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Efficacy of sodium metabisulphite for control of *Aspergillus flavus* and aflatoxin B_1 contamination *in vitro* and in chilli powder and whole red chillies

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Abstract

Chillies are an important spice in many regions of the world. During the production and processing phases, they are prone to infection by mycotoxigenic fungi, especially *Aspergillus* Section *Flavi* species and contamination with aflatoxins. There is significant interest in controlling aflatoxin B_1 (AFB_1) contamination in such spices to ensure they remain below the legislative limits for human consumption. This study initially examined the potential efficacy of sodium metabisulphite (NaMBS, up to 5000 mg/L) for the control of growth and AFB_1 production by *Aspergillus flavus* strains *in vitro* on a chilli-based medium modified to 0.93-0.995 water activity, a_w). Subsequent studies examined efficacy of NaMBS in stored naturally contaminated chilli powder and whole red chillies, or when inoculated with *A. flavus* at 0.70, 0.80, 0.90, 0.95 a_w and 30°C for 20 days. NaMBS was effective *in vitro* in controlling growth of the three strains of *A. flavus* at the different a_w levels examined. No growth occurred with >500-1250 NaMBS mg/L. AFB_1 was only produced at 0.98 a_w with complete inhibition at ≥1250 mg/L of the preservative at 30°C. The *in vitro* ED_{50}, ED_{90} for NaMBS varied with a_w conditions. *In situ* studies with chilli powder and whole red dry chillies (naturally contaminated or + conidial inoculum of *A. flavus*) showed that at 0.70 and 0.80 a_w, NaMBS treatments of 1000 and 2000 mg/L resulted in AFB_1 contamination levels below the legislative limits for spices. However, under wetter conditions at 0.90 and 0.95 a_w, AFB_1 contamination of stored chilli powder, -/+ *A. flavus* inoculum, were above the legislative limits, even with 2000 mg/L NaMBS and 20 days storage at 30°C. Stored whole chillies inoculated with *A. flavus* resulted in a reduction of populations, especially at 2000 mg/L NaMBS. Control of AFB_1 was achieved only with 2000 mg/L NaMBS treatment after 10 and 20 days storage. Studies with commercial laminated sheets containing immobilised NaMBS with slow release of SO_2 properties significantly reduced fungal populations and effectively controlled AFB_1 contamination of the stored and packaged whole red chillies.

Key words: Abiotic factors, control, chillies, mycotoxins, preservatives, storage

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1. Introduction

Chillies are an important aromatic spice in many regions of the world. It is an economically important ingredient as a fresh spice, dried whole chillies, chilli flakes or in a powder form. The dry forms are quite hygroscopic and can adsorb water easily during transport and storage that can affect quality. This can make them prone to colonisation by mycotoxigenic fungi, especially *Aspergillus flavus*, a species in the *Aspergillus* section *Flavi* group (Costa et al., 2019). This can result in contamination with aflatoxins (AFs). Indeed, in many countries, including the European Union, there are strict legislative limits on the maximum allowable contamination levels with aflatoxin B_1 (AFB_1) and total AFs in spices. There is thus significant interest in developing food-grade preservation systems that can effectively minimise or prevent fungal spoilage and mycotoxin contamination of chillies. A common group of compounds used for fungal control in foods and beverages are the sulphites, especially potassium or sodium metabisulphite NaMBS), i.e., the salts of sulphurous acid, with known antimicrobial action. Sulphites are extensively used for the preservation of fresh and dried fruit and vegetables, juices and wines (Doughas, Heyes & Smallfield 2005; Jay 2000; Pateraki et al., 2007; Türkkan & Erper 2014) and the European Commission, with the Regulation 1129/2011, set limits for sulphites use in foods and beverages (European Commission 2011). A recent review of sulphur dioxide (SO_2) and other sulphite preservatives clarified that NaMBS contains about 67% SO_2 (EFSA ANS Panel, 2016). It can be used in concentrations of up to a maximum of 2000 mg/L available SO_2 in a range of dried fruits and between 200 and 500 mg/L in a range of other foodstuffs. However, it has not been used in spices such as chillies and chilli-based products previously (EFSA ANS Panel, 2016).

It should of course be noted that many of the food-grade preservatives which can be applied to such commodities are fungistats and not fungicides. While NaMBS and indeed other aliphatic acids and their salts have been examined for the control of spoilage and mycotoxigenic fungi in a range of commodities, few if any, have been focused on control of *A. flavus* and associated AFB_1 contamination of chillies (Costa et al., 2019).

Previous studies with NaMBS (0-2000 mg/L) showed efficacy against a range of pure cultures of mycotoxigenic and spoilage moulds in defined semi-solid media (Ioannidis et al., 2018). In these studies two *Penicillium* species were particularly sensitive and had MIC concentrations of <100 mg/L, with *A. carbonarius* having a MIC of <1300 mg/L. *Aspergillus flavus* appeared to have moderate sensitivity to NaMBS in short term experiments for 48-72 hrs using a micro-well titre assay system. Lopez-Malo, Alzamora & Palou (2005) using defined media found the MIC for *A. flavus* was 900 mg/L at 0.99 a_w and pH 4.5 and 700 mg/l at 0.95 a_w and the same pH. Magan & Medina (2020) found that NaMBS and other aliphatic acids significantly controlled *A. flavus* populations in stored peanuts, with some reductions in AFB_1 contamination.

Other more specific studies on the efficacy of SO_2 on growth of *A. flavus* strains have predominantly focused on mycelial growth only, and not on AFs production. For example, Santos et al. (2013) suggested that *in vitro* growth of *A. flavus* isolated from marine shrimp was inhibited when exposed to 10% NaMBS. Chourasia (1993) found that mycelial growth of *Aspergillus parasiticus* was completely inhibited by 0.1% of NaMBS but no account of abiotic factors, especially temperature x a_w, were taken into account. Studies on other mycotoxigenic species have found differential results. Pateraki et al. (2007) found MIC values of between 750-1000 mg/L NaMBS depending on the prevailing a_w level for ochratoxigenic strains of *A. carbonarius* on grape juice-

based media. Ioannidis et al. (2015) also using grape juice-based media and strains of *A. carbonarius* found MIC values of 458 mg/L under optimum temperature (31.7°C) and a_w (0.965) conditions. However, practically no studies have examined the effect of food-grade preservatives on the control of growth and AFB_1 production by *A. flavus* strains with food-grade preservatives such as NaMBS in chillies (Costa et al., 2019).

The objectives of this study were to: (a) screen concentrations of NaMBS for *in vitro* efficacy to control growth and AFB_1 production by strains of *A.flavus* under different a_w conditions at 30°C on a chilli-based medium and (b) calculate the relative ED_{50} and ED_{90} concentrations for efficacy of NaMBS for control of growth and AFB_1 control. These studies were complimented with *in situ* studies to examine (i) effects of NaMBS on colonisation and AFB_1 contamination of chilli powder naturally contaminated or inoculated with *A.flavus* conidia and stored at different a_w levels for 20 days at 30°C, (ii) control of *A. flavus* and AFB_1 of naturally contaminated whole red chillies or with additional *A. flavus* conidial inoculum on fungal populations and AFB_1 contamination when stored at 0.90 and 0.95 a_w for 20 days at 30°C, and (iii) efficacy of commercial laminated NaMBS treated sheets with slow release of SO_2 concentrations on control of total and *A. flavus* fungal populations and AFB_1 production in whole red chillies inoculated with *A. flavus* conidia and stored for 20 days at 30°C.

2. Materials and methods

2.1 Fungal strains

Thee AFB_1 producing strains of *A. flavus* (DAJ_1, DAJ_2 and DAJ_4) isolated from Iraqi chilli samples were used in the *in vitro* studies. These were all confirmed using molecular analyses, and were all AFB_1 producers (Al-Jaza et al., 2021). They were maintained on a 10% chilli powder-based agar medium.

2.2 Basal media preparation

A 10% red chilli powder-based medium (10% chilli powder; 2% technical agar (Thermo Fisher Scientific, Hemel Hempstead, Herts, U.K., Technical Agar No. 3, Code: LP0013); water) was used as the basic medium in these studies (0.995 water activity, a_w). This was modified to 0.98 and 0.93 a_w by substituting water with appropriate glycerol/water solutions.

2.3 Screening of sodium metabisulphite for efficacy against growth and aflatoxin B_1 production by A. flavus *strains* in vitro

The analytical grade sodium metabisulphite ($Na_2S_2O_5$; NaMBS; E223 was obtained from Merck Ltd. (Merck Life Science, Gillingham, Dorset, UK). One gram of the NaMBS was dissolved in 10 ml of distilled water to obtain a stock solution (100,000 mg/L). The final stock solution was filtered through a 0.22 µ filter cartridge using a sterile syringe into a sterile 25 ml plastic Universal bottle and kept at 4ºC until use.

The required concentrations (500, 1250, 2500 and 5000 mg/L) were prepared by mixing the appropriate amount of stock solution of each compound with sterilized molten 10% chilli media at each of the different a_w levels when cooled to about 50ºC. Each treatment medium was shaken thoroughly and then poured into 9 cm Petri plates (approx. 17.5 mls per plate), in the sterile flow bench and allowed to cool. The different a_w treatment were stored in separate sealed bags at 4ºC until use. Control plates at each a_w level were also prepared using the 10% basic chilli medium only. The final a_w of each treatment was checked using the Aqualab TE4 (Labcell Ltd., Medstead, Hants., U.K.) and found to be within 0.003 of the treatment levels.

A spore suspension from a growing colony of each *A. flavus* strain was made by lightly scraping the culture surface of 7 day-old cultures on 10% chilli medium with a surface sterilised loop. The conidia were placed in 10 mls of sterile water containing 0.001% tween 80 in 25 ml glass Universal bottles. These were shaken to obtain a conidial spore suspension. The concentration of the spore suspension was quantified using a haemocytometer and diluted with sterile water to obtain 10^6 spores/ml.

The treatment and replicate plates were all centrally inoculated with a sterile loop of the spore suspension. Each a_w treatment was kept in different sealed bags and these were all incubated 30ºC for ten days. The diametric growth rates were measured in two directions at right angles to each other on a daily basis. This data was used to calculate the temporal growth and the regression of the exponential phase of the growth curve used to calculate the diametric growth rate (mm/day). These were plotted and used to calculate the 50% effective dose inhibition (ED_{50}) and 90% effective dose (ED_{90}) concentrations necessary for inhibition of growth. Similarly, this approach was used for subsequent calculation of the concentrations of NaMBS necessary for ED_{50} and ED_{90} values for AFB_1 production control.

2.4 In situ *studies of NaMBS efficacy for control of growth of* A. flavus *and AFB_1 contamination of chilli powder and whole chillies during storage*

2.4.1 Moisture adsorption curves

Chilli powder: A moisture absorption curve was constructed to determine the quantities of water required to add to dry red chilli powder samples to modify the moisture contents to the treatment target a_w values. This curve was prepared by adding different amounts of distilled water to the chilli powder and equilibrating at 4ºC for 24 hrs. After equilibration and returning the samples to 25ºC the a_w was measured with an AquaLab 4TE. The amounts of added water vs a_w levels was plotted to quantify the exact amounts of water required to modify the chilli powder to the treatment a_w levels.

Whole Chillies: Similarly, a moisture absorption curve for whole red dry chillies was prepared as described for chilli powder.

2.5 Effects of NaMBS on naturally contaminated chilli powder or that inoculated with on conidia of A. flavus *on fungal populations and AFB_1 contamination during storage for up to 20 days*

Thirty grams of chilli powder were each weighed into each of 24 solid culture vessels and then closed with plastic lids with an integrated semi-permeable membrane. The samples were divided into four groups of a_w treatment conditions (0.70, 0.80, 0.90 and 0.95 a_w= 18-20; 26-28, 42-44 and 68-70% m.c.). Each group of chillies was adjusted by adding the appropriate volume of sterile distilled water for the control samples based on the moisture absorption curve to obtain the target levels.

NaMBS treatment concentrations were made up in sterile distilled water to modiy the a_w of the treatments. The preservatives (1000 and 2000 mg/L) were added like water to obtain the target treatments. All chilli powder treatments were stored at 4ºC for 24 hrs with periodic shaking to allow absorption and equilibration.

The controls and preservative treatments and replicates were removed from the 4ºC room and inoculated with 0.25 ml of a 10^2 spores/ml aliquot from a conidial suspension made from conidia obtained from a 10-day old culture of *A. flavus* (strain DAJ_1) and thoroughly mixed. Each treatment was then divided into separate replicate

glass jars with microporous lids at each a_w level and stored in environmental chambers at 30ºC for 20 days. Each chamber also contained 2 x 500 mL beakers containing a glycerol/water solution of the same a_w as the treatment to maintain the equilibrium relative humidity (ERH) of the atmosphere. Three replicates were destructively sampled after 10 and 20 days storage. The samples were all stored at -20ºC for subsequent AFB_1 analyses. The experiments were repeated once.

Enumeration of total and A. flavus *populations in the stored chilli powder samples:* Sub-samples of each treatment and replicate were cut into pieces and 1 g of chilli powder weighed and mixed with 9 ml of sterile distilled water (with 0.01% Tween 80) to obtain the 10-1 dilution. All samples were serially diluted after vigorous shaking at each dilution using a vortex mixer. A 200µl of each dilution was spread-plated with a surface sterilised L shaped glass spreader onto Malt Extract Agar (MEA; ThermoFisher Scientific, Hemel Hempstead, Herts., U.K., Code: CM0059) in 90 mm Petri plates. The three replicate plates of each dilution for each sample were incubated at 30ºC for seven days and the dilutions with 5-50 fungal colonies enumerated. The *A. flavus* colonies were enumerated with reference to the strains used in the *in vitro* experiments.

2.6 Efficacy of NaMBS treatment of whole red chillies inoculated with A. flavus *conidia under different water activity conditions on fungal populations and aflatoxin B_1 when and stored for up to 20 days at 30ºC.*

The same procedure was followed as for chilli powder except that naturally contaminated whole red chillies were used and only two a_w treatments (0.90 and 0.95 = 35-36 and 48-50% m.c.). Either sterile distilled water for the controls or different concentrations of NaMBS (1000, 2000 mg/L) in sterile distilled water were added with reference to the moisture adsorption curve. Again all treatments and replicates were equilibrated at 4ºC for 24 hrs and shaken regularly. The whole red chillies treatments were inoculated with 0.25 ml of a 10^2 spores/ml of *A. flavus* (DAJ_1) at each a_w level as detailed previously. The treatments were shaken thouroughly and equilibrated at 25ºC. The treatments and replicates were then placed in the glass jars with microporous lids in the environmental chambers as detailed previously and stored for up to 20 days with three replicates destructively sampled after 10 and 20 days storage at 30ºC. Treatments were sampled at time 0, 10 and 20 days for fungal enumeration and AFB_1 contamination levels. Those for toxin analyses were stored at -20ºC for subsequent analyses.

Enumeration of total and A. flavus *populations on stored whole red chillies:* Sub-samples of each treatment and replicate were cut into pieces and 1 g of chillies weighed and mixed with 9 ml of sterile distilled water (with 0.01% Tween 80) to obtain the 10^{-1} dilution. The procedure followed was as detailed in Section 2.5.

2.7 Efficacy of laminated sheets of NaMBS with different concentrations of slow release SO_2 for control of fungal populations and AFB_1 production in naturally contaminated whole red chillies inoculated with A. flavus *spores*

Sub-samples of naturally contaminated whole red chillies were weighed into 12 solid culture vessels. These were split into two groups of a_w treatments (0.90 and 0.95 a_w by the addition of sterile water, excluding an inoculum of 0.25 ml of the *A. flavus* conidial suspension. After a_w modification overnight at 4ºC the samples were kept at 25ºC and an inoculum of 0.25 ml of a 10^2 spores/ml of *A.flavus* (DAJ_1) at each a_w level

as detailed previously. The replicates and treatments were then covered with the NaMBS treatment sheets (Tessara PTY, Ltd, Cape Town, South Africa). These laminated sheets had either 40 or 70% immobilised NaMBS and released sulphur dioxide gas (SO_2) rapidly in the first 12-24 hrs and then slowly subsequently over periods of 96 hrs or more. These treatments released approx. 50 mg/L and 110 mg/L SO_2. Each treatment was then enclosed iin a re-sealable plastic bag and incubated. All treatments were incubated at 30ºC for 20 days and after 10 and 20 days, three replicates per treatment were destructively sampled for fungal populations and sub-samples frozen at -20ºC for later AFB_1 toxin analyses.

2.8. Aflatoxin B_1 extraction and quantification

2.8.1 In vitro *studies*

Extraction from fungal cultures: Up to eight agar plugs were taken across the colony with a surface-sterilised 4 mm cork-borer and placed in pre-weighed 2 ml Eppendorf tubes and re-weighed. A 1 ml aliquot of chloroform was added to the agar plugs in the Eppendorf tube which was then shaken on a digital orbital shaker (KS 501; IKA-Werke Gmbh, Staufen, Germany) for 1hr. The supernatant was removed from the Eppendorf tube and the chloroform placed in a new tube and dried overnight.

Derivatization of aflatoxins: For each 2 ml Eppendorf tube containing the residue of a sample, 200 µl of hexane was added plus 50µl trifluoroacetic acid (TFA, Fisher Scientific, UK). The Eppendorf tube was vortexed for 30 seconds and left for 5 min. after this, 950 µl of water:acetonitrile (9:1, v/v) was added and the mixture vortexed for 30 secs then left for 10 min. The mixture separated into two layers, the supernatant containing the hexane and the bottom layers containing the toxin. The hexane was removed (300 µl) and the residue containing the toxin filtered through polythene filters (13 mm, 0.22 µm; Jaytee Biosciences, Ltd, Herne Bay, UK) directly into amber salinized 2 ml HPLC vials (Agilent Technologies, Ltd, Cheadle, UK) for HPLC analysis (Sultan et al., 2010).

Preparation of AFs standards: A 200 µl stock solution of AFs mixed solution (Romer Lab UK Ltd, Cheshire, UK) comprising of 0.2 µg of aflatoxin B_1 (AFB_1), 0.2 µg of aflatoxin G_1 (AFG_1), 0.06 µg of aflatoxin B_2 (AFB_2) and 0.06 µg aflatoxin G_2 (AFG_2) was made. Different amounts of stock solution were transferred into 2 ml Eppendorf tubes then left overnight and allowed to evaporate inside a fume cupboard at 20ºC to dryness. These samples were used for derivatisation as described previously.

HPLC analysis: The HPLC system used was an Agilent 1200 series (Agilent Technologies, Hewlett-Packard-Strasse 8, 76337 Waldbronn, Germany) reversed-phase with a fluorescence detector (λ_{exc} 36 nm; λ_{em} 440 nm). The mobile phase was methanol (30%):water (60%):acetonitrile (10%). A C18 column was used (Phenomenex Gemini; 150 x 4.6, 3 µm particle size; this was preceded by a Phenomenex Gemini C18 column, 3 mm, 3µm Guard cartridge (Phenomenex, CA, USA). The column temperature was 25ºC; flow rate 1.0 ml/min; Injection volume 50 µl; Stop time 15 min; Ret. Time 10 min; Excitation 360 nm; Emission 440 nm.

2.8.2 Aflatoxin B_1 quantification in chilli powder and whole red chillies

*Immuno-affinity column technique (IAC)***:** Each replicate of the chilli powder or whole red chilli samples from each treatment were analysed for AFB_1 using Immuno-affinity columns (IAC) from Romer PLC ((Afla Star™ R IAC Column Romer Labs®Inc; Tulln, Austria) specifically for chillies and similar pigmented matrices. Three grams of chillies were weighed, and placed in a 30 ml tube. 12 ml of extraction solution

(acetonitrile/water; 6/4 (v/v)) was added to the tube which was closed and then shaken for 1 hour in a shaker. After this, the extract was filtered using a funnel containing a Whatman filter paper (12.5 cm). The extracts were diluted with PBS (Phosphate buffered saline; pH 7.4) until the content of acetonitrile was lower than 5% (v/v) with a pH of 7. The diluted extract was transferred to the IAC and allowed to pass through the column using a syringe barrel as a reservoir. The extract solution was allowed to drip independently (one drop per second) into the collection vessel. The column and extract was kept at room temperature of 20-25°C. The IAC was rinsed with 2 x 10 ml of distilled water. The first portion of the rinse solution was used to rinse the container. The second portion of the rinse solution was applied directly to the Romer IAC column. Any remaining liquid was removed from the column by applying slight pressure on top of the column. 2 ml of methanol was directly added to the column and the eluted liquid collected in a new sterile 2 ml Eppendorf tube. The eluate was evaporated under reduced pressure at 40°C using a rotary evaporator. 1 ml of water:methanol (0.5:0.5) was added to the extract and mixed. This was filtered through a Nylon filter ((13 mm, 0.22 µm; Jaytee Biosciences, UK)), directly into amber HPLC vials and then stored at -20°C until quantification using HPLC.

The HPLC analyses was carried out as described previously. Each batch of samples included a set of derivatized aflatoxin standards. The chilli powder and whole red chillies had a mean background level of 0.018 ng/g chilli powder/chillies. The aflatoxin B_1 results were thus corrected for this initial internal level of contamination.

Preparation of standards: A stock solution of 200 µl of aflatoxin mix solution (Romer Lab UK Ltd, Cheshire, UK) comprising of 0.2 µg each of aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) and 0.06 µg each of aflatoxin B_2 (AFB_2) and aflatoxin G_2 (AFG_2) was made. The stock solution was transferred into 2-ml Eppendorf tube then adds 300µl of acetonitrile to get 800µg stock solutions. Vortex and preparation sequential concentrations (400, 200, 100, 50, 10 and 5) by adding 500µl (acetonitrile: water 50/50) to 500µl of stock solution at each time.

2.9 Statistical analysis

The effects of the NaMBS treatments in vitro and in situ on fungal populations and AFB_1 production were determined by using the Shapiro Wallis Test (Non-normality data) and ANOVA (Normality data). The significant statistical level was at $P<0.05$ for all individual and interacting factors.

3. Results

3.1 In vitro *efficacy of NaMBS on growth and aflatoxin B_1 production by strains of* A. flavus *on a chilli-based medium*

The impact of the interactions between NaMBS concentrations and a_w levels on the mycelial growth of the three *A.flavus* strains (DAJ_1, DAJ_2 and DAJ_4) on a 10% chilli medium at 30°C after 10 days incubation was examined. Fig. 1 shows that mycelial growth was consistently reduced by 500-5000, 1250-5000 and 2500-5000 mg/L of this preservative at 0.93, 0.98 and 0.995 a_w, respectively. Optimum growth was generally at 0.98 a_w and not with freely available water (0.995 a_w).

Fig. 2 shows the relative impact of the NaBMS on AFB_1 production by these three strains on the chilli-based medium. At 0.995 and 0.93 a_w practically no AFB_1 was produced, in both the control or preservative treatments. However, at 0.98 a_w there was a significant production of AFB_1 and this was reduced for all three strains, as the NaMBS concentration was increased. Statistically, concentration of NaMBS, a_w and

their interactions were all statistically significant ($P<0.05$) for the three strains of *A. flavus* using the ANOVA for growth data, and the Shapiro Wallis analyses for the for the AFB_1 data (non-normality data).

These data sets were used to calculate the ED_{50} and ED_{90} concentrations necessary for control of growth and AFB_1 production. Table 1 summarises these concentrations necessary for the different a_w treatments used. Statistically, the effects of NaMBS (mg/L), a_w levels, and concentration x a_w on the growth rate of the three strains of *A.flavus* were all significant ($P \leq 0.05$). For toxin control AFB_1 production was significantly affected ($P \leq 0.05$) by NaMBS concentrations, a_w and their interactions.

3.2 In situ *studies on NaMBS for control of aflatoxin B_1 control in naturally contaminated chilli powder and that inoculated with* A. flavus *conidia under different water activity storage regimes at 30°C*

The total fungal populations and those of *A. flavus* were assayed in both naturally contaminated chilli powder and that inoculated with additional *A. flavus* conidia. The total mycobiota present was Log_{10} CFUs: 2.65±0.55 and that of *A. flavus* populations, Log_{10} CFUs: 0.34±0.18 g/chilli powder. At 0.70 and 0.80 a_w these populations did not change significantly. However, at 0.90 and 0.95 a_w the total fungal populations increased significantly, especially at 0.95 a_w and 20 days storage. Those of *A. flavus* increased slightly, especially in the controls. Suppl. Fig. 1 provides these data. Table 2 provides the statistical summary of significant individual, two or three-way interactions on fungal populations and on AFB_1 production.

Suppl. Fig. 2 shows the *A. flavus* populations isolated from the chilli powder treatments inoculated with additional conidial inoculum of this mycotoxigenic species and stored for up to 20 days. In this case, there were higher populations of the mycotoxigenic species generally. These remained stable at 0.70 a_w. However, at 0.80, 0.90 and 0.95 a_w there was some effect of the NaMBS treatments with a reduction in the *A. flavus* populations (Table 3).

Fig. 3 shows that at 0.70 and 0.80 a_w 1000 and 2000 mg/L NaMBS was effective at reducing AFB_1 levels below the permitted limits after 10 days storage and at 0.80 a_w for 20 days in the naturally contaminated samples. In the wetter sample treatments at 0.95 a_w 1000 and 2000 mg/L NaMBS treatments inhibited AFB_1 production for 10 days in both naturally contaminated and *A. flavus* inoculated samples when compared to the control without NaMBS. After 20 days storage AFB_1 was detected in all the treatments. This suggests that the preservative delayed the production of AFB_1. Statistical analyses showed that for AFB_1 production, concentration, time, concentration x time and concentration x a_w were significant. The a_w and three-way interactions between the factors were not significant.

3.3 Effect of NaMBS on in situ *control in whole red chillies inoculated with A. flavus: effects on total and* A. flavus *populations and AFB_1 contamination in relation to initial water activity and storage periods at 30°C*

Fig. 4 shows the efficacy of two concentrations of NaMBS on the total fungal populations and those of *A. flavus* isolated from whole red chillies inoculated with conidia at two different a_w levels initially and after 10 and 20 days storage. After both 10 and 20 days storage there was a reduction in the total fungal populations and those of A. flavus in both the NaMBS treatments. Statistically, the populations on whole chillies inoculated with *A. flavus* were significantly affected ($P \leq 0.05$) by NaMBS

concentration and the storage time, except in the 2000 mg/L treatment where there was no effect of storage a_w and the interaction between the factors (Table 4).

The effect of storage water availability, preservative treatments and storage time on control of AFB_1 shows that there was a clear effect on the contamination of whole red chillies with this toxin at 0.90 a_w and a more gradual inhibition at 0.95 a_w (Fig. 5). Statistically, AFB_1 production by *A. flavus* was significantly affected ($P<0.05$) by NaMBS concentration, and storage time except after 10 days storage at both a_w levels. The statistical effect of single (concentration, time and a_w) and interacting conditions (concentration x time x a_w) on AFB_1 production are shown in Table 4.

3.4 In situ efficacy of slow release SO_2 from laminated commercial preservative sheets on control of A. flavus *populations and AFB_1 contamination in* A. flavus *inoculated whole red stored chillies*

Fig. 6a,b shows the impact of laminated sheets using 40 and 70% NaMBS immobilisation on the total fungal populations and those of *A. flavus* isolated from the inoculated whole red chillies initially and stored for 10 and 20 days. The populations of *A. flavus* on stored chillies were gradually decreased when using the increased SO_2 treatments (40, 70%) at both a_w levels (0.90 and 0.95 a_w). The efficacy appeared to increase with incubation time. Statistically, there were significant effects of the NaMBS dose, incubation time, a_w and dose x a_w x time on *A. flavus* populations (Table 5).

Fig. 6c,d compares the effect of the treatments on AFB_1 contamination in the whole red chillies pre-inoculated with *A. flavus*. There was a significant impact of the use of such packaging on relative AFB_1 contamination. The AFB_1 contamination was completely inhibited by both NaMBS laminated sheet treatments with contamination levels below the detection limit. Statistically, there was a significant effect of the NaMBS dose and storage time on AFB_1 production, but that this was unaffected by storage a_w and interaction between the factors (concentration x time x a_w). Also, statistical analysis showed that there was no significant difference between the preservative treatments. However, there was a significant difference between the untreated control and these two treatments (see Table 5).

4. DISCUSSION

The present studies have shown the promise of using NaMBS as a food-grade preservative for chilli powder and whole dried chillies. The *in vitro* studies showed that the growth of three strains of *A. flavus,* isolated from chillies*,* was significantly reduced when increasing concentrations, regardless of the a_w level used.

No AFB_1 production was found in the chilli-based medium at 0.995 and 0.93 a_w in both the controls and preservative treatments, probably due to the a_w levels which were marginal for toxin production. However, NaMBS concentrations of ≥1250 mg/L reduced AFB_1 levels at 0.98 a_w. There was also an indication that at lower concentrations of 500 mg/L some stimulation of AFB_1 by some strains occurred at 0.98 a_w. This preservative is a fungistatic compound, and may at low concentration, be perceived as a chemical stress with the response by the *A. flavus* strains resulting in the biosynthesis of secondary metabolites including aflatoxins. This has been previously observed with low levels of other preservatives including anti-oxidants and essential oils (Aldred et al., 2008).

Chourasia (1993) suggested that the growth rate of *A. parasiticus,* also in the *Aspergillus* section *Flavi* group was completely inhibited by 1000 mg/L of NaMBS,

however this was in a defined liquid medium. They also found that using 1000 m/L NaMBS prevented aflatoxins biosynthesis in the liquid broth, but did result in production of aflatoxins and sclerotia on solid media. Unfortunately, without taking into account the natural food matrix and key abiotic parameters, liquid broth studies do not help in determining efficacy accurately. In contrast, Sultan (2010) found that growth of *A. flavus* strains from peanuts were also stimulated by low doses of 500 and 1000 mg/L NaMBS at all a_w conditions examined. Gowda et al. (2004) found that growth of *A. parasiticus* strains was completely inhibited by 1000-5000 mg/L of this preservative. Alam et al. (2010) reported that the growth of *A. parasiticus* was inhibited on Czapek Yeast Extract Agar medium using high concentrations of calcium propionate. Previously, Magan (1993) indicated that the growth rates of *A. flavus*, *A. ochraceus* and *A. terreus* were inhibited by 50 mg/L of SO_2, but on a defined malt extract agar medium at 0.995 and 0.95 a_w.

In both naturally contaminated chilli powder and that inoculated with additional inoculum of *A. flavus* showed that at 0.70 and 0.80 a_w there was relatively good control of AFB_1 contamination. However, at 0.90 and 0.95 a_w, which were more conducive conditions, there was some control of AFB_1 production after 10 days storage but not after 20 days storage, even with 2000 mg/L NaMBS. This was especially so where additional inoculum of *A. flavus* was used. This suggests that when there is a high inoculum pressure due to contamination with *A. flavus* then under conducive conditions, especially at 0.95 a_w there was higher AFB_1 production occurring which would result in rejection of the chilli powder.

Subsequent studies with whole red chillies inoculated with conidia of *A. flavus* were stored at 0.90 and 0.95 a_w to examine the changes in both fungal populations and AFB_1 contamination. This showed that after inoculation the populations of *A. flavus* became predominant during the first 10 days of storage. The NaMBS treatment reduced the total fungal population and those of *A. flavus* by approx. $\log_{10}$ 1.5 CFUs g/chillies, especially at 0.95 a_w and after 20 days storage.

The AFB_1 contamination clearly showed that contamination was highest at whole red chillies stored at 0.95 aw and 30°C. Only the use of 2000 mg/L of NaMBS reduced the AFB_1 contamination levels to the EU legislative maximum levels after 20 days storage. There is little comparable studies with this or other food-grade preservatives in relation to control of fungal populations and AFB_1 contamination of chillies. Studies on stored peanuts by Sultan (2011) compared different salts of aliphatic acids and some anti-oxidants for control of *A. flavus* populations and AFB_1 contamination stored at different aw levels at 25°C. While NaMBS was not included the results showed that the anti-oxidant octyl gallate was very effective. At 0.90 and 0.93 a_w peanuts treated with this compound and stored at 25°C for 15 days showed that the best results were obtained with 1000-2000 mg/L with a $\log_{10}$ reduction of 1.5-2.0, especially after 7 days storage. Also, at these doses there was a significant inhibition of AFB_1 contamination of stored peanut after 7 days storage at 0.93 a_w. However, after 14 days storage there was little difference between treatments. Thus, it is difficult with some of these food grade compounds to maintain efficacy during storage, especially under conducive abiotic conditions. Perhaps, hurdle technology approaches would be beneficial by combining such food-grade preservatives with modified atmosphere storage or the use of active packaging systems (Erkmen and Bozoglu, 2016).

This is the first time that laminated sheets containing immobilised NaMBS have been examined for use in commodities such as chillies. The results suggest that at the higher concentration (70%) there was a reduction in both the total fungal populations and those of *A. flavus* after 10 and 20 days storage. The fact that AFB_1 was below he

limit of detection or quantification in the two NaMBS impregnated treatments certainly suggests some promise in developing this approach. The 40% laminated sheet treatment results in the initial release of about 40-50 ppm SO_2 over the first 12-24 hrs and then this decreases, with some residual production of 5-10 ppm SO_2 subsequnetly. For the 70% treatment, the initial release is about 100-110 ppm SO_2 and then this deceases to about 50 ppm after 24 hrs and then to about 10 ppm over the following 3-4 days (Tessara Ltd, personal communication). It may thus be possible to modify the relative immobilised NaMBS treatment in the laminated sheets for potential applications for the storage of spices and perhaps other commodities. More research is needed on whether this approach could be used for both dry chillies and indeed in fresh chillies which have a very short shelf life and where such packaging may have potential applications.

No previous studies have examined in detail the use of this food grade preservative for minimising AFB_1 contamination of chillies or indeed other related spices (Costa et al., 2019). There are some studies of the efficacy of NaMBS as anti-fungal agents for controlling fungal decay in grapes, wine, cereals and dried fruits but not to minimise mycotoxin contamination. For example, Jiang et al. (2015) examined the effect of SO_2, ethanol, and combinations on the fungal profile and OTA production by *A. carbonarius* in wine and found significant inhibition of growth and of OTA production after addition of SO_2. In addition, Ahmed et al. (2018) found that the SO_2-releasing sheets were highly effective to reduce the incidence of grey mold in Italian grapes during cold storage for 50 days. From a human health perspective, the use of NaMBS is allowed as a food preservative by the IARC and WHO and there is a threshold limit of the concentration which can be used by law (GRAS) with a maximum of 200 mg/kg. In the present study, the two concentrations used were 1000 and 2000 mg/L NaMBS.

Conclusions

This study has shown that both total and *A. flavus* fungal populations and AFB_1 contamination of chilli powder and whole dry red chillies can be effectively reduced by using NaMBS when stored under conducive conditions of 0.90-0.95 a_w and 30°C. The use of laminated sheets containing immobilised NaMBS certainly shows promise and needs to be examined in more detail to identify and translate such approaches into practical preservation products for chillies and their derived products and other spices to minimise the potential colonisation by *A. flavus* and AFB_1 contamination.

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Table 1. (a) Effective doses (ED_{50} and ED_{90}) of NaMBS (mg/L) required for *in vitro* inhibition of growth of the three strains of *A. flavus* (DAJ_1, DAJ_2 and DAJ_4) and (b) on AFB_1 production (ng/g medium) on a 10% chilli powder medium at 30°C.

(a) **Growth inhibition**

	ED_{50} (mg/L)			ED_{90} (mg/L)		
A. flavus strains	**DAJ1**	**DAJ2**	**DAJ4**	**DAJ1**	**DAJ2**	**DAJ4**
Water activity						
0.995	800	860	900	1175	1175	1180
0.98	1550	1600	1550	2300	2380	2310
0.93	250	250	275	450	450	450

(b) **Aflatoxin B_1 inhibition (ng/g medium)**

A. flavus strains	**DAJ1**	**DAJ2**	**DAJ4**	**DAJ1**	**DAJ2**	**DAJ4**
Water activity						
0.995	420	525	300	460	1180	800
0.98	250	255	300	410	800	800
0.93	240	250	250	410	440	460

Table 2. Statistical analyses of the effects of NaMBS (mg/L) on the fungal populations and AFB_1 contamination in naturally contaminated stored chilli powder at 30°C, at different a_w levels for 10 and 20 days.

Factor	**Effect**	**Response**
Concentration (C)	*S*[a]	Fungal populations (Log_{10} CFUs/g)
	S[a]	Log_{10} AFB_1 (µg/kg)
Time (t)	*NS*[b]	Fungal populations (Log_{10} CFUs/g)
	S[a]	Log_{10} AFB_1 (µg/kg)
Water activity (a_w)	*S*[b]	Fungal populations (Log_{10} CFUs/g)
	NS[a]	Log_{10} AFB_1 (µg/kg)
C x t	*S*[a]	Fungal populations (Log_{10} CFUs/g)
	S[a]	Log_{10} AFB_1 (µg/kg)
C x a_w	*S*[a]	Fungal populations (Log_{10} CFUs/g)
	S[a]	Log_{10} AFB_1 (µg/kg)
C x t x a_w	*S*[a]	Fungal populations (Log_{10} CFUs/g)
	NS[b]	Log_{10} AFB_1 (µg/kg)

[a]ANOVA test; [b]Shapiro Wills test

Table 3. Statistical analyses of effects of NaMBS (mg/L) on isolation of *A. flavus* populations from chilli powder initially inoculated with conidia of this mycotoxigenic species and on aflatoxin B_1 contamination after storage for up to 20 days at 30°C at different a_w levels (0.70, 0.80, 0.90 and 0.95 a_w).

Factor	**Effect**	**Response**
Concentration (C)	*S*[a]	*A. flavus* populations (Log_{10} CFUs/g)
	S[a]	Log_{10} AFB_1 (µg/kg)
Time (t)	*S*[b]	*A. flavus* populations (Log_{10} CFUs/g)
	S[b]	Log_{10} AFB_1 (µg/kg)
Water activity (a_w)	*S*[b]	*A. flavus* populations (Log_{10} CFUs/g)
	S[a]	Log_{10} AFB_1 (µg/kg)
C x t	*S*[b]	*A. flavus* populations (Log_{10} CFUs/g)
	NS[b]	Log_{10} AFB_1 (µg/kg)
C x a_w	S[b]	*A. flavus* populations (Log_{10} CFUs/g)
	NS[b]	Log_{10} AFB_1 (µg/kg)
Time x a_w	S[b]	*A. flavus* populations (Log_{10} CFUs/g)
	S[b]	Log_{10} AFB_1 (µg/kg)
C x t x a_w	S[a]	*A. flavus* populations (Log_{10} CFUs/g)
	NS[a]	Log_{10} AFB_1 (µg/kg)

[a]ANOVA test; [b]Shapiro Wills test

Table 4. Statistical analyses of the effect of NaMBS (mg/L) on *in situ* populations of *A. flavus* inoculated whole red chillies and AFB_1 contamination when stored at 30°C and 0.90 and 0.95 water activity for 10 and 20 days.

Factor	**Effect**	**Response**
Concentration (C)	*S*[a]	Fungal populations (Log_{10} CFUs/g)
	S[b]	Log_{10} AFB_1 (µg/kg)
Time (t)	*S*	Fungal populations (Log_{10} CFUs/g)
	S	Log_{10} AFB_1 (µg/kg)
Water activity (a_w)	*NS*	Fungal populations (Log_{10} CFUs/g)
	NS	Log_{10} AFB_1 (µg/kg)
C x t	*S*	Fungal populations (Log_{10} CFUs/g)
	NS	Log_{10} AFB_1 (µg/kg)

C x a_w	*S* *S*	Fungal populations (Log_{10} CFUs/g) Log_{10} AFB_1 (µg/kg)
C x t x a_w	*NS* *NS*	Fungal populations (Log_{10} CFUs/g) Log_{10} AFB_1 (µg/kg)

[a]ANOVA test; [b]Shapiro Wills test

Figures

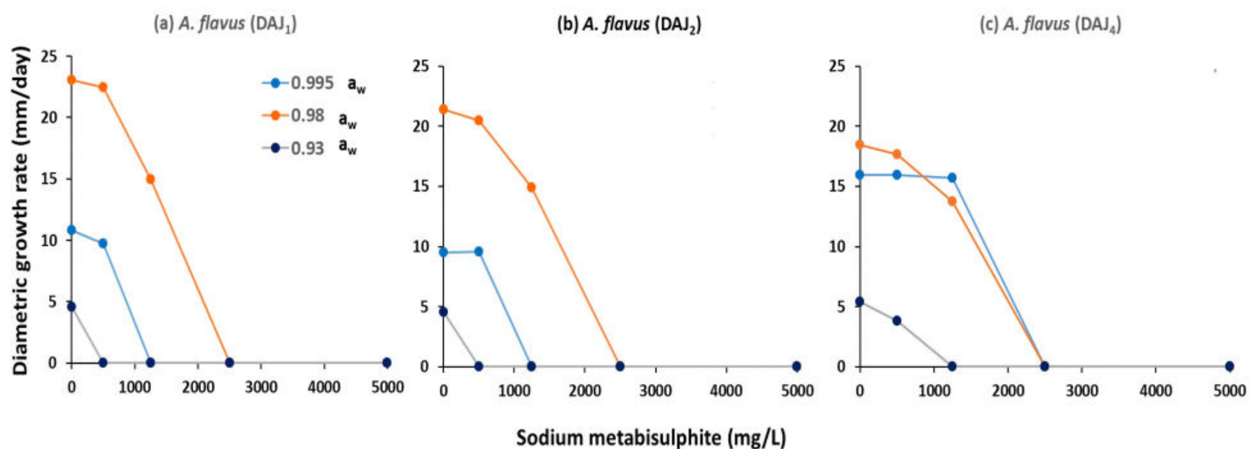


Fig 1a-c. Effect of sodium metabisulphite (NaMBS) concentrations (mg/L) on mycelial growth rate of three strains of *A. flavus* (DAJ_1, DAJ_2 and DAJ_4) on a 10% chilli powder medium modified to three a_w levels (0.93, 0.98 and 0.995 a_w) at 30ºC for 10 days. Bars represent the standard error. Where there are no bars, they are smaller than the symbols.

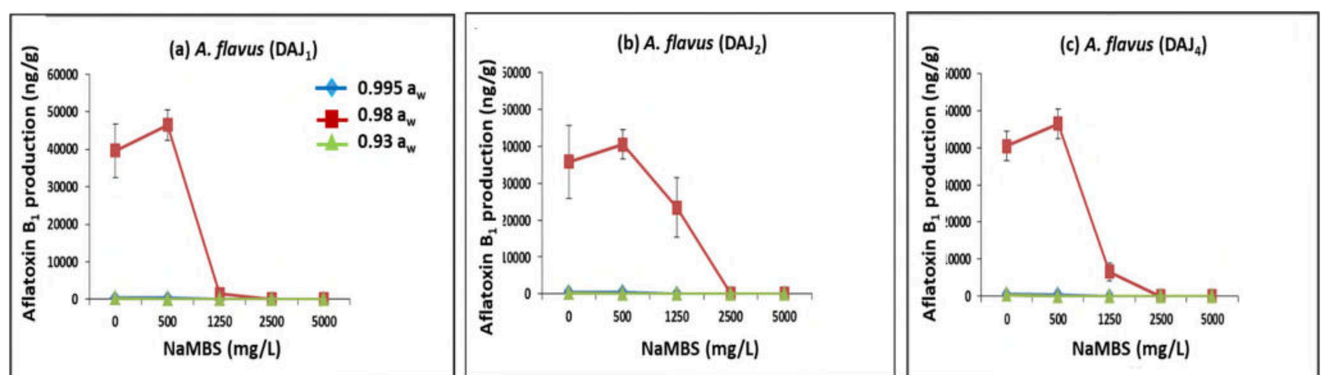


Fig. 2a-c. Effect of sodium metabisulphite (NaMBS; mg/L) on AFB_1 of *A. flavus* strains (DAJ_1, DAJ_2 and DAJ_4) on a 10% chilli powder medium at three a_w levels at 30ºC for 10 days. Bars are the standard errors of the mean.

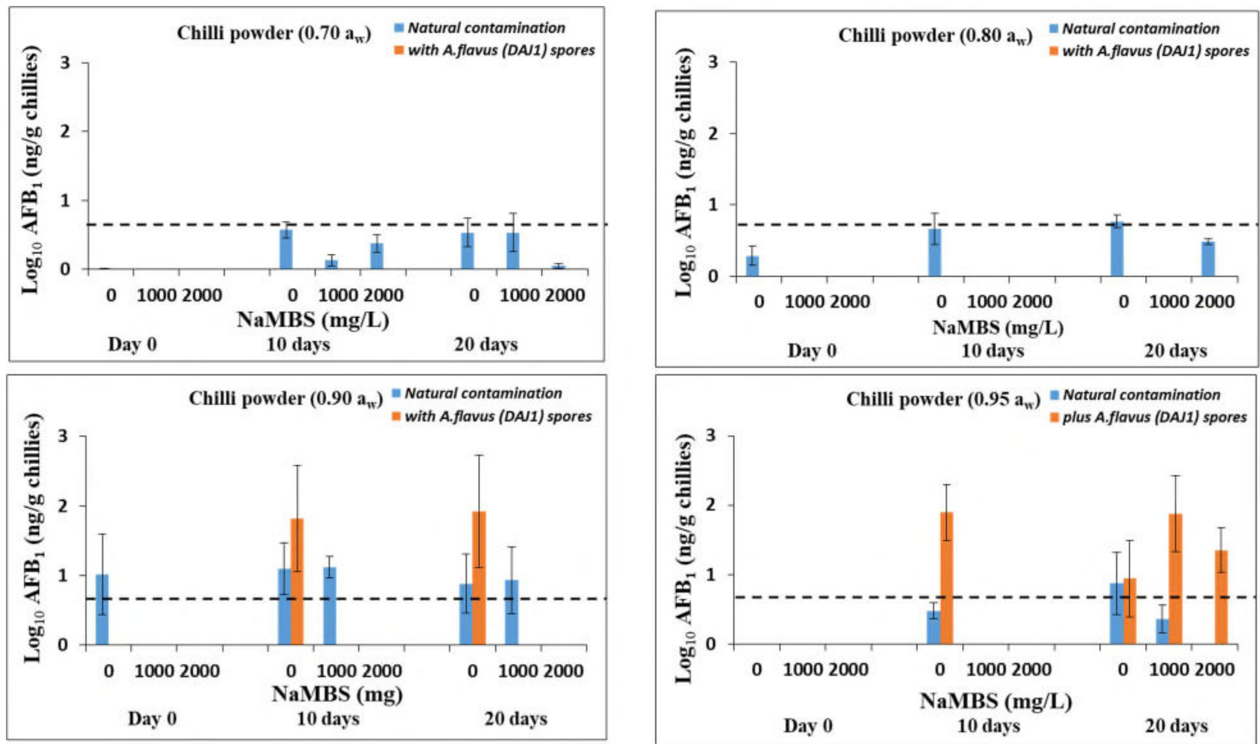


Fig. 3. Effect of NaMBS (mg/L) on AFB_1 contamination in naturally contaminated, or + *A. flavus* inoculum of stored chilli powder at 30ºC at four different a_w levels (0.70 to 0.95 a_w) for 10 and 20 days. Bars indicate standard error of the means. The dotted line indicates the EU legislative limits for AFB_1 in spices including chillies (Log_{10} 5 µg/kg = 0.699 µg/kg.

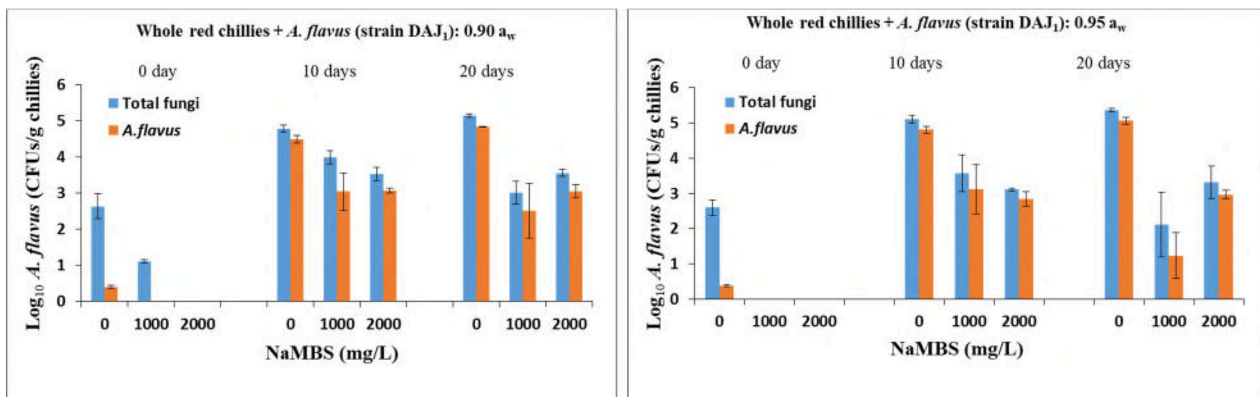


Fig. 4. Efficacy of NaMBS (mg/L) on total fungal populations and of *A. flavus* (Log_{10} CFUS/g) isolated from whole chillies at 30ºC and (0.90 and 0.95 a_w) after 10 and 20 days storage. Bars indicate standard errors of the means.

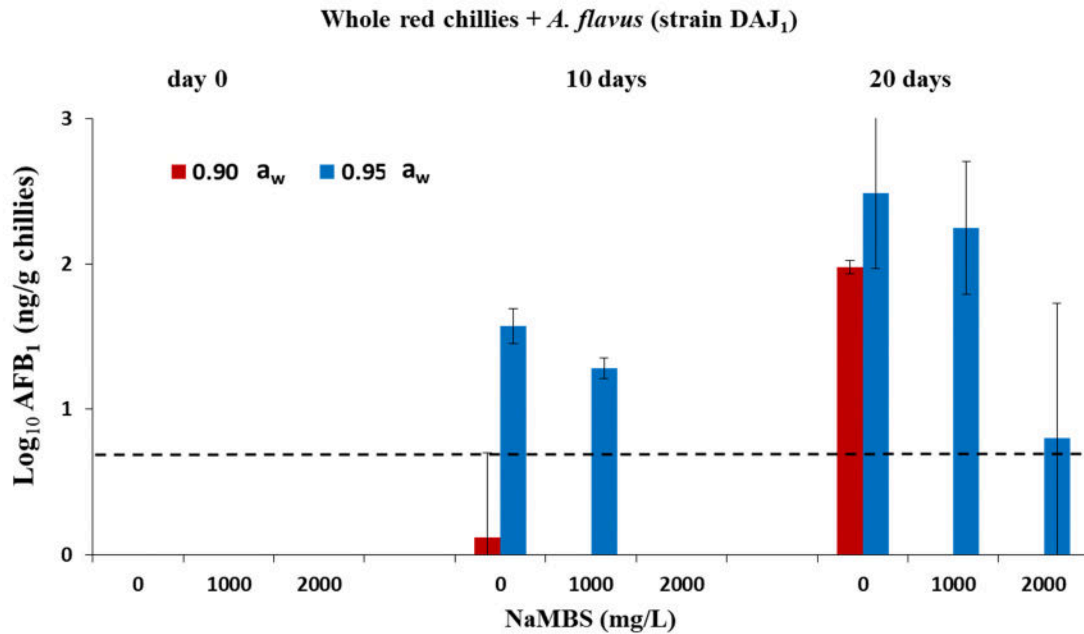


Fig. 5. Effect of NaMBS (mg/L) on AFB_1 contamination (Log_{10} AFB_1 (µg/kg) by *A. flavus* in stored whole chillies inoculated with *A. flavus* conidia at 30ºC and two a_w levels for 10 and 20 days. Bars indicate standard error of the means. The dotted line indicates the EU legislative limits for AFB_1 in spices including chillies (Log_{10} 5 µg/kg = 0.699 µg/kg.

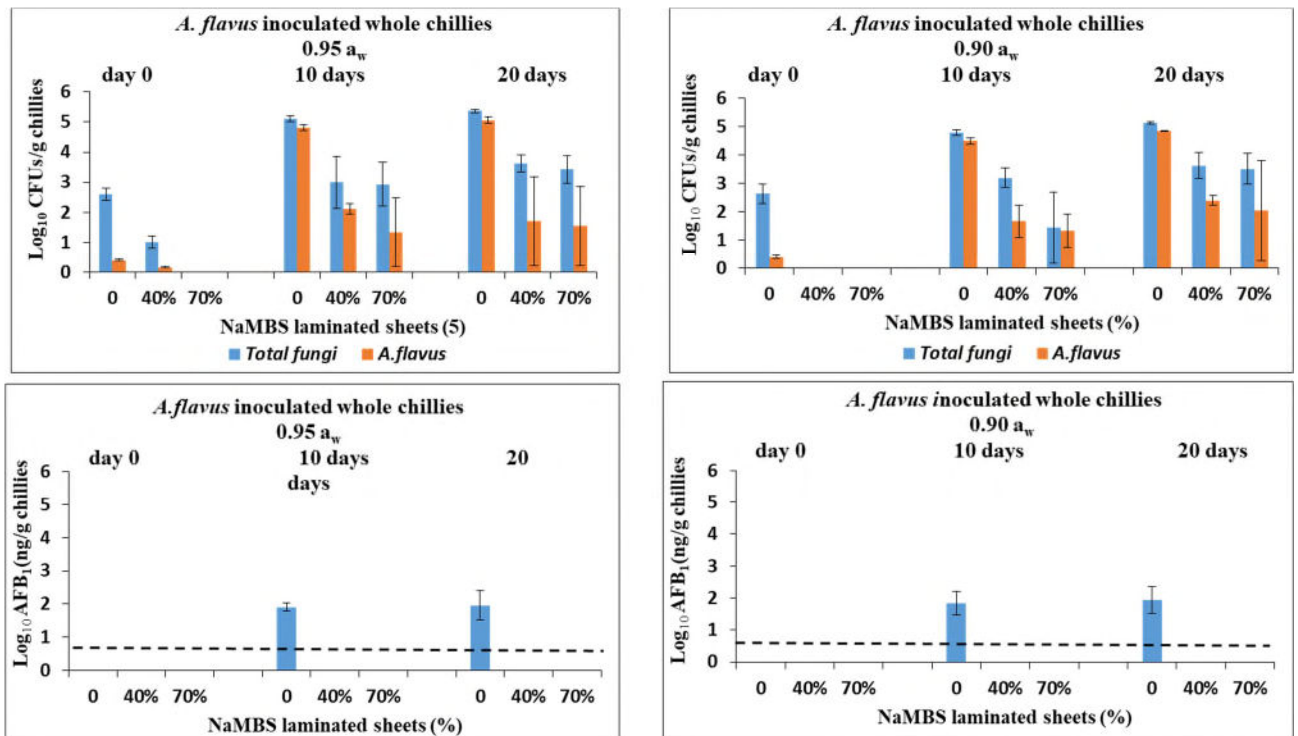
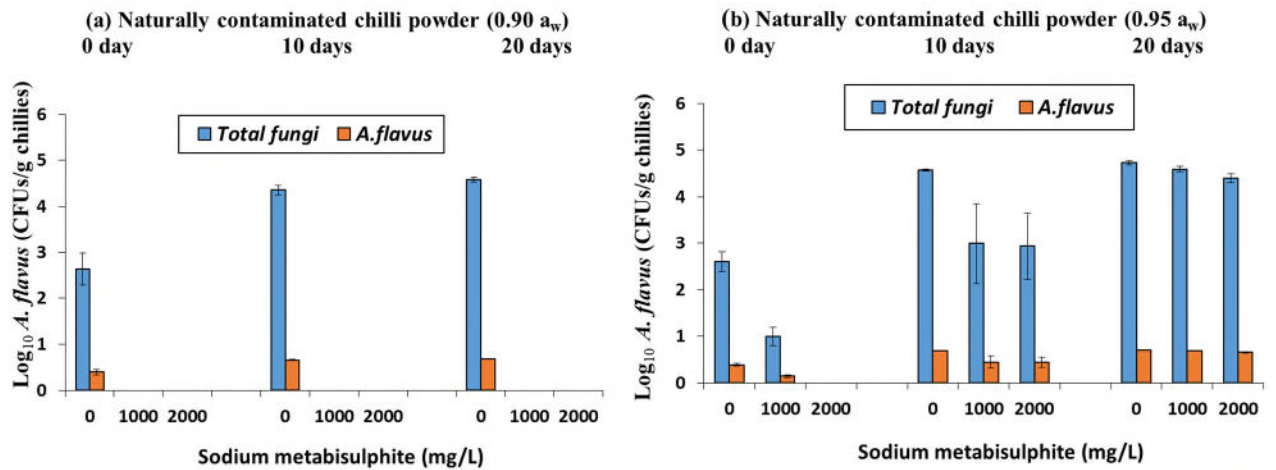


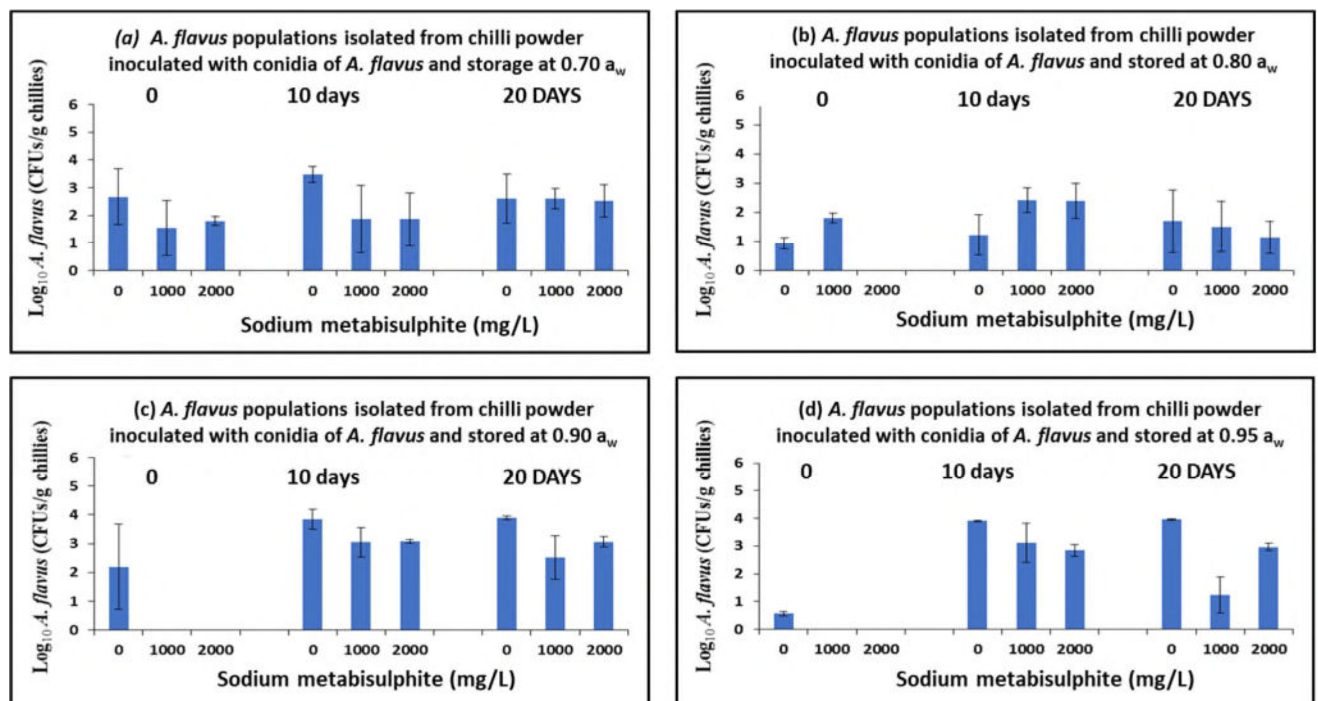
Fig. 6. Effect of laminated sheets with different NaMBS concentrations on (a, b) total fungal populations and those of *A. flavus* (Log_{10} CFUs/g) and (c-d) aflatoxin B_1 contamination of whole red chillies stored at 30ºC at 0.95 and 0.90 water activity (a_w) for 10 and 20 days. Bars indicate standard errors of the means. The dotted line

indicates the EU legislative limits for AFB_1 in spices including chillies (Log_{10} 5 µg/kg = 0.699 µg/kg.

Suppl. Figures



Suppl. Fig. 1. Total fungal populations and those of *A. flavus* isolated from naturally contaminated chilli powder treated with different concentrations of sodium metabisulphite and stored for up to 20 days at 30ºC at (a) 0.90 and (b) 0.95 a_w. Bars represent S.E.M.



Suppl. Fig. 2. Isolated *A. flavus* populations from chilli powder inoculated with conidia of this species and treated with sodium metabisulphite and stored for 10 and 20 days at 30ºC and (a) 0.70, (b) 0.80, (c) 0.90, and (d) 0.95 a_w. Bars represent S.E.M.

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Efficacy of sodium metabisulphite for control of Aspergillus flavus and aflatoxin B1 contamination in vitro and in chilli powder and whole red chillies

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