letters in the same line are significantly different ($p < 0.05$).

Table 2
Production (μg/kg) of aflatoxins in *A. parasiticus strains.*

	Control	*S.* Muenster	*S.* Yoruba	*S.* Javiana	*S.* Typhimurium	*S.* Oranienburg	*S.* Miami	*S.* Glostrup
Aflatoxin B_1	89,267.4 ± 9160.4	26,690.1 ± 2164.1 (70.1%)	31,077.6 ± 7399.6 (65.2%)	30,280.7 ± 7738.0 (66.1%)	29,365.4 ± 3561.7 (67.1%)	30,270.5 ± 2000.5 (66.1%)	24,443.0 ± 7358.3 (72.6%)	29,289.1 ± 10,318.0 (67.2%)
Aflatoxin B_2	1910.3 ± 235.7	471.6 ± 80.1 (75.3%)	452.7 ± 66.3 (76.3%)	492.9 ± 92.4 (74.2%)	415.5 ± 31.3 (78.2%)	496.5 ± 9.7 (74.0%)	399.9 ± 112.8 (79.1%)	420.2 ± 158.2 (78.0%)
Aflatoxin G_1	1238.0 ± 219.7	396.0 ± 47.4 (68.0%)	299.0 ± 55.4 (75.8%)	243.6 ± 60.5 (80.3%)	319.0 ± 30.3 (74.2%)	213.9 ± 25.6 (82.7%)	178.7 ± 64.8 (84.8%)	311.2 ± 199.5 (74.9%)
Aflatoxin G_2	43.0 ± 23.6	7.6 ± 0.8 (82.3%)	6.0 ± 1.0 (86.4%)	5.0 ± 0.5 (88.4%)	7.5 ± 0.8 (82.5%)	4.4 ± 1.4 (89.8%)	4.9 ± 1.7 (88.6%)	6.7 ± 2.1 (84.4%)
Total[a]	92,458.7a ± 9594.8	27,565.3b ± 2198.5 (70.2%)	31,835.3b ± 7518.6 (65.6%)	31,022.1b ± 7872.8 (66.4%)	30,107.4b ± 3618.1 (67.4%)	30,985.3b ± 1979.9 (66.5%)	25,026.5b ± 7526.6 (72.9%)	30,027.1b ± 10,654.6 (67.5%)

Values between brackets represent the aflatoxin reduction in percentage.
LOD (limit of detection of total aflatoxins) = 1.7 μg/kg and LOQ (limit of quantification of total aflatoxins) = 5.7 μg/kg.
Recovery percentage of total aflatoxins = 81.3%.
[a] Values obtained from three independent trials with standard deviation. Means with different letters in the same line are significantly different ($p < 0.05$).

Furthermore, *Salmonella* had a greater impact on the aflatoxin group B production by *A. flavus* when compared to *A. parasiticus*. On the other hand, *A. parasiticus* sporulation was more affected than *A. flavus*. The inhibition of aflatoxin biosynthesis by probiotic bacteria and yeasts occurs due to specific bacterial metabolites or because of a binding process that takes place on the cell wall with an efficiency depending on the bacterial strain (Gratz et al., 2004; Peltonen et al., 2001). Hamad et al. (2017) showed that the microbe surface is able to adsorb the toxic particles of the mycotoxins and concluded that not only the strain specificity affects the removal of the aflatoxin in contaminated media but also the strain concentration affects the removal of the mycotoxin. The fact that the co-cultivation with the seven serotypes of *Salmonella* caused a substantial reduction in aflatoxin production of a minimum of 66.4% (*A. parasiticus* with *S.* Javiana) (Table 2) brings new information for monitoring and assessment studies in the mycotoxin field. However, further studies are needed to better understand what happens in the interaction of these two microorganisms.

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.

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