The in vitro Efficacy of Two Microbial Strains and Physicochemical Effects on their Aflatoxin Decontamination in Poultry Feeds

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Research

Keywords: Aflatoxin decontamination, Yeast, Bacillus spp, commercial binder, animal feeds, pH, Temperature

Posted Date: May 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-27273/v1

Abstract

Background: Contamination of animal feeds with aflatoxigenic fungi is a challenge to livestock farmers worldwide. Aflatoxins are very toxic fungal metabolites that are associated with carcinogenic, mutagenic, teratogenic, and estrogenic effects. The toxins affect animal productivity and may lead to deaths, causing enormous economic losses. Aflatoxin decontamination is a challenge to the feed industry, despite the several approaches available. This study investigated the efficacy of two microbial isolates, Bacillus spp (B285) and Yeast strain (Y833), in reducing Aflatoxin concentration in poultry feeds in comparison with a commonly used commercial chemical binder, Bentonite. The influence of the poultry feed matrix, pH, and temperature on the aflatoxin reducing activity by the two microorganisms was also explored.

Results: The in vitro studies showed that the two microorganisms and the chemical binder reduced aflatoxins by over 74% of the original concentration. The chemical binder registered the highest reduction at 93.4%; followed by Y833 (83.6%), then the combination of Y833 and B285 (77.9%); and lastly B285 (74.9%). There was no significant ($p>0.05$) influence of temperature on the toxin reducing capacity of all the agents tested. The pHs 4.5 and 6.5 did not have a significant effect on the performance of both chemical binder and biological agents, however, the former performed better at pH 6.5 with 95% aflatoxin reduction compared to the microorganisms. The aflatoxin reducing activity was lower in presence of feeds compared to that in Phosphate Buffered Saline except for Y833 where no difference was observed.

Conclusions: Although the feed components affected the aflatoxin reducing capacity of the test materials, the chemical binder was more effective than the microbial agents. Yeast strain was more effective than the bacterial strain in reducing the aflatoxin levels, however, both are promising strategies for countering the aflatoxin challenges in animal feeds. In response to the advocacy for use of biological control agents, there is need for more investigations to establish the safety of the microorganisms, the mechanism of decontamination and safety of the products; the optimum concentrations that can reduce aflatoxins in feeds to permissible levels and the effect of the toxin contamination levels on microbial efficiency.

Introduction

Consumption of contaminated foods of animal origin has been linked to increasing incidences of human illnesses and deaths affecting about 1 in 10 people worldwide [1]. The safety of food and feed is compromised by contamination with different materials including chemically active fungal metabolites, especially aflatoxins. Aflatoxins are a large group of secondary metabolites that are highly toxic and carcinogenic. The toxins are produced by specific species of fungi, that is, *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*; which are ubiquitous in soil, decaying vegetation, hay and grains [2]. Aflatoxins that cause major detrimental effects are B_1, B_2, G_1, and G_2; with aflatoxin B_1 being the most potent hepatocarcinogen known [3]. Aflatoxin B_1 are also reported to be mutagenic, teratogenic and estrogenic [4].

Aflatoxins occur in most of the staple foods produced in Africa such as maize, sorghum, groundnuts, peanuts and cottonseed [5]. The contamination is associated with high moisture content; 18–18.5% in the grains, yet subsequent processing steps, such as drying and boiling, do not reduce the aflatoxin levels [4, 6]. The European Economic Community (EEC) as well as the United States Food and Drug Authority (FDA) set the maximum permitted levels of total aflatoxins in poultry feed as 20 μg/kg [EEC, 1991], while the Uganda National Bureau for Standards (UNBS) set a limit of 10 μg/kg for all foods and feeds but only those intended for export [7].

Consumers in Uganda are continuously exposed due to the persistent occurrence of Aflatoxin contamination, beyond acceptable levels, in selected food and feedstuffs [8, 9]. Although few reports on occurrence of aflatoxins in animal products, such as milk and its products, exist; it infers animal feed contamination. Indeed, a previous study in Uganda revealed poultry feed contamination with aflatoxins at levels between 7.5 ± 0.71 and 393.5 ± 19.09 μg/kg; with only 17.9% passing the FDA limits [10]. Unfortunately, there is limited monitoring and enforcement of the regulations for management of mycotoxin contamination in the animal feed industry in Uganda.

Management of aflatoxin contamination in the food/feed chain calls for improvement in the farming practices, pre- and post-harvest handling; with the latter emphasizing appropriate storage of the grains and processed feeds [11]. These strategies require additional treatments to effectively eliminate aflatoxin contamination. Therefore, further efforts in development of safe, effective, affordable and environmentally safe aflatoxin decontamination methods are required [12]. The most widely used approach in the detoxification of feed and food is the application of sorbents for selective removal of toxins by adsorption during passage through the gastrointestinal tract [13]. Aflatoxin binders adsorb the toxin while *in-situ*, in the feed and in the gut resulting in the excretion of the toxin-binder complex in the faeces [14]. Inorganic binders, such as hydrated sodium calcium aluminosilicates, bentonites, phyllosilicates, smectites, kaolinites, zeolites and activated charcoal are used especially in developed countries [15]. Bentonite is among the most widely used sequestering agents and has been reported to adsorb over 85% of aflatoxins present in the feed [16–18] and was therefore included in the current analyses for comparison purposes. The effectiveness of inorganic substances as binders vary with the toxin type; and in some cases, there is need to eliminate them from the treated feeds before presenting to the animals [19]. Hence, investigations into the effectiveness and feasibility of applications of biological means are recommended as alternative strategies for aflatoxin decontamination in feeds [13].

Several microorganisms including bacteria, yeast, and non-toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* have exhibited aflatoxin detoxification capabilities and are thus promising as alternatives to chemical binders [13]. Biological detoxification, which is due to biotransformation or degradation of the toxin by the microorganism or its enzymes, yield metabolites that are either non-toxic when ingested by animals or less toxic than the parent toxin molecule [14]. Despite considerable research on microbial detoxification and their premise on utilization in feeds and foods [19], they are not widely available, especially in developing countries, such as Uganda. Besides, given the regulatory rigor on introduction of microbes into a country and the biosecurity issues, there is need to develop products from

sources obtained from the local environment. This study aimed at evaluating the efficacy of two locally isolated microbial strains in reducing Aflatoxin concentration in poultry feeds in comparison to the commercial chemical binder (bentonite). The influence of the feed matrix, pH, and temperature on the aflatoxin reducing activity of the microbes and bentonite was also determined.

Results

Reduction of aflatoxin concentration in poultry feeds subjected to the different agents

Results of aflatoxin reducing activities in poultry feeds are presented in Table 1. It was impossible to obtain aflatoxin-free ingredients for feed formulation; thus, the non-spiked feed contained 1.55 ± 0.001 µg/L total aflatoxins. Addition of 0.3% (w/v) bentonite (positive control) to aflatoxin-spiked feed yielded a 93.43% reduction of total aflatoxin concentration, while the addition of the same to a non-spiked feed sample yielded a 100% removal of the toxins present. Mean aflatoxin concentration in presence of bentonite was significantly ($p < 0.00001$) lower compared to the absence of the binder.

Table 1
Mean aflatoxin concentrations in poultry feed exposed to different treatments

Treatment	*Mean AFT (µg/L)		Percentage change (%)	P-value
	Start	End		
Non-AF spiked feed + No Binder	1.55 ± 0.001	1.55 ± 0.001		
Aflatoxin spiked feed + No binder	41.60 ± 0.007	41.60 ± 0.007		
Aflatoxin spiked feed + Bacteria	41.60 ± 0.007	10.41 ± 0.004	75.0 ± 0.01	0.000
Aflatoxin spiked feed + Yeast	41.60 ± 0.007	6.80 ± 0.004	83.7 ± 0.03	
Aflatoxin spiked feed + Bacteria & yeast	41.60 ± 0.007	9.20 ± 0.001	77.9 ± 0.01	
Aflatoxin spiked feed + 0.3% Bentonite	41.58 ± 0.03	2.73 ± 0.003	93.4 ± 0.03	

All treatments were carried out in triplicates (n = 3); *Total Aflatoxin concentration at Start and End of experiment (after 4 h of incubation)

Yeast was the most effective biological agent with a mean aflatoxin reduction from 41.53 ± 0.062 µg/L to 6.80 ± 0.002 µg/L giving a percentage reduction of 83.7%. The combination of yeast and *Bacillus* spp produced a higher percentage reduction (77.8%) compared to inclusion of *Bacillus* spp (74.9%) only. The aflatoxin reduction by biological agents followed the trend of Yeast > Yeast + *Bacillus* spp > *Bacillus* spp.

In non-spiked feeds, the biological agents reduced the aflatoxins to undetectable levels and hence produced 100% reduction from 1.549 ± 0.001 to 0.00 (undetectable levels).

Comparing the efficacy of chemical versus biological agents, all test materials reduced aflatoxin concentration in feeds by more than 74%. However, the commercial binder, bentonite, had the highest reduction percentage (93.4%) followed by the Yeast isolate with 83.7%; and lower values were recorded where bacteria were added.

Effect of Feed Matrix on aflatoxin reduction capacities of the test agents

Results indicating the effects of feed matrix on aflatoxin reduction activity of the different test agents are presented in Table 2. Aflatoxin reducing activity was lower by 3% and 8% in the presence of feeds as compared to Phosphate Buffered Saline (PBS), for bacteria and bacteria plus yeast test materials, respectively. No differences were observed in case of the yeast isolate while bentonite performed better in presence of feeds than in PBS. The bacterial strain had lower activity compared to the yeast strain, when used singly; however higher performance was observed in PBS, when combined with the yeast. Performance of bentonite was higher than the biological materials by 9–18% and by 5–13% in presence of feeds and in PBS, respectively. There were significant differences in aflatoxin reducing activity in the presence of feed ($p < 0.001$) and in PBS ($p < 0.0001$).

Table 2
The effect of feed matrix on the aflatoxin-reducing activity of two microbial strains (bacteria and yeast) and bentonite

Treatment	Feed matrix		Phosphate buffered saline	
	Aflatoxin content (µg/L)	% Aflatoxin reduction	Aflatoxin content (µg/L)	% Aflatoxin reduction
AF without binder	41.57 ± 0.017	-	40.00 ± 0.000	-
AF with Bentonite	2.73 ± 0.003	93	3.57 ± 0.001	91
AF with *Bacillus*	10.41 ± 0.002	75	8.80 ± 0.005	78
AF with yeast	6.79 ± 0.002	84	6.23 ± 0.000	84
AF with *Bacillus* and yeast	9.19 ± 0.001	78	5.58 ± 0.001	86
P-value	0.0001		0.00001	
AF: Aflatoxin spiked, All treatments were carried out in triplicates (n = 3)				

Effect of pH and Temperature on aflatoxin reduction capacities of the agents

Aflatoxin reduction activities of the test agents at various temperatures and pH are presented in Fig. 1. The percentage reduction ranged from 74–96%. Generally, the aflatoxin reduction activity for all the materials at different physicochemical conditions was above 70% with the commercial binder having > 90% in all cases. The yeast strain performed second best, with 83% – 86% reduction; and the *Bacillus* spp showed the least activity (74% – 78%) except at 42 °C, where its reduction was at 96%. The bacteria and yeast combination exhibited 77–80% reduction. Aflatoxin reduction increased with rise in temperature, although it was not significant ($p = 0.0940$).

There was no significant effect ($p = 0.9817$) of pH on aflatoxin reduction by the various test materials. However, bentonite had the highest activity (95%) at pH 6.5. For the other treatments, there was a 1% reduction in activity at pH 6.5 compared to that at pH 4.5.

Discussion

The continuous search for effective and appropriate mycotoxin control strategies has biological control as a promising strategy due to the associated efficiency, minimal effect on feed and/or food quality and safety; and environmental friendliness. This study screened two microbial agents for *in vitro* aflatoxin reduction activity at a single concentration. The selected strains, one of each of bacteria (*Bacillus*) and yeast strain reduced aflatoxin concentrations in contaminated poultry feed by 75% – 83.7%. The duration of exposure used was informed by the transit time of feed on ingestion, while the total aflatoxin concentration was twice the FDA recommended levels.

The yeast strain Y833 exhibited similar reduction activity in both feed and PBS, which fits in aflatoxin decontamination activities of 40% – 99.3% reported by previous researchers [20–22]. Various species including *Saccharomyces cerevisiae* and *Candida krusei* were shown to bind > 60% of aflatoxins in a study to determine the binding efficacy of the yeast isolates [23]. The reduction activity of Y833 was comparable to what was reported for the yeast cell wall and brewery dehydrated residues; but lower than the autolysed and the dried cane sugar yeasts at almost similar conditions as the current study [22]. Lower reduction activity was reported by some researchers, but the measurements were made at or beyond 6 hours post exposure, such as 60% – 72.8% AFB1 reduction by selected *Saccharomyces* spp [24–26]. The decontamination activity depends on the yeast strains, pH and temperature conditions, duration of exposure and initial aflatoxin concentration [27–29]. The reversible binding of Aflatoxins by the yeast influences the detoxification levels, which have been reported to reduce with time [22]. An alternative mode of decontamination, through both intracellular and extracellular biodegradation by yeasts, such as *Candida versatilis* was reported [30]. The current study did not evaluate the activity over a period of time, which could have offered a clue on the mechanism of decontamination, hence, there is need for determining the mode of action of the selected isolate.

The aflatoxin reducing activity of the *Bacillus* strain B 285 from this study is in agreement with findings by previous researchers who reported activities up to 97.3%, depending on the bacterial strain as well as the conditions and duration of experiment [31–33]. Our findings are similar to those of Petchkongkaew et al [31]; who reported *Bacillus lichenformis* exhibiting 74% while *B. subtilis* had 85% reduction of AFB1 at 37 °C after 48 h of exposure, although the current exposure lasted 4 h only. The mode of activity by the *Bacillus* spp, is more of biodegradation than adsorption, which despite the different duration of exposure, results in comparable level of activities. However, like for the yeast studies, the analyses did not explore the effect of time on the decontamination activity.

Combined microbial activity (77.8%) was lower than that exhibited by the yeast strain (83.7%) but higher than that for bacteria (75%). The lower activity of the combined microbes than yeast is due to the reduced amount of yeast cells in the preparation, however, this contributed to a higher activity than what was experienced by the bacteria. A higher effect compared to that by each strain was expected but the different mode of aflatoxin decontamination, and reduced concentration of each microbe probably affected their activity. The current findings contrast those from a previous study where degradation rates of 38.38% and 21.08% when *Bacillus subtilis* and *Candida utilis* were used singly, respectively; and a much higher rate of 45.49% by a combination of the two species [30]. The lower activity compared to yeast alone, was probably due to some growth-inhibitory activity of *Bacillus* spp against the yeast in addition to the lower concentration, thus affecting the aflatoxins decontamination. Determining the mode of decontamination, and the level of aflatoxins against time and microbial cell population can elucidate the combined microbial activity to obtain conclusive explanations.

Bentonite exhibited highest reduction, followed by yeast, then a combination of yeast and bacteria, while bacteria was the least active. Compared to bentonite, the microbes exhibited lower reduction activity. Vekiru et al [34] reported similar findings where sodium bentonite showed the strongest binding capacity for aflatoxin B1. Bentonite is one of the inorganic toxin binders that are more effective in binding AFB1 than others, which is due to the purification of the inorganic clays [35]. The surface area and chemical affinities between adsorbent and mycotoxin have been reported to increase the process of binding [36]. The latter explains why bentonite had higher aflatoxin reduction activity than the microbes. The performance of yeast was next to that of bentonite, probably because of the similar mode of action, that is, adsorption of the aflatoxins. The lower performance of bacteria is most likely due to the differences in mode of action; the biodegradation effect, where enzyme induction and production needs more time than what was used in this study. The experimental feed contained some aflatoxins because it was impossible to obtain toxin-free ingredients. Removal of the basal aflatoxins in the non-spiked feed sample to undetectable levels by both the bentonite and the microbes; is probably an indication that the efficiency depends on the levels of contamination. However, the decontamination activity at different aflatoxin levels calls for further evaluation. Previous researchers used AFB1 for the decontamination experiments. Similar reduction activities probably point to presence of the AFB1 as the major type in the in-house aflatoxin extract used during the current study.

Generally, in-feed aflatoxin decontamination was lower than *in vitro* activity in PBS for the bacteria; and for the yeast and bacteria combination. This could have been influenced by the substrate, the feed that did not offer a conducive environment, which probably affected the growth and therefore aflatoxin biodegradation process. The current finding contrasts with that by Siahmoshteh et al [32] where *Bacillus subtilis* had 85.66% reduction in AFB1 toxin in broth culture compared to 95% in the pistachio nuts. The latter was probably because the bacteria can grow luxuriantly in presence of the nuts. Bentonite exhibited higher activity in feed than in PBS and there was no difference in case of yeast, which was probably influenced by the mode of action. Shetty and Jespersen [23] reported that live yeast strains and heat-treated preparations were efficient at binding AFB1 on mixing as feed additives, thus emphasizing that adsorption was not affected by the media where the toxin was suspended. Significant differences on the effect of feed matrix between the test agents were observed, but not on the decontamination activity of each test item, this attribute needs to be evaluated further before availing the product to the processors.

There were no significant differences in the aflatoxin decontamination at various pH. The same trend of reduction of bentonite being the highest, followed by yeast, then a combination of yeast and bacteria; and lastly bacteria, was observed. Bentonite performed better at pH 6.5 than at pH 4.5; which contrasted to the microbial agents' activity. Yeast, just like other fungi, prefer acidic pH for growth and the adsorption mode of action, which depends on the cell wall components does not depend on cell viability is probably reason for the observed activity. Similar findings were reported by Gonçalves et al [22]. Dogi et al [37] demonstrated three yeast strains that survived under gastrointestinal conditions and effectively adsorbed AFB1 at different pH levels, which is similar to the findings of the current study. The bacterial agent was also not affected by pH since *Bacillus* spp have a wide range of pH at which they survive or grow. It is likely that the enzyme that is involved in biodegradation is resistant to the low pH, hence the comparable activity observed in this study. The 1% decrease in aflatoxin reducing activity at pH 6.5 compared to that at pH 4.5 contrasts Jouany et al [38] findings of greatest degradation at pH 5–6.5, probably due to differences in the microorganisms analysed, which was *Lactobacillus*, a member of the Lactic Acid Bacteria group that are acidophilic.

The trends of decontamination by the test agents was the similar at the same temperature, except at 42 °C where B285 performed better than the others, including bentonite. Alteration of temperatures in the present study did not have any significant effect ($p > 0.05$) on the efficacy of bentonite on aflatoxin reduction; like findings [39]. This finding contrasts earlier reports, where a significant decrease in the adsorbed AFB1 by bentonite was recorded when the temperature was elevated from 25 °C to 45 °C [40]. The latter can be explained by the ability of the *Bacillus* spp to grow at high temperatures; hence there could have been increased cell populations and/or their enzymatic activity. Having been isolated from chicken droppings and given the body temperature of the birds, the *Bacillus* spp B285 was already adapted to such thermal conditions. The current findings agree with what Farzaneh et al [41] reported, that optimum temperature for *B. subtilis* cell free supernatant was 35 °C – 40 °C. Besides, an earlier study indicated that bacterial adsorption of aflatoxins was both temperature and concentration dependent [42]. Since yeast and other eukaryotic cells' optimum temperature for growth is 37 °C, lower activity would be expected at higher temperatures. However, the yeast activity was highest at 42 °C because non-viable

cells can also adsorb the toxins. The high activity at 42 °C, pH 6.5 and at pH 4.5 implies that these microbial strains can impart their effect *in vivo*, since these conditions simulated those of chicken. The crop and the proventriculus have a pH of about 4.5 whereas the small and large intestines have a pH of about 6.5 [43].

Identification of potential mycotoxin-detoxifying agents is based on the ability to adsorb more than 80% aflatoxins available in solution; and determining the mechanisms and conditions favorable for adsorption [44, 45]. These are considered the minimum for passing the *in vivo* tests. Basing on this, the yeast strain should be analyzed further to establish the mechanism of action, the appropriate concentrations for *in vivo* application.

Conclusions

This study demonstrated the *in vitro* aflatoxin reducing activity of two microbial strains. From the results, it can be concluded that Bentonite is more effective in adsorbing aflatoxins from poultry feed compared to their biological counterparts. Yeast strain Y833 was more effective than the bacterial strain B285 (*Bacillus* spp) in decreasing the aflatoxin concentrations. It can also be concluded that alternation of temperatures between 25 °C through 37 °C to 42 °C has no significant effect on the aflatoxin reduction capacity of the tested agents. Likewise, changing pH from 4.5 to 6.5 has insignificant influence on the aflatoxin reducing capacity of both biological and commercial mineral binders. Determining the mode of decontamination; reduction activity in relation to aflatoxin concentration, the duration of exposure and microbial cell population can elucidate the combined microbial activity to obtain conclusive explanations before recommendation for use by the feed processors.

Methods

Study design and source of microorganisms

An experimental laboratory-based study was carried out to establish the efficacy of two microbial agents in reducing aflatoxin concentrations in poultry feeds. The microbial agents were obtained from previous studies that involved *in vitro* screening for aflatoxin reduction activity of isolates from various sources. A commercial binder, Bentonite, was included as the positive control. Bentonite, as well as the specifications for its use, were obtained from local traders in Kampala. The microbes included a bacterial strain (*Bacillus* spp B285) and a yeast strain (Y833); that were isolated from poultry droppings and brewer's waste, respectively.

Preparation of the aflatoxin-reducing microbial test materials

Stock cultures of the microbial isolates, *Bacillus* spp (B285) and a yeast strain (Y833) were resuscitated in Trypticase Soy Broth (TSB) and Czapek Dox Broth (CDB), respectively. Purity of the isolates was confirmed by sub-culturing on Trypticase Soy Agar (TSA) and Potato dextrose agar (PDA) for the bacteria and yeast, respectively. All media were manufactured by Laboratorios Conda S.A, Spain. Working cultures were prepared by inoculation into the respective broth medium and incubated at 37 ºC, for 18 hours and 5 days, for bacteria and yeast, respectively.

From the overnight microbial broth culture, 2.0 mls were centrifuged at 3000 rpm (Zentrifugen, Germany) for 15 min at 10 ºC. The cells were washed twice with Phosphate Buffered Saline (pH 7.3) and once with double distilled water; and the cell pellet was suspended in 1.0 ml of sterile double distilled water. The microbial preparation was standardized to about 10^6 CFU/ml for yeast and 10^8 CFU/ml for bacteria by comparing with McFarland standard No.5. The viable bacterial and yeast cell concentration was confirmed by surface spread method and expressed as colony-forming units per milliliter (CFU/ml).

Compounding of the poultry feed

Freshly supplied raw materials for feed preparation were purchased from produce dealers in Kampala. The ingredients were used in compounding of about 2 kgs of broiler starter feed following an in-house formulation at the Nutrition laboratory, College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University. The ingredients and proportions included: cotton seed cake – 300 g, silver fish meal (*Rastrineobola argentea*, locally known as “mukene”) – 240 g, bones – 120 g, maize – 1230 g, salt – 10 g and soya – 100 g. The ingredients were ground separately and mixed to obtain the required compounded feed. From the compounded feed, 250 g were ground further using a laboratory mill to pass No.20 and then No.14 sieve. The feed sample was mixed thoroughly for homogeneity and placed in paper bags and stored at refrigeration temperature. The compounded feed was used for the aflatoxin - reducing experiments.

Source of Aflatoxins for feed sample spiking

In-house production of aflatoxins was carried out following the methods described by [46] with modifications. An aflatoxigenic *Aspergillus* strain, Y20, was obtained from the National Agricultural Research Laboratory, Kawanda. Coarsely ground rice was used as the substrate. The inoculated sterile rice slurry was incubated for 7 days at 25 °C in the dark.

Aflatoxin extraction from multiple 50 g samples was made following the procedure by [47]; with modifications; and all procedures were carried out in a fume hood. For purification, the extracts were pooled and filtered through Whatman No. 1 filter paper. To 125 ml of the filtrate, 30 ml of 2% Sodium Chloride solution and 500 ml n-hexane were added, placed in a separating funnel and shaken vigorously for 5 minutes. The n-hexane layer was discarded and the lower methanolic layer was placed into another separating funnel to which 40 ml of chloroform was added; shaken and allowed to rest undisturbed for separation of layers. The chloroform layer was collected in a flask containing 5 g of Cupric carbonate, agitated and allowed to settle down. The extract was filtered through a bed of anhydrous Sodium sulphate over a filter paper; the various portions were pooled and then evaporated to dryness on a heat block. The residue was dissolved or reconstituted in 1 ml acetonitrile, to have a crude extract that was kept in a refrigerator until further analysis or use. Presence of aflatoxins in the crude extract was confirmed using Thin Layer Chromatography (TLC) plates (Sigma- Aldrich, Germany) alongside 0.04 µg/ml AFB1, 0.024 µg/ml AFB2, 0.03 µg/ml AFG1 and 0.04 µg/ml AFG2 (Romer Labs, UK) as the aflatoxin standards. The bands were visualised under UV light at 365 nm wavelength.

Further purification of the aflatoxin extract was achieved by passing it through an Aflatest Immunoaffinity column (VICAM, USA) following the manufacturer's instructions. The column was washed with Phosphate Buffered Saline (PBS) and the toxins eluted with 100% HPLC grade

methanol. Quantification of aflatoxin content was achieved using VICAM®, a fluorimetric method following the manufacturer's instructions.

Preparation of the Aflatoxin working solution involved evaporation of the methanol by keeping the loosely closed vials at 50 °C for overnight, after which the concentration was adjusted to 1 µg/ml using PBS. The preparation was kept in an amber bottle in the refrigerator.

Preparation of Aflatoxin spiked feeds and non-spiked feeds

Preparation of the feeds was as described by [48], with modifications. To 10 g of the compounded poultry feed, 0.8 g of Sodium chloride were added and mixed properly. A slurry was prepared by adding 10 ml PBS (pH 7.2) and then autoclaved. To 10 ml of the feed slurry, the aflatoxin working solution was added to obtain a concentration of about 40 µg/L [49], that is, 400 µL of 1 µg/ml aflatoxin working solution. To ensure proper distribution, small amounts of the feed slurry were continuously mixed with the Aflatoxin working solution and homogenised. For non-spiked feeds, instead of aflatoxin solution, 400 µL of PBS was added.

Determination of the effect of feed matrix, pH and temperature on aflatoxin reduction

A factorial study design was used to evaluate the aflatoxin reducing efficacy of the two selected microorganisms and the control (Bentonite) in aflatoxin-contaminated feed *in vitro* and under simulated *in situ* gastrointestinal tract environment (pH and temperature) of chicken according to [48]. For each treatment, the toxin reduction was tested in triplicates at pH levels of 4.5 and 6.5; and at 37 ºC, 42 ºC and at room temperature; after which the residual aflatoxin concentrations were determined.

To the Aflatoxin-spiked and non-spiked feed samples, the appropriate test reducing agents were added. For bentonite, 0.3% w/v [50] added according to the instructions given by the local agrochemical supplier. For the microbial agents, 5 mL of the standardized yeast and bacterial cell suspensions with concentration 1.0×10^6 CFU/mL and 1.0×10^8 CFU/mL, respectively, were used. In order to determine the effect of the feed matrix, PBS instead of feeds was used. The tests were carried out in triplicates and incubated at the appropriate temperatures for four (4) hours. The experimental set-up is detailed below and summarized in Table 3.

Table 3
The experimental set up for determination of aflatoxin reduction in poultry feeds, and the effect of feed matrix, pH and temperature

Reducing agent	Aflatoxin (40 µg/L) status			
	Feeds		PBS*	
	Spiked	Non-spiked	Spiked	Non-spiked
5 ml 1 × 10^6 cfu/ml of yeast	1	2	11	12
5 ml 1 × 10^8 cfu/ml of *Bacillus* spp	3	4	13	14
2.5 ml 1 × 10^6 cfu/ml yeast + 2.5 ml 1 × 10^8 cfu/ml *Bacillus* spp	5	6	15	16
Bentonite (Positive control)	7	8	17	18
None (Negative control)	9	10	19	20

PBS – Phosphate Buffered Saline. Arabic numerals 1–20 indicate the treatment groups. *The same set-up for PBS was followed for evaluating the effect of pH and temperature on Aflatoxin reduction.

For comparison of the activity of the test materials, the treatment groups included aflatoxin spiked feeds with yeast (Group 1); non-spiked feed with yeast (Group 2); spiked feed with bacteria (Group 3); non-spiked feeds with bacteria (Group 4); spiked feeds with 50% yeast and 50% bacteria (Group 5); and non-spiked feeds with 50% yeast and 50% bacteria (Group 6). In order to determine the effect of the feed matrix, the same set up was used, however, PBS instead of feeds was used. For positive control, bentonite was added, while for the negative control, nothing was added to the spiked or non-spiked feeds.

For determination of the effect of pH on aflatoxin reduction, the pH of PBS was adjusted to 4.5 and to 6.5; one portion was spiked with aflatoxins, while the other was not. The test materials were then added following the same set-up as for the feed experiments. For the effect of temperature, PBS experimental set up as described above was employed. Two sets, one for incubation at 37 °C and the other at 42 °C were put-up. Positive and negative controls involved addition of bentonite (positive) or nothing (negative) to the spiked and non-spiked preparations (Groups 7, 8, 9, 10; and Groups 17, 18, 19 and 20).

Residual Aflatoxin concentration was determined after 4 hours of exposure. For each treatment, Aflatoxin extraction and quantification followed a procedure described by [47], using the VICAM ® fluorometric method according to the manufacturer's instructions. Absolute values of the Aflatoxin concentrations for all the treatments were recorded.

Data Analysis

Using the absolute concentration values, percent aflatoxin reduction was calculated from the amount of unbound (residual) aflatoxins extracted after the experiments compared to negative control (without

reducing agent). The formula below was employed.

$$A = 100\left[1 - {}^{A_1}/_{A_0}\right]$$

$A = \%\ of\ AF\ bound$

$A_1 = Amount\ of\ AF\ in\ supernatant\ solution\ after\ binding\ assay$

$A_0 = Amount\ of\ AF\ in\ negative\ control$

Data were analyzed by STATA v12 and the differences between the treatments were determined using ANOVA.

For safety precautions, the cultures for aflatoxin production were autoclaved and later treated with 2% Sodium Hypochlorite before incineration. Likewise, all the plasticware and glassware were soaked overnight in Sodium Hypochlorite before disposal or washing for reuse.

List Of Abbreviations

AFB1: Aflatoxin B1

CAES: College of agriculture and environmental sciences

CDB: Czapek Dox Broth

COVAB: College of veterinary medicine, animal resources and biosecurity

EEC: European Economic Community

EFSA: European Food Safety Authority

FDA: US Food and Drug authority

HPLC: High performance Liquid chromatography

HSCAS: Hydrated sodium calcium aluminosilicates

PBS: Phosphate buffered saline

PDA: Potato dextrose agar

TSA: Trypticase Soy agar

TSB: Trypticase Soy Broth

UNBS: Uganda National Bureau of Standards

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this article.

Competing interests

The authors declare no conflict of interests at all

Funding

This study was part of the research project entitled “Development of a biological mycotoxin-binder for improving the safety of the livestock feeds and animal productivity”, referenced as CGS/2/17/14. The project was supported under the National Agricultural Research Systems-Competitive Grant Scheme 2013/2014.

Authors Contributions

JLN, ANK, MMN and BG conceptualized the research proposal and study design; MMN and ZT were involved in experimental feed formulation; JLN and BG guided in-house Aflatoxin production and purification; ZT and ANK were involved in laboratory analyses and interpretation; ZT, JLN and PB were involved in the first manuscript draft preparation; PB carried out the statistical analyses, while PB and JLN prepared the subsequent versions of the manuscript. All the authors read and approved the final version for submission.

Acknowledgements

We are thankful to the National Agricultural Research Systems-Competitive Grant Scheme 2013/2014 for funding this work. The laboratory assistance offered by Ritah Nabunje, and Tom Bbosa is greatly acknowledged.

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Figures

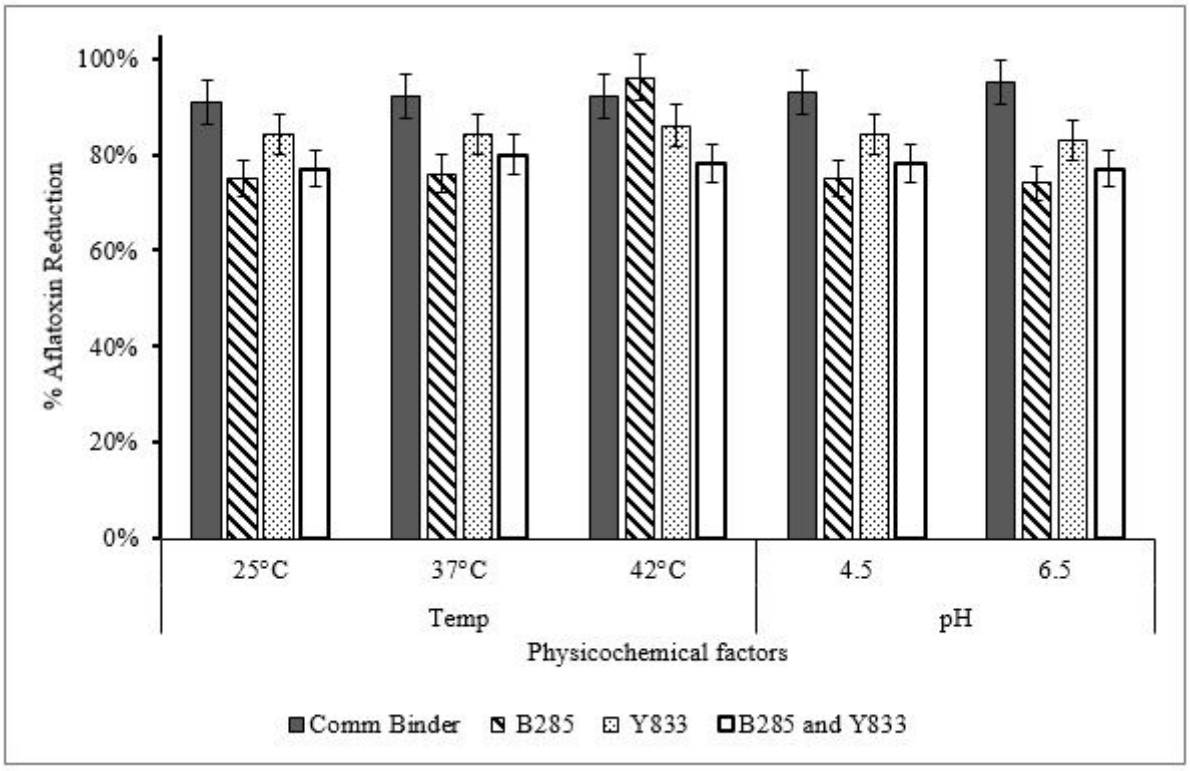

Figure 1

The effect of temperature and pH on the aflatoxin reducing activity of Bacillus spp strain B285 and Yeast strain Y833 in comparison with a commercial aflatoxin binder, bentonite