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Full Length Article

Mineralogically Well Characterized Bentonite Sources Controlled Aflatoxin Contamination in Poultry

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Abstract

Aflatoxin in poultry feed reduces production, and is a health hazard to the consumers. Its toxicity is reduced through addition of clays. Evaluation of the country's bentonites may promote their use as toxin binder. After detailed mineralogical and aflatoxin adsorption characteristics of large number of indigenous quarries effectiveness of two bentonites was determined through feeding trial. Bentonites from Bhimber and Dina quarries were applied in three replications at 1 and 2% (w/w) with positive and negative controls to a feed containing 250 μg kg^{-1} aflatoxin B1. Ten birds initially fed on clean starter feed for two weeks, fed on the experimental feeds and slaughtered after recording live weight on day 35 and dress weight along with internal organs weight were recorded with any apparent disorders. While addition of the bentonites had no detrimental effect, aflatoxin contamination had the lowest body weight and weight gain rate, reduced feed intake and feed conversion ratio with apparent disordered liver. The live body weight and weight gain rate increased with 2% bentonite addition to the contaminated feed from both the sources and was statistically similar to the clean feed though the values for these parameters were highest when the addition was to the clean feed. A 42% reduction occurred in weight gain without smectite addition in the toxin feed. The toxin feed had the lowest absolute liver and heart weight with dark coloration. The bentonites use in feed industry may reduce aflatoxin incidences at the application rate of 2%.

Keywords: Bentonite; Aflatoxin; Poultry; Contaminated feed; Adsorption; Growth performance

Introduction

Aflatoxins are carcinogenic mycotoxins (IARC 2002) that occur in poultry feed, reduce production and pose health risk to the consumers (Sana *et al.* 2019). The critical limit of aflatoxin in feed is 20 μg kg^{-1} (Stoloff *et al*. 1991). The incidence rate of feed contamination as high as 83% has been reported in Pakistan (Khan *et al*. 2013; Iqbal *et al*. 2014) and the level of contamination increases during rainy months (Anjum *et al*. 2012). Aflatoxin contamination beyond the regulatory level may induce economic losses (Kana *et al*. 2010) through immune-suppression, poor growth with high feed intake and low efficiency for feed conversion (Naseem *et al*. 2018; Al-Ruwaili *et al*. 2018).

Smectite containing geologically occurring clays are called bentonites and their addition in the contaminated animal and poultry feed is an effective control measure (Pasha *et al*. 2007). Adsorption of aflatoxin from the gastro-intestinal tract occurs in the interlayer of smectite through ion-dipole interaction and H-bonding (Deng *et al*. 2010). The amendment rate and mineralogical characteristics of the clays have been reviewed (Huwing *et al*. 2001; Dixon *et al*. 2008). The mineral purity and mineralogical properties that leads to fast kinetics of adsorption with high binding strength and no dissociation in the gastrointestinal tract are important features for considering for use as a feed additive (Li *et al*. 2010). Several bentonite based binders including Novasil plus, Astra ben 20A and hydrated sodium and calcium aluminosilicates (HSCAS) are commercially available (Bailey *et al.* 2006) while Mycofix, the first bentonite based binder recommended by European Commission, possess > 90% adsorption efficiency (European Commission 2013). The bentonite with dominance of calcium on exchange sites is more effective as aflatoxin adsorbent than those with sodium (McClure *et al*. 2014). Bentonite improves serum enzymatic activity in an incidence of severe aflatoxicosis in broiler birds (Bhatti *et al*. 2016).

Smectite application at the rate of 0.25 to 2% effectively reduce aflatoxin toxicity in poultry and animals (Phillips *et al*. 2008; Kermanshahi *et al*. 2009). Addition of calcium bentonite reduces accumulation of aflatoxin B1 residues in liver (Fowler *et al*. 2015) and improves humoral

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immunity against Newcastle Disease (Wafaa *et al.* 2013). Smectite is a superior feed additive than palygorskite and zeolite (Dixon *et al.* 2008).

Due to complex gut conditions the feeding experimental data may differ with the *in vitro* aqueous adsorption calculated from the batch studies and fitting (Kannewischer *et al*. 2006). A large mismatch with estimated adsorption and an effective control determined through feeding trials have been reported (Barrientos-Velazquez and Deng 2020). Aflatoxin adsorption studies using natural or synthetic gastric fluids are limited (Li *et al*. 2010).

Systematic feeding experiments on the bentonites with their complete mineral characterization have limited the use of indigenous sources as a potential aflatoxin adsorbent. The study evaluates the efficacy of indigenous bentonite selected after detailed characterization from a large number of quarries from the country. While detailed mineralogical characteristics and adsorption potential are available, the study evaluates two application rates on weight gain, feed consumption and disorders in internal organs for the two bentonites.

Table 1: Experimental poultry feed composition

Feed ingredients	Application Rate (kg 100 kg^{-1})
Corn	45
Soybean meal	17
Soybean grain	5
Wheat	5
Canola meal	6
Rice polish	5
Wheat bran	5
Di-calcium phosphate	1
Premix	1
Corn gluten 30%	4
Corn gluten 60%	2
Molasses	3

Table 2: Poultry feeding trial details

Treatments	Treatment ID
Clean feed + no clay no toxin	CF
Toxin feed + no clay	TF
Clean feed + Bhimber bentonite @ 2%	CFB-I
Clean feed + Dina bentonite @ 2%	CFB-II
Toxin feed + Bhimber bentonite @ 1%	TFB-I-1
Toxin feed + Bhimber bentonite @ 2%	TFB-I-2
Toxin feed + Dina bentonite @ 1%	TFB-II-1
Toxin feed + Dina bentonite @ 2%	TFB-II-2

Materials and Methods

Experimental details

The bentonite sources: (i) Bhimber, AJK and (ii) Dina, Punjab were selected for the feeding experiment for which the relevant mineralogical and aflatoxin adsorption characteristics were known. Two feed stocks were prepared: (i) clean feed having aflatoxin contamination < 20 μg kg^{-1} and (ii) contaminated feed with 250 μg kg^{-1} aflatoxin B1 in the final preparation. The feed ingredients were tested for their total and aflatoxin B1 levels using an Enzyme-linked immuno-sorbent assay (Barabolak 1977) and for the feed stock ii, naturally contaminated wheat, maize and soyabean meal were mixed proportionately (Table 1) for the final aflatoxin B1 contamination level of 250 μg kg^{-1}. The bentonite amendments (Table 2) were homogenized by using a feed mixer for 15 min, and remained stored under controlled humidity and temperature conditions.

One day old broiler chicks from a commercial hatchery were reared on the clean feed for the first two week maintaining shed temperature of 32°C and relative humidity of 70%. The shed temperature was reduced 3°C per week until 22°C and was maintained thereafter. Birds were provided with ad libitum access to feed and water throughout the experiment. At day 14, the birds were distributed in the designated treatments with 10 birds per pen in a way that each replicated pen had similar average weight. The birds were fed on the experimental feeds during the following three weeks. The vaccination schedule as recommended by the National Disease Control Committee in Pakistan was followed. Daily health and mortality, weekly body weight gains were recorded during the experimental period. The feed offered, feed retained and body weight per pen were recorded at day 21, 28 and 35. Live weight of randomly selected three birds from each replicated pen was recorded on day 35 and slaughtered. The dress weight and the weight of liver, heart and spleen were recorded. Apparent deformation of internal organs was also examined.

The temporal data for body weight, weight gain, feed consumption and feed conversion ratio were subjected to a multivariate analysis using SAS/STAT® version 9.4. (SAS Institute Inc. 2002) repeated for the weeks. Variation in the absolute and relative weight of the internal organs and dressing percentage was analyzed by an ANOVA under Completely Randomized Design (CRD). Treatment means were compared using Tukey's HSD test at $p < 0.05$.

Results

Both the bentonites from Bhimber and Dina had relatively pure smectite while illite, kaolinite and quartz occurred as traces in Bhimber, and Dina had same component minerals with calcite in moderate amount (Fig. 1). Smectite was identified as dioctahedral montmorillonite with aluminium dominance at octahedral positions. Bhimber had isomorphic substitution of Mg in octahedra while Dina had Fe^{2+}/Fe^{3+} for Al. The maximum aflatoxin adsorption capacity (Q_{max}) of Bhimber was 500 μg g^{-1} while for Dina it was 750 μg g^{-1} of clay. The bentonites amendments to the feed reduced aflatoxin toxic effect as seen through an improved body weight, feed intake and weights of internal organs. The clays as had no negative impact on the broiler health, and caused gain in body weight when added to the clean feed. The results are presented in detail as follows.

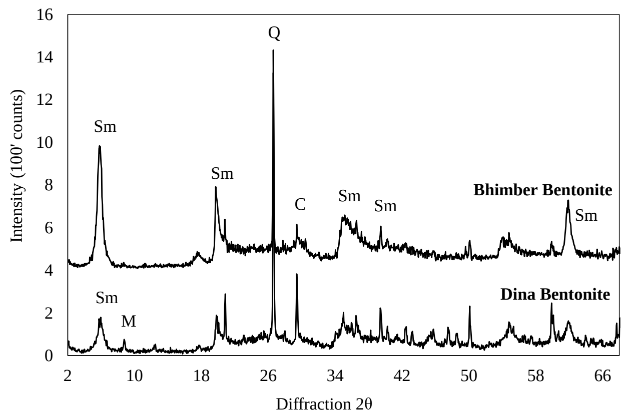

Fig 1: Mineral composition of indigenous bentonite sources having dominant smectite (Sm) with traces of quartz (Q), calcite (C) and mica (M) as identified by X-ray diffraction analysis

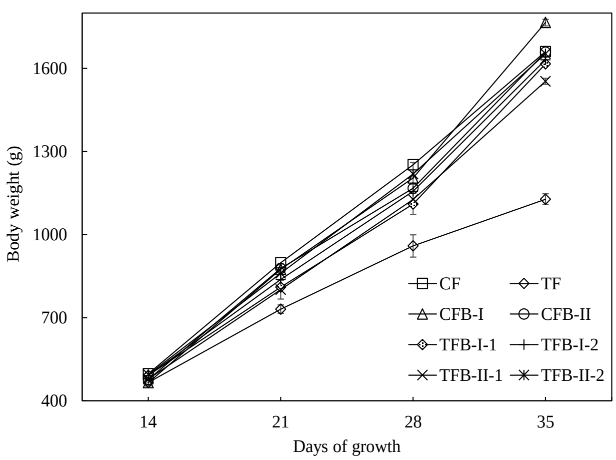

Fig 2: Live body weight over the feeding weeks indicating the clays addition to the contaminated feed at 2 % reduced toxic effect

Body weight

Live weight in a particular pen recorded on weekly basis divided with the number of living birds in each pen was an average body weight per bird (Fig. 2). The body weight varied with the treatment differently over the experimental feeding weeks as the hypothesis of no week × treatment effect was rejected (MANOVA test criteria Wilks' Lambda $p \geq 0.0001$).

At week 1 on experimental diets, (day 21) the body weight changed significantly ($p < 0.0001$) and was the highest, 898 g bird^{-1}, with the clean feed followed by the clean feed with the bentonite from both sources. The toxin containing feed had the lowest body weight, 731 g bird^{-1} that was similar to the toxin feed with 1% of the two bentonite sources. At day 28, the body weight in the clean feed was at par with the toxin feed with bentonites addition from both the sources at 1 and 2%. The toxin feed had the lowest body weight, 959 g bird^{-1}, that was significantly different with all the other treatment combinations ($p = 0.0073$). At day 35, the highest body weight of 1765 g bird^{-1} was with the clean feed with Bhimber bentonite and was significantly different than all other treatment combinations. Both bentonite sources at 1% were at par and only higher to the toxin feed that had the lowest body weight of broilers, 1127 g bird^{-1}. The clean feed had similar body weight as in the toxin feed with 2% application of both bentonites. Overall, the toxic effect of aflatoxin contamination was reduced by the bentonite sources at 2% and the clays addition in the clean feed had no negative impact on broilers body weight.

Weight gain rate

Body weight gain in broilers during the three weeks of experimental feedings had significant variation ($p < 0.0001$) due to the treatment combinations (Fig. 3). The aflatoxin containing feed had 42% lower body weight gain than with the bentonite addition at 2% from any of the two clays. The clean feed with bentonite from Bhimber was significantly different than all the other combinations, and had the highest weight gain (1300 g bird^{-1}). The birds fed on the contaminated feed gained only 661 g bird^{-1} during the experimental period resulting in a reduced production. At 1% application rate both bentonite sources in toxin feed were only better in weight gain than contaminated feed suggesting 1% was as a low application level. Both the bentonites at 2% application and the clean feed had similar weight gain.

Weight gain over the experimental feeding weeks changed significantly. After first week the higher body weight gain recorded in the clean feed whether or not the clay was added which differed significantly from the toxin feed. Aflatoxin contamination reduced weight gain to only 228 g bird^{-1} during second week and 168 g bird^{-1} during third week. During third week 2% clays addition in contaminated feed gained lower weights than in second week. Overall, both the bentonite sources improved growth and weight gain in birds in the clean as well as the toxin containing feed.

Feed intake

The average feed consumed in each pen was recorded weekly after subtracting the amount of feed retained from the feed offered, and the feed consumed divided with the number of living birds in a particular pen was an average feed consumed per bird (Fig. 4). The hypothesis of no week × treatment effect was rejected through the test statistics Wilks' Lambda from the MANOVA ($p = 0.006$). Feed consumption varied significantly ($p = 0.0002$) with treatments at different weeks and was also significantly correlated.

While the feed intake at day 21 was non-significant, feed consumption in week 2 on experimental diets (28 day) varied significantly with the treatment combinations (p <

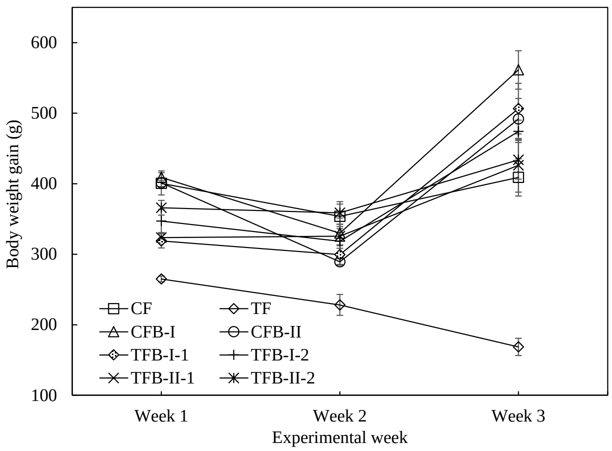

Fig 3: Body weight gain over three experimental weeks indicating the lowest weight gain on the toxin feed and increase under the bentonite addition to the contaminated feed

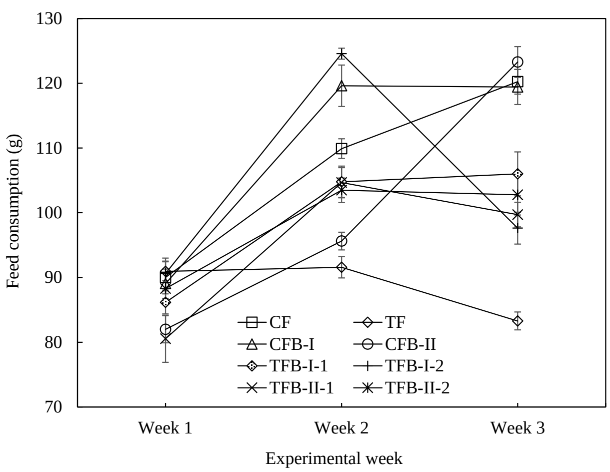

Fig 4: Weekly feed consumption representing the lowest feed intake with the toxin feed whereas the clean feed with and without bentonite had the highest feed consumption

0.0001). The toxin feed with Bhimber bentonite at 2% rate of application had the highest feed intake that was similar to the clean feed with the same bentonite. The lowest feed intake was observed in the toxin feed and was at par with clean feed with Dina bentonite. The treatment means also varied significantly for feed intake on week 3 ($p < 0.0001$). The clean feed with Dina bentonite had the highest feed consumption that was similar to the clean feed and the clean feed with Bhimber bentonite. The toxin feed with both bentonite sources at 1 and 2% were statistically at par. The lowest feed intake was recorded in the toxin feed that differed significantly with all the other treatment combinations.

Feed conversion ratio

The efficiency of broilers in converting consumed feed to body weight was demonstrated by their feed conversion ratio (Fig. 5). Feed conversion ratio varied significantly ($p < 0.0001$) with treatment combinations at different weeks. After first week on experimental feeds, day 21, the treatment combinations differed significantly for the feed conversion ratio ($p < 0.0001$). The highest feed conversion ratio, 2.4, was determined in the toxin feed that significantly varied with all the other feed combinations. Both the bentonite sources at 1 and 2% in the toxin containing feed were similar. The clean feed with Dina bentonite had the lowest feed conversion ratio and was at par with the clean feed along with the clean feed with Bhimber bentonite suggesting better conversion of feed to body mass. The feed conversion ratios generally increased on second week of experimental feedings irrespective of feed combinations. At 28 day the highest feed conversion ratio was associated with the toxin feed that was similar to the toxin feed with Bhimber bentonite at both levels (1 and 2%) and the clean feed with both bentonite sources. At the final week, day 35, the toxin feed had the highest feed conversion ratio, 3.49, that varied significantly with all the other treatment combinations. The lowest feed conversion ratio was in the toxin feed with Bhimber bentonite and was at par with all other treatments except the toxin feed. Overall, the toxin containing feed had higher feed conversion ratio at each week and the clay sources addition in both the clean feed and the toxin feed demonstrated lower feed conversion ratio suggesting improved feed conversion efficiency in the experimental birds.

Dressing percentage

The treatment effect on the dressing percentage was significant (F 9.53, df 7; $p < 0.0001$) (Fig. 6). The toxin feed with Dina bentonite at 2% yielded the highest dressing percentage, 65.9%, followed by the clean feed with any of the two bentonites. The dressing percentage for all other feed combinations was non-significant except the toxin feed. The toxin feed had the lowest dressing percentage, 57.3% which was significantly lower than all the other treatment combinations.

Internal organs weight

Absolute and relative weights of liver, heart and spleen (Table 3) were compared. The treatment combinations had significant effect on absolute liver (F 5.69, df 7; $p < 0.0019$) and heart weights (F 2.94 df 7; $p < 0.0351$). The highest liver weight was in the clean feed with Bhimber bentonite. It had lesions that was similar to that of in the toxin feed with Dina bentonite at 2% and the toxin feed with bentonite from Bhimber at 1%. The highest heart weight, 13.6 g, was in the toxin feed with 1% Bhimber bentonite which was at par with the toxin feed with 2% bentonite from Dina and the clean feed. The lowest liver, 30.9 g, and heart weight, 9.4 g, were observed in the toxin feed. The toxin containing feed

Table 3: Absolute and relative weight of internal organs

	Absolute weight (g)			Relative weight (%)		
Treatments	Liver	Heart	Spleen	Liver	Heart	Spleen
	……………………..… grams ………………………			……………………….. % …………………………		
CF	42.63 ± 7.61 ^{b}	11.62 ± 0.88 ^{ab}	2.09 ± 0.30 ^{a}	2.30 ± 0.42 ^{b}	0.63 ± 0.04 ^{bc}	0.11 ± 0.01 ^{a}
TF	30.90 ± 3.28 ^{c}	9.40 ± 0.72 ^{c}	1.69 ± 0.38 ^{a}	2.28 ± 0.15 ^{b}	0.70 ± 0.07 ^{ab}	0.12 ± 0.03 ^{a}
CFB-I	54.25 ± 0.77 ^{a}	11.33 ± 0.75 ^{bc}	2.49 ± 0.38 ^{a}	2.83 ± 0.10 ^{a}	0.59 ± 0.02 ^{c}	0.13 ± 0.02 ^{a}
CFB-II	43.32 ± 5.65 ^{b}	11.53 ± 1.97 ^{b}	2.23 ± 0.24 ^{a}	2.32 ± 0.10 ^{b}	0.62 ± 0.07 ^{bc}	0.12 ± 0.01 ^{a}
TFB-I-1	46.09 ± 2.39 ^{ab}	13.68 ± 1.65 ^{a}	2.53 ± 0.71 ^{a}	2.47 ± 0.07 ^{ab}	0.73 ± 0.05 ^{a}	0.14 ± 0.04 ^{a}
TFB-I-2	41.78 ± 7.65 ^{b}	11.02 ± 0.37 ^{bc}	2.62 ± 1.05 ^{a}	2.39 ± 0.40 b	0.63 ± 0.01 ^{bc}	0.15 ± 0.06 ^{a}
TFB-II-1	41.39 ± 0.75 ^{b}	10.92 ± 1.09 ^{bc}	2.12 ± 0.13 ^{a}	2.44 ± 0.13 ^{ab}	0.64 ± 0.02 ^{abc}	0.12 ± 0.01 ^{a}
TFB-II-2	47.34 ± 4.09 ^{ab}	11.76 ± 1.25 ^{ab}	1.88 ± 0.48 ^{a}	2.58 ± 0.15 ^{ab}	0.64 ± 0.06 ^{abc}	0.10 ± 0.02 ^{a}

Values (Mean ± SD) in each row followed by different letters are significantly different ($p \leq 0.05$). For analysis, 3 birds were selected randomly from each replicate of treatment combinations at day 35 and the data collected were analyzed by applying ANOVA

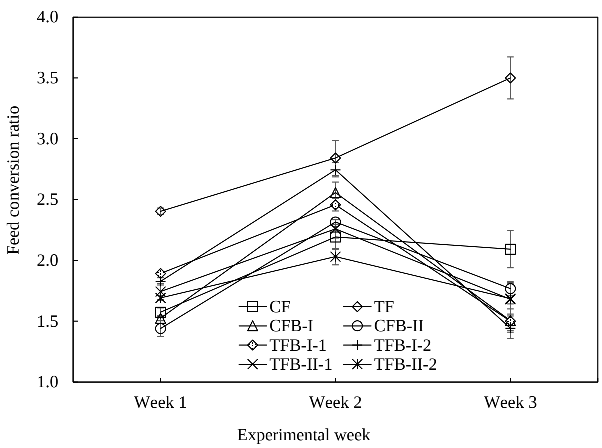

Fig 5: Weekly feed conversion ratio indicating detrimental effect of the toxin feeding while the lower FCR was with the clay's addition in both the clean feed and the toxin feed

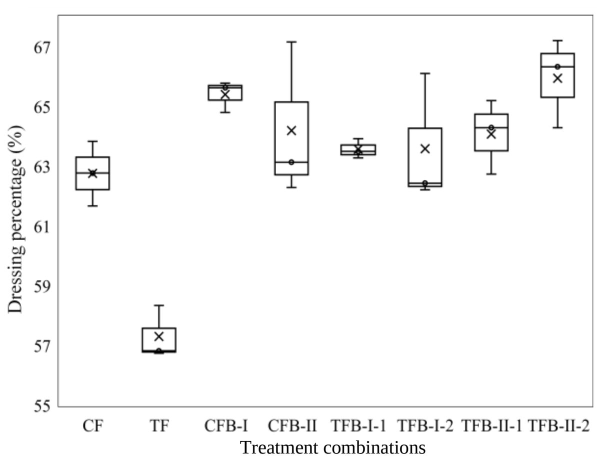

Fig 6: Dressing percentage with the lowest values in the toxin feed that improved with the clay's addition at both applied levels from the two sources in the clean and the toxin feed

with Bhimber bentonite at 1% had liver morphology similar to the clean feed. The spleen in terms of its absolute and relative weight was non-significant. Therefore, among the internal organs absolute liver and heart weight varied while the relative weight of all the three internal organs (liver, heart and spleen) did not change suggesting equal effect of toxin feeding on the internal organs weight.

Discussion

The lowest body weight at each experimental week in the toxin containing feed explained the toxic effect of contaminated feedings. Aflatoxin toxicity affects the body weight gain, feed intake and broilers health suggesting interference of aflatoxin in the digestive system (Hassan *et al*. 2012) and results in reduced poultry production (Pasha *et al*. 2007; Naseem *et al*. 2018). The toxin feed reduce synthesis of protein and lipids loss with droppings, reduction in nutrients assimilation and less production of digestive glands (Verma *et al*. 2002). Aflatoxin effect started from the first week on experimental feeds resulting in the lowest body weight in the toxin containing feed. Therefore, the kinetics of aflatoxin effect on body weight is fast though the repeat effect occurred from the second feeding. Broilers growth and production is reduced by aflatoxin contamination above critical level (Kana *et al*. 2010). Body weight and body weight gain is also affected by toxin level and exposure time (Heba and Hesham 2004; Mahmood *et al*. 2017). Feeding aflatoxin results in higher feed conversion ratio with lower body weight gain, and is generally agreed (Pasha *et al*. 2007). Reduction in the weight gain may be related to the inability of the birds fed on the toxin feed to consume and digest dry ration and amino acids. Contaminated diet results in high feed conversion ratio during experimental period and is related to reduced efficiency of broilers. Our findings were consistent with the toxic effects of feeding aflatoxin contaminated feed in broilers (Shi *et al*. 2006).

A low weight gain at each experimental week suggest progressive reduction and is related to dose and prolonged exposure to aflatoxin feeding (Mahmood *et al*. 2017). Inconsistent weight gain reduction has also been reported mainly due to the duration and level of the aflatoxin exposure. The toxin results in stunted and ruffled chickens with a reduction in weight gain (Bhatti *et al*. 2018; Naseem *et al.* 2018). Aflatoxin feeding also reduce feed consumption and efficiency of feed conversion (Mahmood *et al.* 2017) and is in agreement with the previous studies (Heba and Hesham 2004; Pasha *et al.* 2007).

A variability occurs between the adsorption capacity measured in a laboratory and the feeding trial results (Jaynes *et al.* 2007) and indicates the effectiveness of mycotoxin binders in real gut conditions. The clays addition up to 2% in the clean feed results in better weight gain and health with improved feed conversion ratio suggesting positive impact of clays even at low or no aflatoxin contamination. The improvement of body weight in the clean feed with clays addition may be associated with adsorption of any harmful bacteria or mycotoxin other than aflatoxin present in the clean feed (Xia *et al.* 2004). Increase in body weight by the clays addition in the clean feed with no and/or low contamination levels occur (Pappas *et al.* 2014). Contradictory results for improved weight gain with bentonites addition in the clean feed relates to the non-selective adsorption of smectite (Bailey *et al.* 2006). Our findings suggest safe use of both the bentonite sources at 1 and 2% with no negative impact on weight gain and feed consumption.

Quality smectites have been found effective in reducing the toxicity of aflatoxin in the feed. Bentonites with high adsorption in the laboratory estimation reduces the toxin effect (Boudergue *et al*. 2009). The toxic effect of aflatoxin in broilers is reduced by adding bentonites (Wafaa *et al*. 2013). Bentonite addition in the contaminated feed is not limited to aflatoxin adsorption and is also reported to boost enzymatic activities in the gastro-intestinal tract of the birds (Xia *et al.* 2004). Complete recovery from the toxic effects of aflatoxin contamination can occur by 5% application of hydrated sodium calcium aluminosilicate (Sehu *et al*. 2007). Bentonites addition at 2% ameliorated the negative effect of aflatoxin. Addition of clay binders at concentration higher than 5% may result in nutritional deficiency and lower production in birds (Mabbett 2005).

Lower body weight, high mortality, poor feed conversion ratio, immunosuppression and clinical disorders are related to aflatoxin feeding (Naseem *et al*. 2018; Bhatti *et al.* 2018; Saleemi *et al.* 2020). A reduction in the feed intake occur in response to the contaminated diets in broilers (Rauber *et al*. 2007), with liver lesions as basic symptoms of aflatoxin toxicity (Johri and Majumdar 1990). Aflatoxin fed broilers demonstrate gross and histopathological lesions on the liver, kidney and bursa of Fabricius (Ortatatli and Oguz 2001). The experimental feed combinations affect the absolute liver and heart weight with higher weight in the contaminated feed with 1% bentonite from Bhimber and 2% Dina bentonite. Aflatoxin feed results in the lower absolute weight of liver and heart. However, in most studies enlarged liver and kidney have been reported due to aflatoxin feedings (Miazzo *et al*. 2005; Wafaa *et al*. 2013). Internal organs especially liver respond to aflatoxin feedings with deformation in morphology and absolute weight. In our study darkish liver with small size in the toxin fed birds may be attributed to severe immune suppression (Kubena *et al.* 1990). In contradiction, enlarged liver have been reported in the toxin fed birds over the clean feed (Tessari *et al*. 2006). In addition to liver the internal organs including kidney, heart and spleen may be affected by feeding contaminated feed to broilers (Quezada *et al*. 2000). However, non-significant difference for spleen weight among the treatments with greater variability among the replicates suggest inconsistent response of internal organs towards toxin feedings which may be due to lower level of aflatoxin contamination compared to other studies where high aflatoxin doses have been tested. Non-significant variation in the relative weight of internal organs may be associated with the low application levels of clays as the internal organs weight is non sensitive at clay level below 5% (Barrientos-Velazquez and Deng 2020).

Conclusion

The feeding experiment suggested both bentonite sources were effective in reducing the toxic effect of aflatoxin. Addition of both bentonites had no negative impact on broilers growth and production suggesting safe use of these clays as toxin binder. Smectite addition in the feed containing 250 μg aflatoxin kg^{-1} had similar body weight, internal organs morphology, and feed conversion ratio as that of clean feed where without smectite 42% body weight reduction had occurred. Use of indigenous smectite clays as aflatoxin binder appears to have great potential that can reduce import of binders and control aflatoxicosis in poultry industry of Pakistan.

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Author Contributions

MSA, TA and AK planned the experiment, AK and SA carried out the trial and interpreted the results, MSA and KSK guided during writeup, NM and TA supervised and guided during the trial, MSA helped during statistical analysis, and AK made illustrations and completed writeup

Conflicts of Interest

The authors declare no conflict of interest.

Date Availability

The data will be available on a fair request.

Ethics Approval

All the ethical policies regarding animal health approved from the University Ethics Committee were adhered and the research meet the standards for the protection and use of animals for scientific purposes.

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