Food and Chemical Toxicology 90 (2016) 142–150

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

The toxic effects of combined aflatoxins and zearalenone in naturally contaminated diets on laying performance, egg quality and mycotoxins residues in eggs of layers and the protective effect of *Bacillus subtilis* biodegradation product

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ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form
7 February 2016
Accepted 10 February 2016
Available online 15 February 2016

Keywords:
Aflatoxin
Bacillus subtilis
Egg quality
Laying performance
Residue
Zearalenone

ABSTRACT

The toxic effect of aflatoxins (AF) and zearalenone (ZEA) and their combination on laying performance, egg quality and toxins residues in eggs, as well as the efficacy of *Bacillus subtilis* biodegradation product (BDP) for ameliorating these effects in layers were evaluated. Layers were submitted to a two phase experiment. The first phase was an intoxication period (18–23 wk) with birds fed 7 ($3 \times 2 + 1$) diets (3 treatments with mycotoxins: AF (123.0 μg/kg), ZEA (260.2 μg/kg), or AF + ZEA (123.0 + 260.2 μg/kg); 2 treatments with or without BDP (1000 g/t); and a control group contained no toxins nor BDP). The next phase was a recovery period (24–29 wk) in which birds were fed a toxin-free diet. In the intoxication period, AF and AF + ZEA groups exhibited lower egg production, feed intake and shell thickness, and higher AFB_1, AFB_2 and AFM_1 residues as compared with the control group. In addition, AF and ZEA exerted synergistic effects on egg production and feed intake. Moreover, AF alone or combined with ZEA had a continuous toxic effect on laying performance in the recovery phase. Addition of BDP offset these negative effects, showing that BDP has a protective effect on layers fed contaminated diets.

1. Introduction

Eggs are important international food commodity. China accounts for roughly a quarter of the world's egg production at approximately 39.68 billion pounds, with an estimated value of nearly US$ 20.3 billion (Food and Agriculture Organization, FAO). However, significant economic losses may occur due to the presence of natural feed contaminants, such as mycotoxins, which are secondary metabolites produced by certain toxigenic species of fungi. Among these, aflatoxins (AF) are the most widely distributed and toxic to poultry. AF are mainly produced by the fungi *Aspergillus parasiticus*, *A. flavus*, and *A. nomius* (Ito et al., 2001), and exhibits teratogenic, carcinogenic and mutagenic effects (Yunus et al., 2011). Additionally, acute or chronic aflatoxicosis in poultry results in retarded growth, decreased production and egg quality, impaired immune response, increased mortality and liver and intestine damage (Danicke et al., 2002, Pandey and Chauhan, 2007). Moreover, the carry-over of AF through animal-derived products, such as meat and eggs, into the human food chain is a potential threaten to human health (Aly and Anwer, 2009; Christofidou et al., 2015; Herzallah, 2013 and Iqbal et al., 2014).

Another mycotoxin that has captured the attention of poultry producers is zearalenone (ZEA), produced by *Fusarium spp* (Zinedine et al., 2007). ZEA is regular contaminant of cereal crops worldwide, and is produced in the field rather than during storage (Bennett and Klich, 2003). Moreover, ZEA has also been shown to be hepatotoxic, immunotoxic and genotoxic (Abbès et al., 2006). Exposure to ZEA produces symptoms of reproduction disturbance and genital organ alterations particularly in pigs, due to capability of ZEA to bind the oestrogen receptors (Fink-Gremmels and Malekinejad, 2007).

It is worth noting that most studies on feed contamination describe the effects of individual mycotoxins, and do not consider the natural processes where multiple mycotoxins can be produced.

Abbreviations: AF, aflatoxins; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AFM_1, aflatoxin M_1; ZEA, zearalenone; BDP, *B. subtilis* biodegradation product; HPLC, high performance liquid chromatography; ND, not detected; Pooled SEM, standard error of the mean.

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http://dx.doi.org/10.1016/j.fct.2016.02.010
0278-6915/

The interactions among mycotoxins are complex and may cause different effects. Sometimes chronic exposure to a combination of toxins does not elicit important clinical signs like decreased body weight or hematology, but induced microscopic lesions and altered the immune response (Sypecka et al., 2004). Feeding laying hens diets simultaneous contaminated with AF, ochratoxins, T-2 toxins, fumonisin and deoxynivalenol have been reported to exert synergistic, additive or antagonistic effects in some cases (Hassan et al., 2012; Lee et al., 2012 and Raju and Devegowda, 2000) while information on the combined effects of AF and ZEA is lacking.

In order to degrade mycotoxins, extensive research has been conducted to counter mycotoxicosis by physical, chemical and biological approaches. However, most of these methods are impractical or potentially unsafe, due to losses in the nutritional value, high equipment costs, and formation of toxic residues or derivatives (CAST, 2007; Kabak et al., 2006 and Moshtaghian et al., 1991). The use of adsorbents has shown considerable promise in countering AF (Kumar et al., 2015). However, many of these agents lack a similar effect against other mycotoxins of practical importance (Edrington et al., 1997). Therefore, new specific, practical and effective methods are required. One of the most promising methods is biological detoxification using microorganisms or enzymatic preparations (Taylor and Draughon, 2001). Some species of microbes, such as *Rhodococcus erythropolis* (Alberts et al., 2006), *Armillariella tabescens* (Cao et al., 2011, Liu et al., 1998) and *myxococcus fulvus* (Zhao et al., 2011) have been reported to have different AF-degrading ability. *Rhizopus oryzae* (Varga et al., 2005), *Bacillus licheniformis* (Yi et al., 2011) and *Pseudomonas sp* (Altalhi and El-Deeb, 2009) have demonstrated their abilities to degrade ZEA. However, there is little research concerning elimination of both AF and ZEA by microbial biotransformation. Considering that, our lab has screened two strains of the probiotic bacteria *Bacillus subtilis*, which can be directly applied in the feedstuffs. It has been reported that two strains can degrade 81.5% AFB_1 and 85% ZEA in naturally contaminated feed *in vitro* (Gao et al., 2011, Lei et al., 2014). In addition, previous studies from our laboratory have found that *B. subtilis* had protective effects against aflatoxicosis in layers and broilers fed naturally AF-contaminated diets (Fan et al., 2013, 2015 and Ma et al., 2012) and also ameliorated ZEA toxicosis in pre-pubertal gilts when fed diets containing ZEA (Zhao et al., 2014).

The objective of the present study was to evaluate the adverse effects of AF and ZEA and their combination on laying performance, egg quality and toxins residues in egg of layers, and to determine the efficacy of a combination of the two *B. subtilis* strains in reducing or eliminating these effects. In addition, we investigated further the toxic effects of AF and ZEA on the above indices through a toxin-free period.

2. Materials and methods

2.1. Microbes and preparation

B. subtilis ANSB060 and *B. subtilis* ANSB01G were originally isolated from the fish gut (Gao et al., 2011) and broiler intestinal chyme (Lei et al., 2014), respectively. In this experiment, the biodegradation product was composed of 40% *B. subtilis* ANSB060 (1×10^9 colony forming unit (CFU)/g), 40% *B. subtilis* ANSB01G (1×10^9 CFU/g) and 20% carrier (rice husk meal) using industrial fermentation and dry-processing technologies, partially modified using the method of Schallmey et al. (2004). In addition, for the examination of CFU, plate count method was used according to the instruction of the ISO 4833: 2003 standard (2003).

2.2. Animals, diets and managements

After an adaptation period of two weeks, a total of 336 healthy Hy-Line Brown laying hens (BW = 1.43 kg, 18 weeks of age) with an average egg production of 4.17% and average egg weight of 39.85 g were randomly allocated to one of 7 dietary treatments with 8 replicates each. Each of the 8 replicates used 2 stainless steel suspended cages (40 × 37 × 35 cm, length × width × height) containing 3 hens each. A completely randomized experimental design was applied in a 1 + 3 × 2 factorial arrangement, namely a control diet (C); three treatments with mycotoxin addition: AF, ZEA, and AF + ZEA; with or without *B. subtilis* biodegradation product (BDP).

The experiment included two phases. The first phase was an intoxication period, from 18 to 23 weeks in which the birds were fed 7 different diets. The diets were: C (a basal diet containing 21% normal peanut meal and 57.7% normal corn meal); AF (the basal diet containing 21% moldy peanut meal and 57.7% normal corn meal); ZEA (the basal diet containing 21% normal peanut meal and 57.7% moldy corn meal); AF + ZEA (the basal diet containing 21% moldy peanut and 57.7% moldy corn meal); and AF1000, ZEA1000 and AF + ZEA1000 (supplementation of 1000 g/t of BDP in AF, ZEA and AF + ZEA diets, respectively). The second phase was a toxin-free period, from 24 to 29 weeks in which all birds were fed a toxin-free basal feed until the termination of experiment. The basal diets (Table 1) were formulated to contain adequate concentrations of all nutrients required for laying hens according to the National Research Council (NRC, 1994).

Throughout the study, feed and water were provided *ad libitum*. The birds were housed at average 18 °C under 16 h of lighting. The animal care protocol was approved by the Animal Welfare Committee of China Agricultural University. Laying performance such as egg production, average egg weight, feed intake and feed conversion ratio were measured on a replicate basis during the two stages of the study.

2.3. Determination of mycotoxins content

A total of 1099 samples of feed ingredients were collected across China. The contents of the AFB_1, AFB_2, AFG_1, AFG_2, ZEA, deoxynivalenol, and ochratoxin A in those samples were determined. The detection method for mycotoxins was according to Binder et al. (2007). The naturally moldy peanut meal mainly contaminated with AF from north of China and the naturally moldy corn mainly contaminated with ZEA from northwest of China were selected to collocate the toxin-treated diets. In the naturally moldy peanut meal, the contents of AFB_1, AFB_2, AFG_1, and AFG_2 were 418.6, 96.4, 43.5 and 9.7 μg/kg, respectively, and there were no other mycotoxins. The concentration of ZEA was 516.7 μg/kg and other mycotoxins were determined to be at concentrations below detection limits in the naturally moldy corn. In addition, the contents of AF, ZEA and other mycotoxins were below 0.5 ppb in the normal peanut meal, normal corn meal and other ingredients in the diet.

The seven diets were prepared according to the proportion of feed ingredients described in 2.2. Samples of all seven diets were collected to analyze the contents of mycotoxins. The concentrations of AFB_1, AFB_2, AFG_1 and AFG_2 in groups AF, AF + ZEA, AF1000 and AF + ZEA1000's diets were on average 89.5, 20.2, 9.6, and 3.7 μg/kg, respectively. In groups ZEA, AF + ZEA, ZEA1000 and AF + ZEA1000 the content of ZEA in diets was on average 260.2 μg/kg. The contents of AF, ZEA and other mycotoxins were below detection limits in the control group.

2.4. Determination of egg quality

In the last week of each stage (23 and 29 weeks old), 8 eggs from