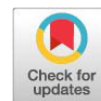


Research Article



Individual and Synergistic Effect of Adding Zeolite and Yeast to Reduce the Negative Impact of Aflatoxin B1 on the Physiological and Immune Performance of Laying Hens

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Abstract | This study was carried out to investigate the impact of incorporating zeolite and yeast (*Saccharomyces cerevisiae*) in laying hen diets contaminated with aflatoxin B1 (AFB1) on some physiological traits and immunological responses. Seventy-two laying hens, 45 weeks old (Lohman Brown), were randomly assigned to six treatments with four replicates per treatment (three hen/replicate). The experiment was carried out in cages. Birds in T1 fed basal diet without addition, birds in T2 fed basal diet contaminated with AFB1 200 ppb, birds in T3 fed basal diet contaminated with AFB1 200 ppb + commercial anti-fungal, birds in T4 fed diet contaminated with AFB1 200 ppb + yeast 4 gm/kg feed, birds in T5 fed diet contaminated with AFB1 200 ppb + zeolite 20 gm/kg feed, birds in T6 fed diet contaminated with AFB1 200 ppb + zeolite 20 gm/kg + yeast 4 gm/kg feed. Results indicated that adding zeolite and yeast synergistic to laying hen diets contaminated with AFB1 (T6) had a positive impact on RBC, PCV, Hb, H/L ratio, and HI titer against Newcastle diseases with a significant increase ($P \leq 0.05$) as compared with T2 and T3. No significant differences ($P > 0.05$) were recorded between treatments in WBC. Also, immunological results observed that Adenosine Deaminase (ADA) in the cecal tonsil and serum exhibited high values by adding a combination of zeolite and yeast to laying hen diets contaminated with AFB1 compared with T2 and T3. Finally, our results indicated that dietary supplements were effective in reducing the negative effects of aflatoxin B1. Findings suggest that adding zeolite and yeast to laying hen diets could be a promising strategy for improving the health and productivity of aflatoxin B1-contaminated flocks.

Keywords | Aflatoxin B1, immunity, laying hens, physiological performance, yeast, zeolite

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INTRODUCTION

Aflatoxin B₁ (AFB₁) is a potent carcinogenic mycotoxin that is produced by certain strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. It is a naturally occurring toxin that can contaminate a wide range

of food commodities, including cereals, nuts, spices, and milk (Alameri *et al.*, 2023). The toxin can enter the food chain through contaminated crops, and can also be found in animal feedstuffs, which can lead to the accumulation of AFB₁ in animal products (Kumar *et al.*, 2021). AFB₁ is highly stable and resistant to many forms of food process-

ing, making it difficult to remove from contaminated feeds (Ndagijimana *et al.*, 2020). Chronic exposure to AFB₁ can lead to the development of hepatocellular carcinoma (HCC), a type of liver cancer that is responsible for over 80% of all liver cancer cases worldwide (Kucukcakan and Hayrulai-Musliu, 2015). Mycotoxins especially (AFB₁) are also implicated in other health problems, including stunted growth and immune suppression (Sun *et al.*, 2023). Several strategies have been carried out to minimize the effects of aflatoxins toxic on laying hens and the transfer of its residues to products, such as physical, chemical, and biological methods, These include implementing good agricultural practices to prevent the growth of *Aspergillus fungi* on crops, such as crop rotation, irrigation management, and use of fungicides (Hameed and Mousa, 2025).

Zeolite is a naturally occurring mineral with a unique porous structure that has been found to have several beneficial properties, including the ability to adsorb contaminants such as aflatoxins (Vasconcelos *et al.*, 2023). The use of zeolite in animal feed is a promising strategy to minimize the adverse effects of aflatoxin B₁ (Miazzo *et al.*, 2000). Zeolite can bind to and trap AFB₁ in its porous structure, preventing it from being absorbed by animals and reducing its toxicity (Kraljević *et al.*, 2018). In addition, zeolite has been shown to improve animal health and performance in the presence of aflatoxin B₁ (Alharthi *et al.*, 2022). The results (Mijailović *et al.*, 2022) demonstrated that zeolites have a microporous structure that forms a large internal surface and is associated with their high cation exchange capacity (Ca²⁺) that makes zeolites efficiently adsorb polar molecules such as AFB₁. Zeolite has also been shown to improve immune system function and reduce oxidative stress in animals exposed to aflatoxin B₁, which can help to minimize the negative effects of this mycotoxin on animal health (Mavrommatis *et al.*, 2021). Yeast is a single-celled organism that is widely used in food and feed production (Amara and El-Baky, 2023). There are several mechanisms by which yeast can help minimize the effects of AFB₁. One of the main mechanisms involves the ability of yeast to bind to AFB₁ and prevent its absorption in the digestive tract (Yiannikouris *et al.*, 2021). This reduces the amount of AFB₁ that is available to be absorbed into the bloodstream and distributed throughout the body. In addition, yeast has been found to stimulate the immune system and improve gut health, which can help animals cope with the toxic effects of AFB₁ (Perricone *et al.*, 2022). Another mechanism by which yeast can help minimize the effects of AFB₁ is through the production of enzymes that can break down the toxin (Guan *et al.*, 2021). Certain strains of yeast, such as *Saccharomyces cerevisiae*, have been found to produce enzymes that can degrade AFB₁ into less toxic metabolites. Several studies have demonstrated the effectiveness of yeast in reducing the toxicity of AFB₁ in poultry feed (Stanley *et al.*, 2004; Abdullah *et al.*, 2020; Kolawole

et al., 2022; Bozzo *et al.*, 2023). However, further research is needed to optimize the use of zeolite and yeast in laying hens fed AFB₁-contaminated diet and to determine its long-term effects on the physiological system and growth rate. This study was carried out to show the role of zeolites and yeast individually or combined to reduce the negative effects of mycotoxins in some physiological and immunological traits of laying hens.

Table 1: Calculated and analyzed nutrient contents, as well as the composition percentages of experimental diets of laying hens during 45-65 weeks.

Ingredients	%
Corn	35.4
Wheat	30
Soy bean (48%)	23
Premix*	2.5
Oil	0.5
Limestone	7.5
Salt	0.1
Di calcium phosphate	1
Total	100
Chemical analysis **	
ME kcal/kg	2752
CP %	16.77
Lysine %	0.93
Met. + Cys. %	0.76
Ca %	3.75
Available Phosphor %	0.39

* Premix contains 4.95% protein, kcal / kg 3500 represented energy, 78.95% ash, 6% phosphorus, 11.80% available phosphorus, 5.81% calcium, 1.70% lysine, 4.31% methionine; ** The chemical analysis of diets components was carried out according to NRC (1994).

MATERIALS AND METHODS

This study was conducted in the poultry farm which belonged to the Department of Animal Production/College of Agriculture/University of Anbar from lasted for 112 days from 1st February to 20th June, 2023 (28 days/period), to study the effect of adding zeolite and yeast to laying hens diets contaminated with AFB₁. Seventy-two-layer hens of Lohmann Brown Hybrid, aged 45 weeks were used in this experiment. Laying hens were distributed randomly to six experiment treatments with 4 replicates per treatment (3 laying hens/replicate) in Cages. Laying hens were fed according to basal diet, as shown in Table 1, in equal amounts (NRC, 1994). Cages were provided with a nipple system and, a lighting period of 15.5 hours per day according to the bird's guide. Experimental treatments were as follows: T1 birds fed basal diet without any additions (con-

trol), T2 birds fed basal diet contaminated with AFB₁ 200 ppb, T3 birds fed basal diet contaminated with AFB₁ 200 ppb + commercial anti-fungal (Zero Max 1/2 cm per liter), T4 birds fed basal diet contaminated with AFB₁ 200 ppb + yeast 4 gm/kg feed, T5 birds fed basal diet contaminated with AFB₁ 200 ppb + zeolite 20 gm/kg feed, T6 birds fed basal diet contaminated with AFB₁ 200 ppb + zeolite 20 gm/kg + yeast 4 gm /kg feed. The additives were adopted based on the findings of previous studies and sources, while addition AFB₁ was used and approved considering level of contamination and its non-lethal nature (Alameri *et al.*, 2023).

PRODUCTION OF AFLATOXIN B₁

The study started by isolating *Aspergillus flavus* from the College of Veterinary Medicine, Diseases Branch, University of Baghdad, and from the Food Safety Branch at the Ministry of Science and Technology. The isolation method followed the approach of Shotwell *et al.* (1966) and was modified by West *et al.* (1973) for mushroom development. The fungus was activated using Potato Dextrose Agar (PDA) medium and then grown on corn grains as a primary development medium, while rice was used as the main development medium. The study also used *Saccharomyces cerevisiae* produced by Lesaffre, a French industrial company.

HEMATOLOGICAL TESTS

Hematological parameters were assessed by collecting samples from the wing vein (five birds from each treatment) at the end of the experiment. The blood was taken in capillary tubes containing an anticoagulant, then closed one end of the tube with industrial clay and then placed in a centrifuge for five minutes. The hematological parameters included PCV (Archer, 1965), RBC and WBC counting (Natt and Herrick, 1952) and H/L ratio (Burton and Guion, 1968).

DETERMINATION OF IMMUNOLOGICAL TESTS

The immune response was evaluated by measuring the humoral immunity using the hemagglutination inhibition (HI) test against Newcastle disease (Beard, 1989).

ADENOSINE DEAMINASE ENZYME ACTIVITY (ADA) IN CECAL TISSUE AND SERUM

Enzyme activity was calculated in the extract of the cecum of each bird individually. A portion of the cecum was taken, weighed, and mixed with an equivalent weight of Phosphate Buffer Solution (PBS) solution. The tissue was then homogenized at 4°C and the resulting solution was centrifuged at 2000 rpm for ten minutes with zero-degree cooling. The supernatant was discarded and the sediment was washed with PBS solution three times. The sediment was then placed in 2 mL plastic containers with an equal amount of PBS solution, and to obtain the extract, the cells were broken down through a series of successive grinding

and dissolution steps, repeated three times. The extract was then placed in plastic containers and centrifuged in a Microfuge centrifuge at 14000 rpm for ten minutes. The resulting supernatant was used as a source to measure enzyme activity by measuring the ratio of liberated ammonia to the reaction using a spectrophotometer at a wavelength of 624 nanometers, (Giusti, 1981).

STATISTICAL ANALYSIS

The experiment was a completely randomized design with four replicates of three laying hens assigned to each of the six dietary treatments. Data were subjected to Statistical analysis using the general linear models procedure of SAS software (SAS Institute, 2012). Variable means for treatments showing significant differences in the One-way ANOVA were compared using Duncan's multiple-range test (Duncan, 1955).

RESULTS AND DISCUSSION

Figure 1, showed a significant decrease ($P \leq 0.05$) in red blood cells for T2 which recorded 3.25 as compared with T1, T3, T4, T5, and T6. Also, significant differences ($P \leq 0.05$) were observed for treatments T1, T4, and T6 which recorded 4.4, 4.125, and 4.35 $\times 10^6$ cells/ ml, respectively in comparison with T3 which recorded 3.675 $\times 10^6$ cell/ ml blood.

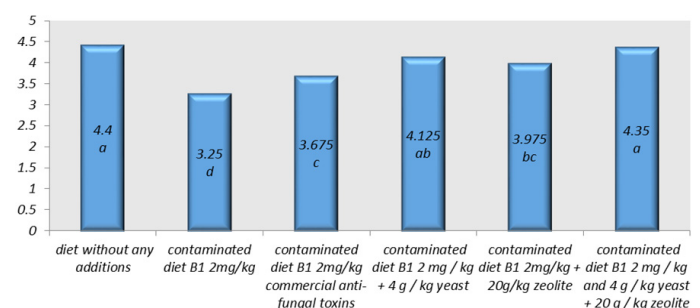


Figure 1: Effect of AFB₁ combined with zeolite or yeast on RBC count (10⁶).

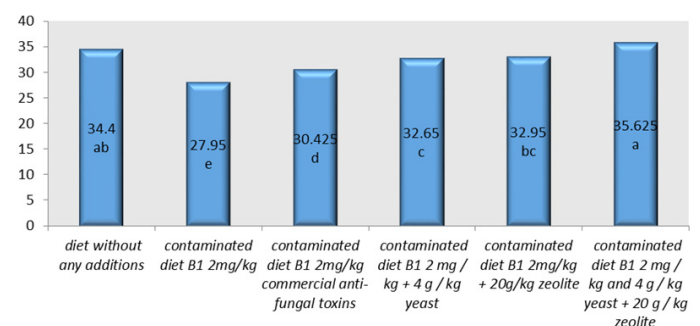


Figure 2: Effect of AFB₁ combined with zeolite or yeast on packed cell volume (PCV%).

The effect of adding zeolite and yeast to a laying hens diet in packed cell volume was shown in Figure 2. T6 and T1

recorded high values in PCV significantly (35.625 and 34.4) as compared with T2, T3, and T4 (27.95, 30.425, and 32.65 %) respectively. Data in Figure 2 showed that the addition of zeolite and yeast individually (T4 and T5) or synergistically (T6) had significant effects ($P \leq 0.05$) in PCV compared with T2 and T3. T2 recorded the lowest value in PCV (27.95 %).

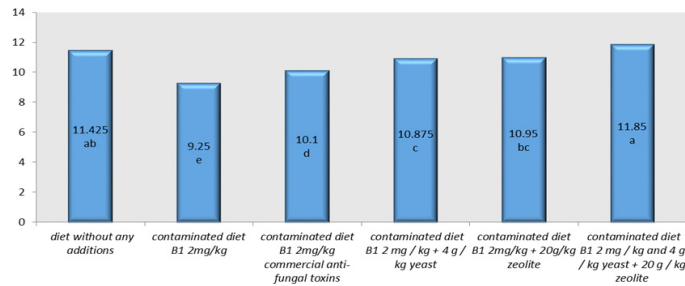


Figure 3: Effect of AFB1 combined with zeolite or yeast on hemoglobin concentration (Hb; g/dl).

Figure 3 showed that there were significant differences ($P \leq 0.05$) between treatments in hemoglobin concentrate of laying hens fed diets contaminated with AFB₁ and supplemented with zeolite and yeast synergistic and individually. Results showed that T2 recorded a significant decrease in hemoglobin concentration as compare with all treatments (9.25 gm/dl), while T1 and T6 recorded high values (11.425 and 11.85 gm/dl respectively). The results of the statistical analysis shown in Figure 4, indicated that no significant differences ($P > 0.05$) were observed between treatments in white blood cells. The heterophil/lymphocyte cell ratio was represented in Figure 5, T2 recorded a high value of 0.60 with a significant increase ($P \leq 0.05$) as compared with T1, T3, T4, T5, and T6 (0.44, 0.47, 0.45, 0.48, 0.40 %, respectively).

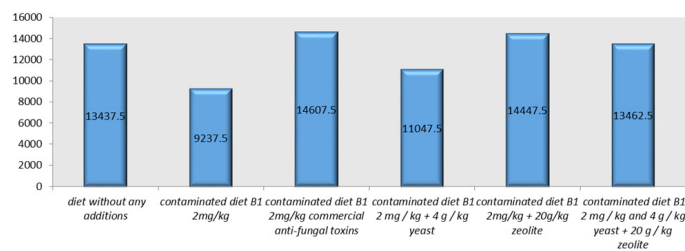


Figure 4: Effect of AFB1 combined with zeolite or yeast on the number of white blood cells (WBC; 10³/ml).

The level of antibodies against Newcastle disease that indicate the development of immune response in laying hens after vaccination is shown in Figure 6, it can be observed that the highest value of antibodies against Newcastle disease was observed in T1 and T6, with a significant difference ($P \leq 0.05$) as compared with experimental treatments. On the other hand, T2, which had only fungal toxins added to the basal diet showed the lowest value of antibodies against Newcastle disease (59.5 mg/ μ L), with a significant

decrease ($P \leq 0.05$) compared with T3, T4, and T5 (111.5, 115.3, 107.8 mg/ μ L) respectively. Also, birds in treatments T5 and T3 and the lasted didn't differ from T4. According to observation data from Figures 7 and 8, the highest ADA enzyme activity was in T1 and T6, with a significant difference ($P \leq 0.05$) compared to the experimental treatments. The least effective enzyme activity was shown in T2, which fed a contaminated diet with fungi. Furthermore, birds in T4 recorded a significant increase ($P \leq 0.05$) as compared with birds in T3.

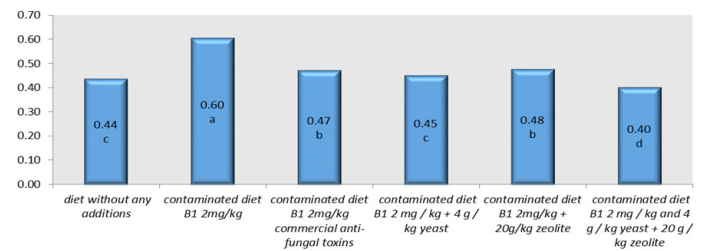


Figure 5: Effect of AFB1 combined with zeolite or yeast on H/L ratio.

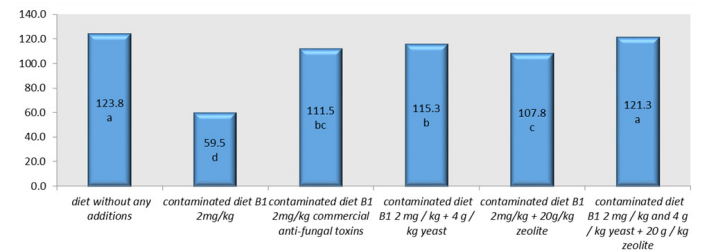


Figure 6: Effect of AFB1 combined with zeolite or yeast on the titer (mg/ μ L) of antibodies to Newcastle Disease.

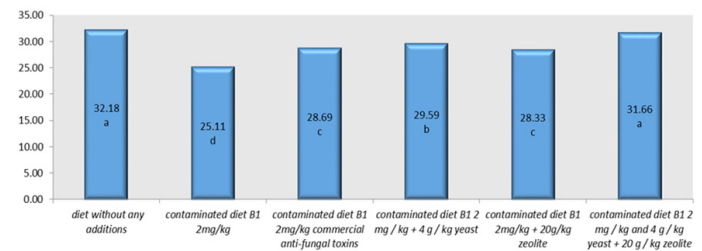


Figure 7: Effect of AFB1 combined with zeolite or yeast on the activity of the immunostimulant enzyme ADA in serum (u/mg $\times 10^{-3}$).

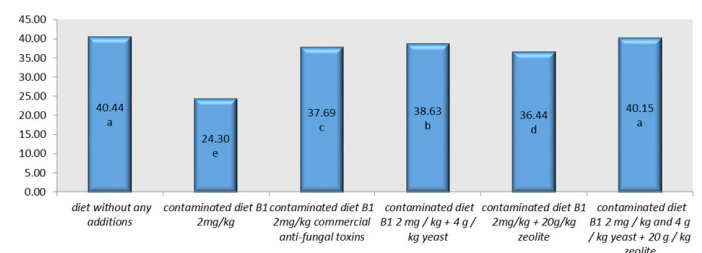


Figure 8: Effect of AFB1 combined with zeolite or yeast on the activity of immune enzyme ADA in cecal tonsil (u/mg $\times 10^{-3}$).

Fungal toxins such as AFB₁ affect cellular traits of laying hens' blood, and exposure of birds to this toxin can lead to decrease in red blood cells and hemoglobin levels which leads to decrease in productive performance and deterioration of chicken health (Ochieng *et al.*, 2021). AFB₁ can also affect the immune system, leading to increased inflammation and decreased immunity against diseases (Chen *et al.*, 2017). To reduce the effects of fungal toxins, natural materials such as zeolites and yeast can be used, the significant improvement in blood characteristics, such as the number of red blood cells, the size of packed cell volume and the blood smear values can be attributed to the addition of zeolites to laying hen diets, which can reduce the harmful effects of fungal toxins on the number of red blood cells and improve the immune system (Stefanović *et al.*, 2023). The improvement may also be due to the role of zeolites in improving antioxidant status, reducing inflammation and enhancing the immune response to disease-causing agents and viral infections (Oggiano *et al.*, 2023). Zeolites and yeast work to improve blood characteristics through various mechanisms, zeolites have a high binding capacity for AFB₁ toxins due to their porous structure and high surface area, and they can eliminate heavy toxins before they are absorbed by the bird's intestines, which in turn reduces the harmful effects of fungal toxins on blood characteristics and improves gut health (Hernández-Ramírez *et al.*, 2021). The improvement in the addition of zeolites may also be due to their ability to increase the availability of nutrients, which helps to improve blood traits (Cataldo *et al.*, 2021). The improvement may also be due to the role of yeast in improving blood characteristics of laying hens by increasing the internal production of antibiotics, amino acids, vitamins, and amino acids with various health benefits, including enhancing digestion, improving immune function and reducing inflammation (Wang *et al.*, 2022).

Yeast is also a rich source of beta-glucans and complex carbohydrates that have various positive effects, which help to enhance the physiological and immune performance of laying hens (Siloto *et al.*, 2021). The improvement in T6 may be due to the synergistic effect of the additives in improving blood characteristics of laying hens, where zeolites work to reduce the harmful effects of fungal toxins on blood characteristics, while yeast works to improve blood traits, increase white blood cell count, and improve immunity. Overall, the additives work to reduce the harmful effects of fungal toxins on the health of laying hens. Results indicated that the synergistic role of zeolite and yeast (T6) reduced the negative effects of fungal toxins and significantly improved the activity and concentration of enzyme Adenosine deaminase (ADA) compared to treatment (T2). Fungal toxins decrease the concentration of this enzyme in the cecum tonsil or serum, resulting in a clear decrease in its concentration in serum and primary and secondary immune organs under immune suppression

or as a result of stress (Aiuti, 2004). Reducing the activity of this enzyme can lead to changes in cellular and functional composition as it is one of the important enzymes that convert adenosine to inosine, which is the most beneficial form for the immune system (Ali and Mousa, 2023). The enzyme is also essential for the proliferation and differentiation of lymphoid cells, especially T cells, and helps in the maturation of lymphoid granules and transformation into macrophage cells, making it one of the essential factors in cellular immune response (Signa *et al.*, 2022). An increase in the concentration of this enzyme in T6 is evidence of a good immune response, and zeolite and yeast addition has been increase cellular immune response.

CONCLUSIONS AND RECOMMENDATIONS

We can conclude from this study that adding zeolite and yeast to the diets of laying hens can be effective in improving health and reducing the toxic effects of AFB₁. This discovery can be useful in developing new techniques to improve food quality and safety and provide food security for consumers.

ACKNOWLEDGEMENTS

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NOVELTY STATEMENT

The novelty of our study has been its strong evidence that adding zeolite and yeast to the diets of laying hens can be effective in improving health and reducing toxic effects of AFB₁ of laying hens, in addition to improving food quality and safety for the first time.

AUTHOR'S CONTRIBUTIONS

All authors contributed equally.

CONFLICT OF INTEREST

None.

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