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The potential of food grade antioxidants in the control of *Aspergillus* section *Flavi*, interrelated mycoflora and aflatoxin B_1 accumulation on peanut grains

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Abstract

The efficacy between analytical and industrial grade antioxidants (butylated hydroxytoluene-BHT, propyl paraben-PP and butylated hydroxyanisole-BHA) at 10 and 20 mmol g^{-1} concentrations on *Aspergillus* section *Flavi* populations, other natural competing mycoflora and aflatoxin B_1 (AFB_1) accumulation at different water activity (a_W) levels during 35 days on peanut grains was compared. Assays were carried out on natural peanut grains conditioned at different a_W (0.982, 0.955, 0.937). Both grades of BHA–PP mixture *M*4 (20 + 20 mM) and BHA–PP–BHT mixtures *M*5 (10 + 10 + 10 mM), *M*6 (10 + 20 + 10 mM), *M*7 (20 + 10 + 10 mM) and *M*8 (20 + 20 + 10 mM) totally inhibited the growth of *Aspergillus* section *Flavi* and peanut mycoflora at all conditions tested. In the same way, BHA–PP mixtures *M*3 (20 + 10 mM), *M*4 and BHA–PP–BHT mixtures *M*5, *M*6, *M*7 and *M*8 completely inhibited the aflatoxin accumulation. The study showed that both antioxidant grades are effective fungal inhibitors to peanut *Aspergillus* section *Flavi* populations, natural competing mycoflora and AFB_1 accumulation in a wide range of a_W during 35 days. The application of the best industrial grade antioxidants mixture tested could be an economic alternative to use in the fungal spoilage control on peanut grains.
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Keywords: Food grade antioxidants; Peanut; Aflatoxigenic fungi; Water activity

1. Introduction

Peanut (*Arachis hypogaea* L.) is a complex food matrix, which is energy giving due to its high fat and protein content and its relatively high fiber content (Elegbede, 1998). These characteristics led the nut to become sensitive to fungal contamination, both pre and post-harvest. As a result of inappropriate processing and storage conditions, peanut and its products may be contaminated with microorganisms. Numerous moulds may be involved in peanut spoilage, such as species of *Aspergillus*, *Penicillium*, *Fusarium* and of *Alternaria* in low percentage (Chulze, 2005). Fungal contamination causes a reduction in grain quality, through the utilization of stock carbohydrates and proteins and producing also oxidative mellowness of the grains (Lacey & Magan, 1991). These phenomena interact with other adverse factors such as immaturity, mechanic injury, unfavorable environmental conditions, incorrect drying, storage and processing factors, all of which contribute to the deterioration of the peanut's quality and flavor, which means a reduction of product quality and economic loss (Ahmed & Pattee, 1987; Ory, Crippen, & Lovegren, 1992; Sanders, Vercellotti, & Grimm, 1993). In 2005, peanut losses in Argentina caused by biotic disease-causing agents at the postharvest level were estimated at 4.1 million tons of grains or 6–8% of total production, this represented a loss of income amounting to $6.1 million to producers (SAGPyA, 2006). *Aspergillus* species are important

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colonizers of peanut because of their ability to produce several mycotoxins such as aflatoxins, ochratoxins, sterigmatocystins, cyclopiazonic acid and patulin. Among the *Aspergillus* species, *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis* and *A. ochraceoroseus* are of concern due to their toxigenic potential for producing aflatoxins (Fente, Jaimez, Vizquez, Franco, & Cepeda, 2001; Horn et al., 1996; Ito, Peterson, Wicklow, & Goto, 2001). Among all classes of aflatoxin, aflatoxin B_1 (AFB_1) is known to be the most significant in terms of animal and human health risk. International Agency for Research on Cancer of WHO included AFB_1 as a primary group of carcinogenic compounds (IARC, 1993). Therefore, raw peanuts now entering the European Economic Community's border must have less than 4 $\mu g\ kg^{-1}$ of total aflatoxins and not more that 2 $\mu g\ kg^{-1}$ of B_1 (Commission of the European Communities, 2006). A previous study has shown that AFB_1 levels in peanut destined to human consumption in Argentina exceed the acceptable maximum level (Barros, Torres, & Chulze, 2003).

One strategy to reduce the entry of aflatoxin into the peanut chain is the use of chemical treatments at post-harvest level to reduce both fungal growth and toxin production. From a human health perspective, the antioxidants such as butylated hydroxyanisole (BHA), propyl paraben (PP) and butylated hydroxytoluene (BHT) are allowed for use as antimicrobial agents by the US Food and Drug Administration (FDA) and are regarded as safe (GRAS) chemicals. The maximum usage level of single or multiple antioxidants approved by the more restricted legislation is 200 $\mu g\ g^{-1}$ based on the weight of the fat or oil (Codex Alimentarius, 2006). Considering that the oil content of peanuts ranges from 40% to 50% (Appelqvist, 1989), therefore, a dose of 100 $\mu g\ g^{-1}$ based on the total nut weight could be applied. In a previous study, we have demonstrated that the residual levels of these additives on peanut grains ranged between n.d. and 174 $mg\ g^{-1}$ after 35 days of incubation (Passone, Funes, Resnik, & Etcheverry, 2006). In another study, the single effect of BHA, PP, BHT and trihydroxybutyrophenone (THB) on *A. flavus* and *A. parasiticus* growth and AFB_1 production over a wide range of concentrations (1–20 mM) at four water activities (a_W) on peanut extract meal agar was tested (Passone, Resnik, & Etcheverry, 2005). This study showed that single food grade antioxidants such as PP and BHT combined with lowered water activity (a_W) could significantly help to control *A. flavus* and *A. parasiticus* growth and AFB_1 accumulation. The application of BHA over the range of 10–20 mM concentrations totally inhibited fungal growth and aflatoxin production. A study conducted by Reynoso et al. (2002) demonstrated that low concentrations of BHA and PP applied on irradiated corn might be more effective together than either one alone in reducing the growth of *Fusarium* section *Liseola*. However, there are no studies that have examined the efficacy of combinations of different concentrations and grades of antioxidants on natural peanut grains to determine whether the effects on control of growth and aflatoxin accumulation might be achieved.

In order to fill this lacuna of information, the aim of the present study was to compare the efficacy between analytical and industrial grade antioxidants (BHA, PP, BHT) on *Aspergillus* section *Flavi* populations, other natural competing mycoflora and AFB_1 accumulation at different a_W levels over 35 days of incubation period on peanut grains.

2. Materials and methods

2.1. Substrate

Natural peanut grains with initial water content of 0.582 a_W and AFB_1 free were used throughout this study and kept at 4 °C. The peanut was rehydrated to achieve the required a_W (0.982, 0.955, 0.937) by addition of sterile distilled water using a moisture absorption curve for the grain. The grain a_W was determined with a Thermoconstanter Novasina TH 200 (Novasina, Zurich, Switzerland).

2.2. Antioxidants

The following antioxidants were used: benzoic acid, 2(3)-*tert*-butyl-4 hydroxyanisole (BHA); *n*-propyl *p*-hydroxybenzoate (PP) and 2,6-di (*tert*-butyl)-*p*-cresol (BHT). Analytical grade antioxidants (99.5–99.9% of purity) were obtained from Sigma Chemical (Dorset, UK) and industrial grade antioxidants were obtained from Eastman Chemical Company. Industrial grade antioxidants, PP and BHT, had a purity of 99% containing as contaminants ash <0.02%, arsenic <3 $\mu g\ g^{-1}$ and heavy metals <10 $\mu g\ g^{-1}$. Butylated hydroxyanisole had a purity of 98.5% containing as trace elements sulfated ash <0.01%, citric acid <2500 $\mu g\ g^{-1}$, arsenic <3 $\mu g\ g^{-1}$ and heavy metals <10 $\mu g\ g^{-1}$. The compounds contaminating industrial grade antioxidants did not exceed the levels allowed by JECFA (1996). Stock solutions of BHA, PP and BHT were prepared in 950 $ml\ l^{-1}$ ethyl alcohol/distilled water. Individual antioxidant treatments of BHA (10 and 20 mM) and double and triple combinations of BHA+each of the other two were tested at 10 and 20 mM concentrations.

2.3. Incubation conditions

Seventy grams of natural peanut grains for human consumption was weighed and dispensed as a monolayer into sterile Petri dishes. The required concentration of each antioxidant was added to reach a final concentration of 10 and 20 $mmol\ g^{-1}$ of peanut grains to obtain the following treatments: BHA (10 mM), (20 mM), BHA–PP mixtures *M*1 (10 + 10 mM), *M*2 (10 + 20 mM), *M*3 (20 + 10 mM), *M*4 (20 + 20 mM), BHA–PP–BHT mixtures *M*5 (10 + 10 + 10 mM), *M*6 (10 + 20 + 10 mM), *M*7 (20 + 10 + 10 mM) and *M*8 (20 + 20 + 10 mM). Controls without antioxidants were also prepared. The grains were conditioned with the appropriate amount of water and kept at

4 °C for 48 h with periodic shaking to allow absorption and equilibrium. Petri dishes containing peanut at the same a_W were enclosed together in sealed plastic containers. Each container had a beaker with a glycerol/water solution at the same a_W as the peanut grains, to maintain the relative humidity constant. All the experiments were carried out with three separate replicates per treatment. The cultures were incubated at 28 °C. Peanut fungal colonization was analyzed at the end of the incubation period (11 and 35 days).

2.4. Fungal populations

The colonization of the grains was assessed as CFU g^{-1} of peanut grains after 11 and 35 days of incubation. A sample (10 g) was taken from each treatment and shaken for 30 minutes with 90 ml of 1 g l^{-1} peptone/distilled water plus 0.06 g l^{-1} of Tween 80. Serial decimal dilutions until 10^{-7} for control samples and until 10^{-4} for treated samples were done. A 0.1 ml aliquot of the three last serial decimal dilutions of each treatment was spread on the surface of three solid media, dichloran/rose bengal/chloramphenicol (DRBC), dichloran/glycerol 18% (DG_{18}) and *Aspergillus flavus* and parasiticus agar (AFPA) by triplicate (Pitt & Hocking, 1997). Plates with AFPA medium were incubated in darkness at 30 °C for 48 h. In this medium, mould species producing orange/yellow reverse colors were counted and the average was reported as colony forming units of *Aspergillus* section *Flavi* per gram (CFU g^{-1}) of peanut. Plates with DRBC and DG_{18} media were incubated in darkness at 28 °C for 5–7 days. At the end of the incubation period, the average number of colonies (triplicate) was done in that plates that ranged between 10 and 100 CFU. The results were expressed as CFU g^{-1} of peanut grains. The identification of *Aspergillus* section *Flavi* and *Aspergillus* section *Nigri* was done according to Klich (2002), the other fungal genera were identified according to Samson, Hoekstra, Frisuad, and Fitenborg (2002).

2.5. Aflatoxin determination

Peanut grain aflatoxin analyses were performed by high-performance liquid chromatography (HPLC) following the methodology proposed by Truckssess, Stack, Nesheim, Albert, and Romer (1994). After 11 and 35 days of incubation, 50 g of peanut samples was analyzed by extracting AFB_1, adding acetonitrile/water (90/10 V/V) and shaking milled peanut grains and solvent for 30 min on an orbital shaker (New Brunswick, Scientific CO., Inc.). The extracts were filtered through a filter paper (Whatman N°4, Whatman International, Maidstone, UK). A 3 ml aliquot of each extract was transferred to a multifunctional column (Mycosep 224 MFC, Romer). A 200 μl aliquot of filtered extract and AFB_1 standard was derivatized with 700 μl of trifluoracetic acid/acetic acid/water (20/10/70 V/V/V). The derivatized aflatoxins (50 μl solution) were analyzed using a reversed-phase HPLC/fluorescence detection system. The HPLC system consisted of a Hewlett Packard 1100 pump (Palo Alto, CA, USA) connected to a HP1046A programmable fluorescence detector, interfaced to a Hewlett Packard Chem Station. Chromatographic separations were performed on a stainless steel C_{18} reverse phase column (150 mm × 4.6 mm i.d., 5 μm particle size, Luna-Phenomenex, Torrance, CA, USA). Water/methanol/acetonitrile (4/1/1 V/V/V) was used as the mobile phase, at a flow rate of 1 ml min^{-1}. Aflatoxin derivative, fluorescence, was recorded at excitation and emission wavelengths of 360 and 440 nm, respectively. Standard curves were constructed with different levels of AFB_1. This toxin was quantified by correlating peak height of sample extracts and those of standard curve. The limit of detection of the analytical method was 1 ng g^{-1}.

2.6. Statistical analyses

Statistical analyses were made using SigmaStat program Version 3.10. Copyright© 2004 Systat Software, Inc. Means of *Aspergillus* section *Flavi* colony forming units grown on AFPA medium were determined by analyses of variance (ANOVA) ($P < 0.001$). The significant differences between the control and treatments were established by using Duncan's new multiple range test at $P = 0.05$ level. Data on fungal populations grown on DRBC and DG_{18} media were often not normally distributed and were therefore analyzed using the non-parametric Kruskall–Wallis test. To evaluate the significant differences between the control and the treatments, Dunn's test ($P = 0.05$) was used. Fisher's LSD test ($P = 0.05$) was applied to compare significant differences between treatment and control means for AFB_1 accumulation.

3. Results

3.1. Effect of antioxidants on peanut Aspergillus section Flavi and interrelated mycoflora

Statistical analyses on total fungal growth, incubation time (I), water activity (a_W), antioxidant grades (G), antioxidant treatments (T) and two-, three- and four-way interactions were statistically significant (Table 1). The major effects were produced by antioxidant treatments followed by the interactions of $a_W \times$ antioxidant grades and by a_W effect.

Figs. 1 and 2a–c show the impact of antioxidant treatments (analytical and industrial grade antioxidants) on propagules count (log CFU g^{-1} of peanut grains) of *Aspergillus* section *Flavi* species at three water availability conditions (0.982, 0.955 and 0.937 a_W) at 11 and 35 days of incubation, respectively. The colony forming units per gram of peanut grain of control samples were affected by the grain humidity, decreasing about 3 log units at driest water condition (0.937 a_W) and at the first incubation time. Similarly, the incubation time (35 days) increased controls count in the order of 1 and 2 log units. Antioxidant

Table 1
Significance of incubation time (I), water activity (a_W), antioxidant grades (G), antioxidant treatments (T) and their interactions on growth of total mycoflora on peanut grains

Factor	DF[a]	MS[b]	F value[c]	$P > F$
I	1	49.93	71.18	0.0001
a_W	2	88.22	125.76	0.0001
G	1	11.93	17.01	0.0001
T	10	676.89	964.97	0.0001
$I \times a_W$	2	18.49	26.35	0.0001
$I \times G$	1	3.05	4.35	0.0376
$I \times T$	10	12.46	17.76	0.0001
$a_W \times G$	2	91.34	130.21	0.0001
$a_W \times T$	20	20.62	29.40	0.0001
$G \times T$	10	15.17	21.62	0.0001
$I \times a_W \times G$	2	14.04	20.01	0.0001
$I \times a_W \times T$	20	7.93	11.31	0.0001
$I \times G \times T$	10	6.42	9.16	0.0001
$a_W \times G \times T$	20	19.36	27.60	0.0001
$I \times a_W \times G \times T$	20	4.07	5.80	0.0001
Error	396	0.70		

[a] DF = degrees of freedom.
[b] MS = mean squares.
[c] Significant at $P < 0.001$.

mixtures showed to be more effective during the first period of incubation (11 days), regardless of a_W. The efficacy of these chemicals increased while a_W level decreased, independently of the preservative quality applied. The following mixtures: BHA–PP $M4$ (20 + 20 mM) and BHA–PP–BHT $M5$ (10 + 10 + 10 mM), $M6$ (10 + 20 + 10 mM), $M7$ (20 + 10 + 10 mM) and $M8$ (20 + 20 + 10 mM) totally inhibited the growth of *Aspergillus* section *Flavi* on peanut grains. The inhibition was independent of chemical quality, this effect was observed at all a_W assayed and the efficacy was extended over all the incubation period (data not shown). The single application of BHA (10 mM) showed the minimum antifungal effect, while the highest dose (20 mM) significantly reduced *Aspergillus* section *Flavi* growth along de assay and totally inhibited fungal development at the lowest a_W condition (0.937). *Aspergillus* section *Flavi* population was found to be significantly affected by binary mixture $M1$ (10 + 10 mM; 0.982 a_W; 35 days), showing reduction percentages of 37.9% (analytical grade) and 24.6% (industrial grade). A significant reduction on fungal count between 2 and 3 log units was observed with analytical grade binary mixture $M1$ at 0.955 a_W, while the same mixture of industrial quality completely inhibited *Aspergillus* section *Flavi* growth.

The efficacy of the two kinds of antioxidants (analytical and industrial grade) on the rest of mycoflora that infect peanut grains was evaluated on DRBC and DG_{18} agar (Tables 2 and 3). The predominance of *Aspergillus* section *Nigri* populations and species of *Penicillium* was observed in the control samples at all a_W tested. These fungal populations were affected by the incubation time, which increased between 1 and 3 log units at 35 days of incubation. The highest water stress condition analyzed (0.937 a_W) mainly affected the growth of genera such as *Fusarium*,

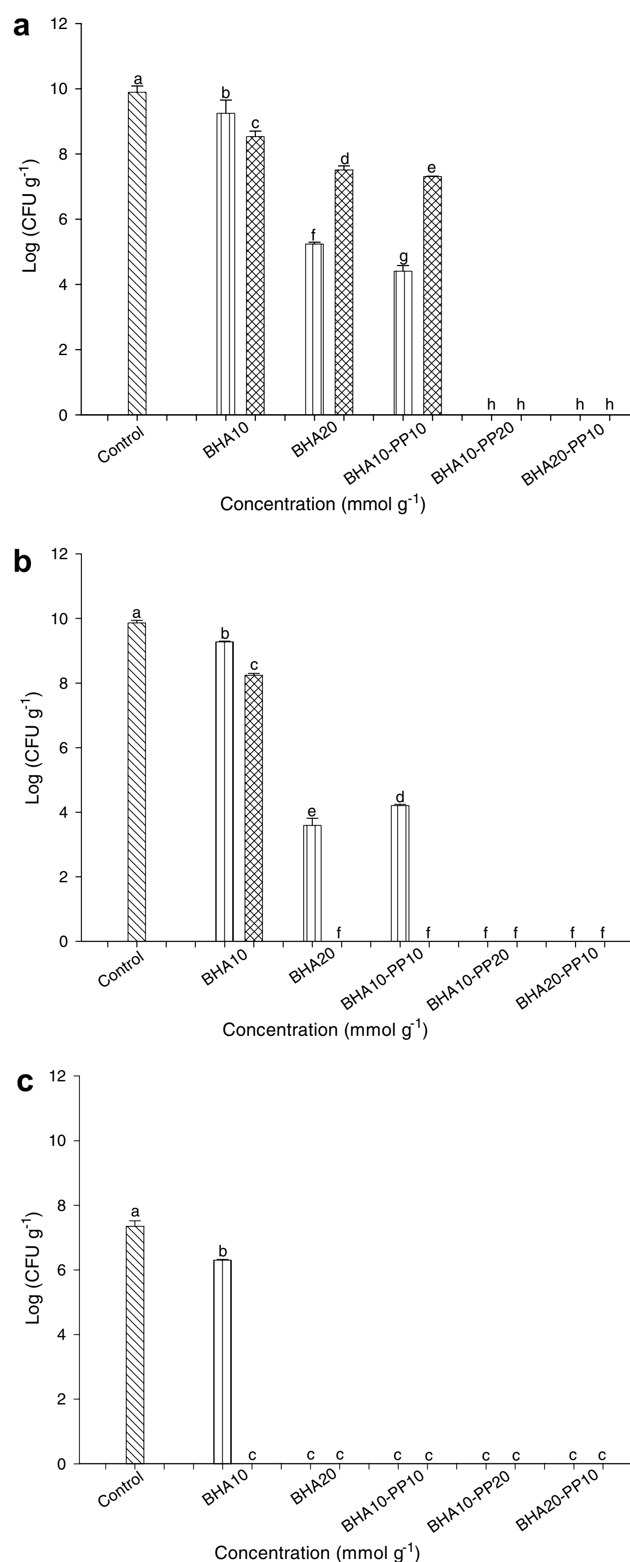

Fig. 1. Effect of antioxidants treatments on the growth (log CFU g^{-1} of peanut grains) of *Aspergillus* section *Flavi* incubated during 11 days and at (a) 0.982 a_W, (b) 0.955 a_W, (c) 0.937 a_W. Analytical grade antioxidants; industrial grade antioxidants.

Trichoderma, *Mucor*, *Rhizopus* and *Absidia* spp. Populations of xerophilic fungi such as *Aspergillus* and *Penicillium* spp. decreased in the order of 2 and 4 log units, at 0.937

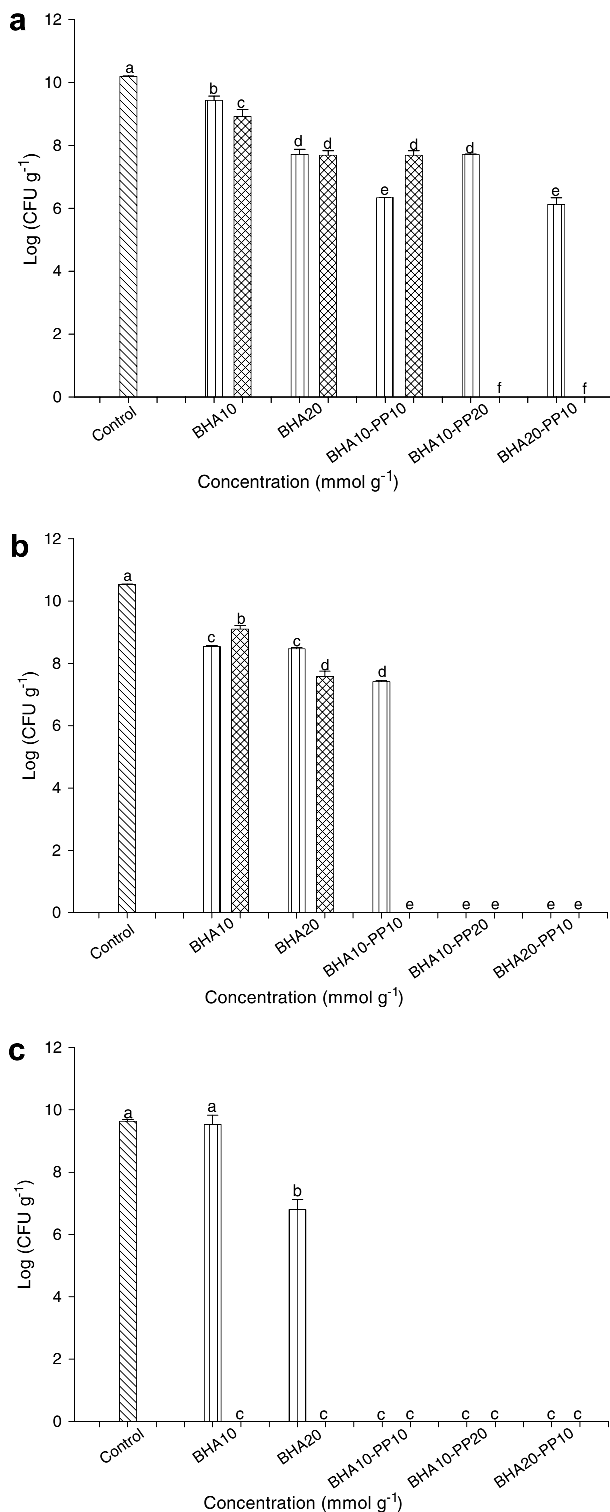

Fig. 2. Effect of antioxidants treatments on the growth (log CFU g^{-1} of peanut grains) of *Aspergillus* section *Flavi* incubated during 35 days and at (a) 0.982 a_W, (b) 0.955 a_W, (c) 0.937 a_W. ▥ Analytical grade antioxidants; ▩ industrial grade antioxidants.

a_W. Peanut fungal populations were inhibited by analytical grade binary mixture $M2$ (10 + 20 mM) after 35 days of incubation. Similarly, binary mixtures $M3$ (20 + 10 mM), $M4$ and ternary mixtures $M5$, $M6$, $M7$ and $M8$ applied on the natural peanut grains totally inhibited fungal growth at all incubation conditions tested, regardless of the antioxidant quality (data not shown). *Aspergillus* section *Nigri* counts decreased between 0.5 and 2 log units at 11 days of incubation after the application of both grades of BHA (10 mM). *Penicillium* species were more sensitive to this chemical (BHA) showing a total growth inhibition at 11 days of incubation, independently of antioxidant grade, dose applied and a_W conditions tested. However, filamentous fungi, especially species of *Mucor*, *Rhizopus* and *Absidia* spp., increased their growth when BHA (10 mM) and binary mixture $M1$ were added to the grains (see Tables 2 and 3).

3.2. Effect of antioxidants on aflatoxin B_1 production

The water condition and the incubation time of the grains showed influence on AFB_1 accumulation (Table 4). It is worth noting that in the control samples, the concentration of this metabolite decreased between 4 and 12 times at 0.955 a_W. At the highest water stress condition assayed (0.937 a_W) and at two incubation periods, there was no detection of AFB_1. Similarly, at the later incubation period (35 days), the AFB_1 accumulation by control samples increased between 2 and 5 times. The effect of antioxidant treatments was variable and dependent on the incubation conditions. Binary mixture $M4$ (20 + 20 mM) and ternary mixtures $M5$ (10 + 10 + 10 mM), $M6$ (10 + 20 + 10 mM), $M7$ (20 + 10 + 10 mM) and $M8$ (20 + 20 + 10 mM) completely inhibited AFB_1 accumulation (data not shown). At the end of the incubation time (35 days) and at both a_W levels, AFB_1 accumulation was reduced between 81.4% and 100% with binary mixture $M2$ (10 + 20 mM). AFB_1 levels quantified in treated samples with binary mixture $M1$ (10 + 10 mM) at 35 days of incubation were higher than control values. Both qualities of BHA (20 mM; 0.982 a_W) reduced aflatoxin accumulation about 34% at 11 days. However, this treatment stimulated the production of this metabolite at the end of the incubation period (35 days). Butylated hydroxyanisole (10 and 20 mM) controlled aflatoxin accumulation at 0.955 a_W at all sampling periods with percentages of reduction ranging between 19% and 56% and between 67% and 37% for analytical and industrial grade antioxidants, respectively.

4. Discussion

In this study, propagules count (CFU g^{-1}) of *Aspergillus* section *Flavi* from peanut was found to be influenced by a_W, showing a decrease of up to 3 log units at the driest treatment (0.937 a_W). The effect of antioxidant treatments was considerably affected by the grains a_W levels and the incubation time. Similarly, Marín et al. (2000) showed that the effect of 0.5 and 1 g kg^{-1} of propionate on maize mycoflora significantly inhibited the population growth at low a_W and at seven days, their efficacy decreased after 14 and 21 days. On the other hand, Magan, Aldred, and

Table 2
Effect of antioxidants on populations of *Aspergillus*, *Penicillium* and other filamentous fungi species on peanut at three water availability conditions and during 11 days of incubation

Treatments	CFU g^{-1a}							
	0.982 a_W			0.955 a_W			0.937 a_W	
	A section *Nigri*	*Penicillium*	Filamentous Fungi[b]	A section *Nigri*	*Penicillium*	Filamentous Fungi	A section *Nigri*	*Penicillium*
Control	4.0×10^9	0.3×10^8	7.8×10^4	5.0×10^8	8.0×10^1	<	6.4×10^7	<
Analytical grade antioxidants								
BHA10 mM	3.8×10^7	<*	1.1×10^6	3.0×10^6	<	<	3.4×10^6	<
BHA20 mM	<*	<*	<*	1.3×10^8	<	<	1.4×10^5	<
*M*1	<*	<*	2.5×10^5	3.0×10^3	<	<	1.5×10^2	<
*M*2	<*	<*	<*	<*	<	<	<*	<
Industrial grade antioxidants								
BHA10 mM	1.9×10^9	<*	<*	6.5×10^6	<	1.0×10^8	<*	<
BHA20 mM	2.3×10^8	<*	<*	<*	<	8.1×10^3	<*	<
*M*1	1.8×10^7	<*	<*	<*	<	2.2×10^3	<*	<
*M*2	<*	<*	<*	<*	<	<	<*	<

Mean in a column with (*) are significantly different from control according to Dunn's Method ($P < 0.05$); BHA, butylated hydroxyanisole; *M*1, BHA–PP (10 + 10 mM); *M*2, BHA–PP (10 + 20 mM); <, minor to 1×10^2 CFU g^{-1}.
[a] Mean values based on triplicate data in DRBC and in DG_{18} medium.
[b] Include the genera: *Aspergillus, Eurotium, Fusarium, Trichoderma, Mucor, Rhizopus, Absidia* and sterile mycelia.

Table 3
Effect of antioxidants on populations of *Aspergillus*, *Penicillium* and other filamentous fungi species on peanut at three water availability conditions and during 35 days of incubation

Treatments	CFU g^{-1a}							
	0.982 a_W			0.955 a_W			0.937 a_W	
	A section *Nigri*	*Penicillium*	Filamentous Fungi[b]	A section *Nigri*	*Penicillium*	Filamentous Fungi	A section *Nigri*	*Penicillium*
Control	2.7×10^{10}	1.0×10^9	5.3×10^5	2.0×10^9	1.0×10^8	<	2.0×10^{10}	2.5×10^5
Analytical grade antioxidants								
BHA10 mM	0.8×10^9	0.1×10^6	5.4×10^7	3.0×10^8	<*	1.0×10^7	1.0×10^{10}	2.5×10^2
BHA20 mM	<*	<*	<*	1.3×10^8	<*	<	6.3×10^9	<
*M*1	<*	<*	8.7×10^7	8.5×10^4	<*	1.0×10^4	5.0×10^9	8.8×10^3
*M*2	<*	<*	<*	<*	<*	<	<*	<
Industrial grade antioxidants								
BHA10 mM	0.6×10^{10}	1.0×10^9	<*	5.0×10^7	2.3×10^8	5.5×10^8	<*	5.0×10^3
BHA20 mM	2.3×10^9	<*	<*	$5.0 \times 10^{3*}$	<*	5.6×10^6	<*	<
*M*1	3.2×10^{10}	4.7×10^{10}	<*	$1.5 \times 10^{2*}$	5.0×10^6	4.0×10^7	1.3×10^4	<
*M*2	<*	<*	<*	<*	3.0×10^6	5.1×10^8	<*	<

Mean in a column with (*) are significantly different from control according to Dunn's Method ($P < 0.05$); BHA, butylated hydroxyanisole; *M*1, BHA–PP (10 + 10 mM); *M*2, BHA–PP (10 + 20 mM); <, minor to 1×10^2 CFU g^{-1}.
[a] Mean values based on triplicate data in DRBC and in DG_{18} medium.
[b] Include the genera: *Aspergillus*, *Eurotium*, *Fusarium*, *Trichoderma*, *Mucor*, *Rhizopus*, *Absidia* and sterile mycelia.

Sanchis (2004) demonstrated that *Aspergillus* and *Penicillium* spp. were able to metabolize a range of food grade preservatives; this behavior may be possible after the first sampling period (seven days). In this study, species of *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri* and other filamentous fungi, especially *Mucor* spp., showed to be quite resistant to BHA (10 and 20 mM) and to the binary mixture *M*1. Recent studies carried out in natural maize grains reported that the percentages of reduction in the log CFU of *Aspergillus*, *Penicillium* and *Fusarium* spp. populations were between 4.7% and 25.6% applying 500 and 1000 ppm of BHA and PP antioxidants (Farnochi, Torres, Magan, & Chulze, 2005).

When we used lower levels of preservatives, such as BHA (10 mM) and binary mixture *M*1, the AFB_1 accumulation was stimulated over the range of environmental conditions assayed. However, with combinations of the highest dose (20 mM) of BHA+each of the other two, AFB_1 accumulation was totally inhibited. Therefore, the determination of optimal application doses of the antifungic substances is very important (Mazzani, Luzán, González, & Quijada, 1995). High levels could produce undesirable effect on the grain organoleptic properties and on its processed products, causing a negative economic impact. On the other hand, sub-optimal levels could cause stimulation on fungal sporulation, growth and secondary metabolism,

Table 4
Effect of antioxidants on aflatoxin B_1 production by *Aspergillus* section *Flavi* at different water activity values at 11 and 35 days of incubation at 28 °C

Time (days)	Aflatoxin B_1 concentration ng g^{-1} (mean ± SD)			
	0.982 a_W		0.955 a_W	
	11	35	11	35
Treatments				
Control	42.2 ± 31.8	78.7 ± 68.6	3.6 ± 1.2	18.3 ± 5.2
Analytical grade antioxidants				
BHA 10 mM	33.5 ± 25.1	79.3 ± 5.3	1.9 ± 0.8	15.9 ± 3.9
BHA 20 mM	16.7 ± 0.3	82.2 ± 5.1	1.7 ± 0.6	7.9 ± 1.4
*M*1	69.2 ± 10.8	139.1 ± 35.5	35.5 ± 10.7	67.9 ± 14.4
*M*2	ND*	14.6 ± 3.9	ND	ND*
*M*3	ND*	12.2 ± 4.3	ND	ND*
Industrial grade antioxidants				
BHA10 mM	83.5 ± 6.9	127.0 ± 68.6	3.4 ± 0.8	11.3 ± 7.3
BHA20 mM	38.9 ± 16.9	132.2 ± 64.3	ND	12.7 ± 7.9
*M*1	35.5 ± 42.6	104.1 ± 23.7	ND	ND*
*M*2	ND*	ND*	ND	ND*

Mean in a column with (*) are significantly different from control according to Fisher LSD test ($P < 0.05$); BHA, butylated hydroxyanisole; *M*1, BHA–PP (10 + 10 mM); *M*2, BHA–PP (10 + 20 mM); *M*3 BHA–PP (20 + 10 mM); ND, below detection limit.

increasing mycotoxin synthesis (Marín et al., 2002). Dusanee, Wongjun, and Muanmai (1992) demonstrated that the application of 0.5, 1.0 and 1.5 kg ton^{-1} of propionic acid in maize increased the aflatoxin accumulation, while with 2 and 2.5 kg ton^{-1} the *A. flavus* growth and its toxin production were considerably reduced.

The current information about action mechanisms of antioxidants on fungal species is limited. It has been suggested that BHA affects the cell membrane, by changing pH values and affecting transduction energy and substrate transport (Aldunate et al., 1992). With regard to the parabens, various mechanisms have been proposed for their antimicrobial action: they may inhibit functions of several enzymes, they dissolve membrane lipids and interfere with the transport of nutrients, they also alter the protein, RNA and DNA synthesis, and in addition, they destroy the membrane potential (Eklund, 1989). Butylated hydroxytoluene acts via a non-specific mechanism involving the perturbation of membrane function (Singer & Wan, 1977). In our study, the single use of BHA for control of mould and aflatoxins accumulation in moist peanut grains does not appear to be feasible. Nevertheless, the application of double and triple combinations of BHA with each of the other food grade antioxidants showed to be more effective than individual one. This behavior could be due to different action mechanisms on fungal cell at diverse target levels. Our results showed that binary mixtures *M*3, *M*4 and ternary mixtures *M*5, *M*6, *M*7 and *M*8 of both analytical and industrial grade antioxidants applied on natural peanut grains inhibited *Aspergillus* section *Flavi* growth and most importantly its AFB_1 accumulation. Considering that the effects of both analytical and industrial grade antioxidants were similar, the other factor to be considered is the application cost. The industrial quality antioxidants are 91% cheaper than the ones with analytical quality; besides, the economic value of this study lies in the simplified technique for control of aflatoxin contamination in agricultural products at stored level. In relation to their environmental cost, it is known that phenolic antioxidants are safe additives for food, while traditional fungicides are dangerous for human and animal health. The finding further emphasizes the fungitoxic impact of industrial grade antioxidant mixtures against fungi attacking stored peanut and strengthens the possibility of using them as alternative to chemicals as effective inhibitors of biodegrading and storage contaminating fungi and aflatoxin occurrence.

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References

Aldunate, J., Coloma Torres, L., Spenser, P., Morello, A., Ojeda, J. M., & Repetto, Y. (1992). Effects of 2-(3)-tert-butyl-4-hydroxyanisole (BHA) on in situ mitochondria of *Tripanosoma cuzi*. *FEBS Letters, 303*, 73–76.

Ahmed, E. M., & Pattee, H. E. (1987). In E. M. Ahmed & H. E. Pattee (Eds.), *Peanut quality: Its assurance and maintenance from the farm to end-product* (pp. 874). University of Florida, Institute of Food and Agricultural Science.

Appelqvist, L. A. (1989). The chemical nature of vegetable oils. In G. Röbbelen, R. K. Downey, & A. Ashri (Eds.), *Oil crops of the world. Their breeding and utilization* (pp. 22–37). McGraw-Hill, Inc.

Barros, G., Torres, A., & Chulze, S. (2003). *Aspergillus* species from section *Flavi* isolated from soil at planting and harvest time in peanut-growing regions of Argentina. *Journal of the Science of Food and Agriculture, 83*, 1303–1307.

Chulze, S. (2005). Aflatoxinas en Maní. In G. J. March & A. D. Marinelli (Eds.), *Enfermedades del Maní en Argentina* (pp. 103–113). Córdoba, Argentina: Biblia Impresores.

Codex alimentarius, (2006). Food additive details. *Update up to the twenty-ninth session of the codex alimentarius commission. The Joint FAO/WHO Committee on Food Additives*. Available in: <www.codex-alimentarius.net/web/jecfa>

Commission of the European communities, (2006). Commission regulation (EC) N° 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal European Union*, L364, 5–24.

Dusanee, T., Wongjun, R., & Muanmai, A. (1992). Effects of sodium chloride, propionic acid and ammonium hydroxide on growth of *Aspergillus flavus* on corn and aflatoxin production. *Asian Food Journal, 7*, 24–29.

Eklund, T. (1989). Organic acids and esters. In G. W. Gould (Ed.), *Mechanisms of action of food preservation procedures* (pp. 181–182). New York: Elsevier Applied Science.

Elegbede, J. A. (1998). Legumes. In A. U. Osagie & O. U. Eka (Eds.), *Nutritional quality of plant foods* (pp. 53–83). Benin City: Post harvest Research Unit, Biochem. University of Benin.

Farnochi, M. C., Torres, A. M., Magan, N., & Chulze, S. N. (2005). Effect of antioxidants and competing mycoflora on *Fusarium verticillioides* and *F. proliferatum* populations and fumonisin production on maize grain. *Journal of Stored Products Research, 41*, 211–219.

Fente, C. A., Jaimez, O. J., Vizquez, B. I., Franco, C. M., & Cepeda, A. (2001). A new additive for culture media for rapid determination of aflatoxin-producing aspergilli strains. *Applied and Environmental Microbiology, 67*(10), 4858–4862.

Horn, B. W., Greene, R. L., Sobolev, V. S., Dorner, J. W., Powell, J. H., & Layton, R. C. (1996). Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus*, *A. parasiticus* and *A. tamarii*. *Mycologia, 88*, 574–587.

International Agency for Research on Cancer (IARC). (1993). *Monograph on the evaluation of carcinogenic risk to human*,(Vol. 56), pp. 257–263. Lyon, France.

Ito, Y., Peterson, S. W., Wicklow, D. T., & Goto, T. (2001). *Aspergillus psudotamarii* a new aflatoxin producing species in *Aspergillus* section *Flavi*. *Mycological Research, 105*, 233–239.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). (1996). *Toxicological evaluation of certain food additives and contaminants in food*. WHO food additives series (N° 35, pp. 3–86). Geneva.

Klich, M. A. (Ed.). (2002). *Identification of common Aspergillus Species*. Utrecht, The Netherlands: Centraalbureau voor Schimmelcultyres.

Lacey, J., & Magan, N. (1991). Fungi in cereal grains: Their occurrence and water and temperature relationships. In J. Chelkowski (Ed.), *Cereal grains micotoxins, fungi and quality in drying and storage* (pp. 77–118). B.V. Amsterdam: Elsevier Science Publisher.

Magan, N., Aldred, D., & Sanchis, V. (2004). The role of spoilage fungi in seed deterioration. In D. K. Arora (Ed.), *Fungal biotechnology in agricultral, food and environmental applications* (pp. 311–323). New York: Marcel Dekker.

Marín, S., Magan, N., Albellana, M., Caenal, R., Ramos, A. J., & Sanchis, V. (2000). Selective effect of propionates and water activity on maize mycoflora and impact on fumonisin B_1 accumulation. *Journal of Stored Products Research, 36*, 203–214.

Marín, S., Guynot, M. E., Neira, P., Bernadó, M., Sanchis, V., & Ramos, A. J. (2002). Risk assessment of the use of sub-optimal levels of weak-acid preservatives in the control of mould growth on bakery products. *International Journal of Food Microbiology, 79*, 203–211.

Mazzani, C., Luzán, O., González, N., & Quijada, P. (1995). Efecto del Shield-Na Plus (propionato de sodio y sobato de potasio) sobre el crecimiento y la esporulación *in vitro* de cinco especies de hongos toxicogénicos en Venezuela. *Fitopatol Venez, 8*, 33–36.

Ory, R. L., Crippen, K. L., & Lovegren, N. V. (1992). Off-flavors in peanuts and peanut products. In G. Charalambous (Ed.), *Off-flavors in foods and beverages* (pp. 57–75). Amsterdam, The Netherlands: Elsevier Publisher, Co.

Passone, M. A., Resnik, S. L., & Etcheverry, M. G. (2005). *In vitro* effect of phenolic antioxidants on germination, growth and aflatoxin B_1 accumulation by peanut *Aspergillus* section *Flavi*. *Journal of Applied Microbiology, 99*, 682–691.

Passone, M. A., Funes, G. J., Resnik, S., & Etcheverry, M. G. (2006). Residues of food grade antioxidants applied on stored peanuts to preserve its mycological quality. In *MYCO-GLOBE Advances in research on toxigenic fungi and mycotoxins in South America ensuring food and feed safety in a myco-globe context* (pp. 146). Córdoba, Argentina: Villa Carlos Paz.

Pitt, J. I., & Hocking, A. D. (Eds.). (1997). *Aspergillus* and related teleomorphs. *Fungi and food spoilage* (pp. 339–417). Australia: CSIRO Division of Food Science and Technology, Sydney Academic Press.

Reynoso, M. M., Torres, A. M., Ramirez, M. L., Rodríguez, M. I., Chulze, S. N., & Magan, N. (2002). Efficacy of antioxidant mixtures on growth, fumonisin production and hydrolytic enzyme production by *Fusarium verticillioides* and *F. proliferatum in vitro* on maize-based media. *Mycological Research, 106*, 1093–1099.

SAGPyA, (2006), Secretaría de Agricultura, Ganadería, Pesca y Alimentación. *Estimaciones Agrícolas Cereales*. Dirección de Coordinación de Delegaciones. Buenos Aires.

Samson, R. A., Hoekstra, E. S., Frisuad, J. C., & Fitenborg, O. (2002). In R. A. Samson & E. S. Hoekstra (Eds.), *Introduction to food-and airborne fungi* (pp. 1–269). Nageningen, The Netherlands: Posen and Looyen.

Sanders, T. H., Vercellotti, J. R., & Grimm, D. T. (1993). Shelf-life peanuts and peanuts products. In G. Charalambous (Ed.), *Shelf life stability of foods and beverages* (pp. 289–309). Amsterdam, The Netherlands: Elsevier Publisher Co.

Singer, M., & Wan, J. (1977). Interaction of butylated hydroxytoluene (BHT) with phospholipid bilayer membranes: Effect on ^{22}Na permeability and membrane fluidity. *Biochemical Pharmacology, 26*, 2259–2268.

Truckssess, M. W., Stack, M. E., Nesheim, S., Albert, R. H., & Romer, T. R. (1994). Multifunctional column coupled with liquid chromatography for determination of aflatoxins B_1, B_2, G_1, G_2 in corn, almonds, Brazil nuts, peanuts and pistacho nuts: Collaborative study. *Journal of AOAC International, 6*, 1512–1521.