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## Evaluation of *Bacillus* spp Candidates for Aflatoxin B1 Biodegradation in Broiler Chickens

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Evaluation of *Bacillus spp* Candidates for Aflatoxin B1 Biodegradation in Broiler Chickens

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science in Poultry Science

By

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University of Buenos Aires  
Bachelor in Veterinary Medicine, 1998

December 2015  
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This thesis is approved for recommendation to the Graduate Council.

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## ABSTRACT

Poultry health has been traditionally maintained by hygienic measures, vaccinations, and the use of antibiotics. Modern husbandry management considers the use of probiotics as a natural way to protect birds against many everyday pathogens. Different strains of *Bacillus spp.* have proved to have beneficial effects in poultry production. However, the most used bacterium in commercial probiotics is *Lactobacillus*, a vegetative cell. In contrast, *Bacillus spp.* are bacterial spores, highly resistant to harsh conditions, which makes them preferable, in some cases, to *Lactobacillus* because of shelf life and storage conditions. There is published information regarding mycotoxin detoxification by bacteria. Mycotoxins are a common threat for the poultry industry and different management strategies have been implemented to avoid their negative impact in the poultry industry. Additionally, not much information is provided on the effect of mycotoxin on intestinal inflammation. In chapter one, the ability of *Bacillus spp.* as direct-fed microbials (DFM) to biodegrade aflatoxin B1 (AFB1) by using an *in vitro* digestive model simulating *in vivo* conditions was evaluated. The experiment was performed with three groups: a) control feed; b) control feed contaminated with 0.01% AFB1; c) control feed contaminated with 0.01% AFB1 supplemented with  $10^9$  spores/g. *In vitro* digestion time was insufficient to confirm biodegradation of AFB1. In chapter two, two experiments were conducted in broilers to evaluate the effect of 3 concentrations of AFB1 (2, 1.5 or 1 ppm of AFB1) on gastrointestinal leakage and liver bacterial translocation (BT). Results from these experiments suggest that AFB1 does not increase gut leakage. In chapter three, three independent experiments were conducted to evaluate the biodegradation potential of previously selected *Bacillus spp.* provided as DFM in broiler chickens consuming feed containing different concentrations of AFB1: a) 2 ppm AFB1; b) 1.5 and 1 ppm AFB1; c) 500 ppb and 50 ppb AFB1. Even though the individual isolates

incorporated in the DFM showed some *in vitro* activity to biodegrade AFB1, when administered in the diets at 5 different concentrations of AFB1, no significant performance differences were observed when compared with their respective control diets.

**Key words:** Aflatoxin B1, *in vitro* digestion, DFM, biodegradation, intestinal inflammation

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## **I. INTRODUCTION**

Mycotoxins are secondary toxic metabolites produced by molds that can be responsible of a toxic condition (mycotoxicosis) when ingested by humans or animals (Binder, 2007). Mycotoxins surveys from period 2004 - 2013 reveal an increase in the contamination percentage of feed components. This rise in the detection of mycotoxins is probably the consequence of the development of more sensitive and user-friendly methods. According to Murugesan et al., (2015), in 2013 there was an increase of 5% in the detection of mycotoxins compared with the average detection from last decade. Reports indicate that an average of 76% of samples from grain and feed tested positive for at least 1 mycotoxin (Murugesan et al., 2015). Moreover, reports indicate that 25% of the world's crop is contaminated by mold or fungal growth (Bryden, 2007). Mycotoxins have a global distribution due to the commercialization as global commodities (Murugesan et al., 2015).

Fungal contamination of crops is the main source of mycotoxin contamination. Once this contamination is produced, it can continue during harvest and storage and thus lead to mycotoxin production. Many factors can influence the growth of fungus. Environmental conditions such as drought, insect activity, humidity, temperature and others are very important factors that can produce plant stress and consequently influence the possibility of pre-harvest parasitism of crop leading to contamination. Likelihood of pre- or post-harvest production of mycotoxin is related to the type of fungus infecting the plant. Aflatoxin is largely known as a post-harvest mycotoxin and it is especially important in maize crops (Wagacha and Muthomi, 2008).

Many management strategies can be followed to diminish fungus proliferation and mycotoxin formation. These strategies take into account all the steps where contamination with mycotoxins takes place. They start with good agricultural practices that include appropriate

harvesting time, control of insects, appropriate drying, storage, and other factors (Lopez-Garcia et al., 1999; Wagacha and Muthomi, 2008; Fink-Grenmels, 1999). However, no technique or strategy can completely avoid mycotoxin formation (Lopez-Garcia et al., 1999).

Detection of mycotoxins is crucial to avoid human consumption or animal feeding of these toxins. Detection is usually performed with the use of chromatographic or immunochemical methods. More modern methods may detect more than one mycotoxin at a time and are more sensitive. However, sampling method may play an important role in mycotoxin detection. Contamination by these toxins can be very heterogeneous. Additionally, mycotoxins are odorless and non visible substances and can be concentrated in “hot spots”, making sampling a great challenge in mycotoxin detection. Moreover, samples are never reported as negative. Many countries have regulations and well-established maximum limits according to the type of toxin and intended consumer of commodities. These limits vary by country. (Binder, 2007; Lopez-Garcia et al., 1999; Murugesan et al., 2015).

Some countries accept dilution of positive samples or transfer of intended use to a less susceptible specie. When these options are not available, many different treatments can be performed to potentially reduce mycotoxin levels in feedstuffs (Lopez-Garcia et al., 1999; Binder, 2007).

This thesis includes three chapters. The first one contains information about the isolation, screening and identification of three strains of *Bacillus spp* candidates as DFM and their performance detoxifying AFB1 in an *in vitro* digestion model. Next, the second chapter shows the effects of aflatoxicosis in 21-day-old chickens suggesting that no gut leakage is produced by this substance when administered at up to 2 ppm in feed. However AFB1 caused an important negative impact in productive parameters, encouraging further research of a viable solution to

this problem. Chapter three concludes with a fusion of chapter one and three showing the results of *in vivo* administration of the selected DFM to day of hatch broilers until the last day of the three independent experiments at 21 day of age.

## **II. LITERATURE REVIEW**

### **A. MYCOTOXINS**

It was not until 1961 with the death of a large population of turkeys in England that the modern concept of mycotoxicology took place (Richard, 2007; Blount, 1961; Cole, 1986). However, that was not a new disease as there is evidence of mycotoxicosis in many episodes of human history. For example, The Salem witchcraft trials in Salem, Massachusetts may describe the effects of ergot alkaloid intoxication (Richard, 2007). Mycotoxins are chemical substances with toxic effects that are produced as a secondary metabolic product of molds. More than 300 mycotoxins are known to date but only a few are known to pose a major risk for animal and human health (Binder, 2007; Fink-Grenmels, 1999; Carvajal and Arroyo, 1997). These molds can grow in feed and food leading to its contamination and some estimations report up to 25% of the global crop production to be contaminated with mycotoxin (Fink-Grenmels, 1999; Wagacha and Muthomi, 2008). There is a geographic distribution related to different types of mycotoxins. This pattern is due to environmental conditions that will positively or negatively affect mold growth. However, mycotoxicosis occurrence has a worldwide impact because contaminated feed and food can reach any country with trade of these commodities (Fink-Grenmels, 1999; Pitt, 2000). Environmental conditions, such as humidity and temperature, have a primary role in modulating toxin production. Droughts or excess of water, insect damage or other situations that produce plants stress increase susceptibility of plants to mold invasion, thus determining mold growth and toxin production rate (Fink-Grenmels, 1999) . Among the identified mycotoxins, aflatoxins, trichothecenes, zearalenone, ochratoxins, fumonisins and ergot alkaloids are considered of major importance due to their potential to harm animals and humans (Richard,

2007; Binder, 2007) . However, toxicity of each mycotoxin depends on species susceptibility together with individual factors such as sex, age and general nutrition conditions (Binder, 2007) .

## **B. AFLATOXIN B1**

Aflatoxins are highly mutagenic and carcinogenic substances known. According to (Binder, 2007), they are classified by the International Agency of Research on Cancer (IARC) as a Class 1 human carcinogen. They are mostly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, contaminating many commodities before or after harvest. They are usually found in corn, peanuts and nuts. Aflatoxins are chemically known as difurocoumarin derivatives and are physically classified as B1, B2, G1 and G2. This classification is associated to their fluorescent color emission after being illuminated with a long wave ultraviolet light. Letter B refers to blue emission and G to green coloration (Agag, 2004). Among these the most important is Aflatoxin B1 because it is considered the most hepatotoxic and carcinogenic. However, the degree of toxicity varies according to animal species, sex, age, duration and dose consumed. Aflatoxins can also be found in milk and meat as a residue of their metabolites, for example, aflatoxin M1 is a hydroxylated metabolite that can be present in milk of mammals which consumed aflatoxins (Binder, 2007; Hussain et al., 2010; Fink-Grenmels, 1999; Andrade et al., 2013).

The poultry industry is concerned about mycotoxins due to economic importance. Aflatoxin ingestion can affect productivity both in broiler and layer chickens with a reduction in weight gain, decreased feed efficiency and also reducing egg quality and productivity. Another important effect is immunosuppression (Murugesan et al., 2015).

## **C. CONTROL METHODS**

To avoid mold growth and thus mycotoxins production is almost impossible. As mentioned above, mycotoxins can be produced not only during crop growth, but also after

harvest, during storage or transportation. Nevertheless, many improvements on these feed and food processes have been achieved to reduce contamination (Fink-Grenmels, 1999; Kumar et al., 2008). To ameliorate aflatoxin B1 effects, different ways of detoxification are commonly practiced in poultry production. These can be classified as physical, chemical or microbiological (Binder, 2007) .

### **Physical methods**

There are some physical methods that can help in the reduction of aflatoxin content in feed. These can be achieved by either removing or inactivating mycotoxins. Moldy grains can be visibly identified and damaged kernels are more susceptible to aflatoxin contamination. In the first case, removal of damaged kernels, hand picking, color separation, along with density segregation are all methods that will not modify the product. Thermal treatment can be adequate for some commodities for which the final product requires a thermal process; such as roasting for peanuts or coffee. However, many mycotoxins can be chemically stable and will not be affected by high temperatures (Lopez-Garcia et al., 1999). A widely used method is the incorporation of clay-based materials. The use of clay as toxin binders has been used for centuries. Certain clays adsorb the toxin in the gastrointestinal tract avoiding absorption and blood distribution (Murugesan et al., 2015; Lopez-Garcia et al., 1999). There are other possible, but less common physical methods such as irradiation (Lopez-Garcia et al., 1999).

### **Chemical methods**

A very effective and safe chemical method used for decontamination of aflatoxin is ammoniation. In some cases, it has been used for more than 20 years, and according to Lopez-Garcia et al., (1999), this method proved to have an effectiveness of more than 99 percent. It was used in many countries including the United States, Mexico and Brazil. Ammoniation is

successfully used for whole or ground maize, peanuts, and cottonseed and meal with two different processes which combine different temperatures and pressures. Ammoniation is a method that can be applied either in a feed mill or in a farm (Richard, 2007; Lopez-Garcia et al., 1999). Some other chemical treatments include the use of monomethylamine. A method called nixtamalization is an alkaline treatment that can produce some toxicity and is not very effective. Other substances recently studied with successful results are hydrogen peroxide and sodium bicarbonate (Lopez-Garcia et al., 1999).

### **Biological methods**

Limitations on physical and chemical methods encouraged researchers to investigate biological methods as an alternative to detoxify not only aflatoxins, but mycotoxins in general. These limitations can be cost effective or can cause detrimental losses in nutritional or organoleptic properties of the grains (Farzaneh et al., 2012; Lopez-Garcia et al., 1999).

Another approach is attempting to reduce the response of animals to mycotoxin ingestion. Those methods that are used as an organ protector are defined as “bio-protectors”. Hepatoprotective drugs are the most common examples and these are usually based on vegetable products (Murugesan et al., 2015). With the use of probiotics, researchers now are more focused on “biodetoxification” methods. Incorporation of microorganisms or purified enzymes into the feed allows a biochemical transformation of the toxin to a non-toxic or less toxic metabolite (Murugesan et al., 2015). Research attempts with this approach were documented as early as 1966, with some promising results (Ciegler et al., 1966; Murugesan et al., 2015). Farzaneh et al., reported a biodetoxification of 85% in nutrient broth culture and 95% in pistachio nuts by a strain of *B. subtilis* (Farzaneh et al., 2012).

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### III. CHAPTER I

#### A. Isolation, screening and identification of *Bacillus* spp. as direct-fed microbial candidates for aflatoxin B1 biodegradation

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## **B. ABSTRACT**

### **Objectives**

To evaluate the ability of *Bacillus* spp. as direct-fed microbials (DFM) to biodegrade aflatoxin B1 (AFB<sub>1</sub>) by using an *in vitro* digestive model simulating *in vivo* conditions.

### **Methods**

Sixty-nine *Bacillus* isolates were obtained from intestines, and soil samples were screened by using a selective media method against 0.25 and 1.0 µg/mL of AFB<sub>1</sub> in modified Czapek-Dox medium. Plates were incubated at 37°C and observed every two days for two weeks. Physiological properties of the three *Bacillus* spp. candidates were characterized biochemically and by 16S rRNA sequence analysis for identification. Tolerance to acidic pH, osmotic concentrations of NaCl, bile salts were tested, and antimicrobial sensitivity profiles were also determined. *Bacillus* candidates were individually sporulated by using a solid fermentation method and combined. Spores were incorporated into 1 of 3 experimental feed groups: 1) Negative control group, with unmedicated starter broiler feed without AFB<sub>1</sub>; 2) Positive control group, with negative control feed contaminated with 0.01 % AFB<sub>1</sub>; 3) DFM treated group, with positive control feed supplemented with 10<sup>9</sup> spores/g. After digestion time (3:15 h), supernatants and digesta were collected for high-performance liquid chromatography fluorescence detection analysis by triplicate.

### **Results**

Three out of those sixty-nine DFM candidates showed ability to biodegrade AFB<sub>1</sub> *in vitro* based on growth as well as reduction of fluorescence and area of clearance around each colony in modified Czapek-Dox medium which was clearly visible under day light after 48 h of evaluation. Analysis of 16S-DNA identified the strains as *B. amyloliquefaciens*, *B. megaterium*

and *B. subtilis*. The three *Bacillus* strains were tolerant to acidic conditions (pH 2.0), tolerant to a high osmotic pressure (NaCl at 6.5%), and were able to tolerate 0.037% bile salts after 24h of incubation. No significant differences ( $P>0.05$ ) were observed in the concentrations of AFB1 in neither the supernatants nor digesta samples evaluated by high-performance liquid chromatography with fluorescence detection between positive control or DFM treated groups.

### **Conclusions**

*In vitro* digestion time was not enough to confirm biodegradation of AFB1. Further studies to evaluate the possible biodegradation effects of the *Bacillus*-DFM, when continuously administered in experimentally contaminated feed with AFB1, are in progress.

**Keywords:** Aflatoxin B1, *Bacillus*, direct-fed microbials, biodegradation, broiler feed

## C. INTRODUCTION

Aflatoxins are naturally occurring mycotoxins that are produced by some strains of *Aspergillus* species which are commonly found in cereals worldwide and bring significant threats to the food industry and animal production (Smith et al., 1976). At least 14 different types of aflatoxin are produced in nature (Abramson et al., 1997; Greco et al., 2014). Aflatoxin B1 (AFB1) is considered the most toxic and is produced by both *Aspergillus flavus* and *Aspergillus parasiticus* (Yunus et al., 2011). Several physical and chemical methods have been developed to reduce aflatoxins (Abramson et al., 1997; Greco et al., 2014). Unfortunately, these methods have restrictions in terms of product nutrition, organoleptic qualities, and adverse health effects, which motivate emphasis on biological methods of degradation of aflatoxins (Ciegler et al., 1966; Farzaneh et al., 2012; Khan and Zahoor, 2014). *Bacillus* spp. are probiotics accepted by human or animals as direct fed microbials (DFM). Our laboratory has showed the safety and efficacy of individual monocultures for prophylactic and/or therapeutic efficacy against *Salmonella* infections under both laboratory and field conditions as well as the development of a novel, cost-effective DFM with potential for widespread utilization and improved production, delivery and clinical efficacy for poultry (Wolfenden et al., 2010; Wolfenden et al., 2011; Shivaramaiah et al., 2011; Tellez et al., 2012; Menconi et al., 2013; Latorre et al., 2014a; Latorre et al., 2014b).

The aim of this study was to screen *Bacillus* candidates capable of biotransforming AFB1. Hence, the DFM candidates could not only be used as probiotics but also as an antidote for aflatoxins.

## D. MATERIAL AND METHODS

### **Isolation and characterization of *Bacillus* spp.**

Previous research conducted in our laboratory focused on isolation of several *Bacillus* spp. from environmental and poultry sources (Wolfenden et al., 2010; Wolfenden et al., 2011; Shivaramaiah et al., 2011; Menconi et al., 2013). Identification was carried out using a bioMerieux API 50 CHB (catalog no. 50430, Biomerieux, Durham, NC) test kit. General recognized as safe (GRAS) was affirmed as described by Wolfenden *et al.* (Wolfenden et al., 2010). For our preliminary experiment, sixty-nine isolates were chosen based on consistent *in vitro* anti- *Salmonella* spp., *Clostridium* spp., and *Campylobacter* spp. activity (Data not shown).

### **In vitro evaluation of biodegradation of AFB1**

A modified Czapek-Dox medium having the following composition per liter proved satisfactory: sucrose, 3.000%; NaNO<sub>3</sub>, 0.300%; K<sub>2</sub>HPO<sub>4</sub>, 0.100%; MgSO<sub>4</sub>, 0.050%; KCl, 0.050%; FeSO<sub>4</sub>, 0.001%; yeast extract (Difco, BD, Becton, Dickinson and Company; Sparks, MD21152 USA; 38800 Le Pont de Clair, France), 0.005%; agar, 2.0% (Ciegler et al., 1966). To evaluate AFB1 (SigmaAldrich, Oakville, ON) inhibition, standard solutions were diluted in chloroform and added to the medium to reach a final concentration of 1 µg/mL of medium, and while it was still hot, the chloroform was drive off. About 30 mL of the medium was added to each Petri dish and allowed to solidify. Sixty nine GRAS isolates were grown in tryptic soy broth (TSB) (catalog no. 211822, Becton Dickinson, Sparks, MD) for 24 h at 37°C and then washed 3 times in 0.9% sterile saline by centrifugation (3900 r/min, 4°C, 15 min). About 10 µl of each isolate was placed on the center of the Petri dish plate with modified Czapek-Dox medium. After point inoculation, the plates were incubated at 37°C and examined at intervals of 1 to 2 days for up to 2 weeks under ultraviolet light (UV) for AFB1 utilization. On initial examination, plates had to be exposed to UV for about 15 min to develop fluorescence. Utilization of toxin was indicated by a zone of non-fluorescence in the colony.

### **Identification of candidate isolates**

Out of the 69 GRAS isolates, three showed capacity to biodegrade AFB1 (data not showed). Those isolates were further identified by 16S rRNA sequence analysis (Microbial ID Inc., Newark, DE 19713, USA). Then, the candidate *Bacillus* strains were chosen for physiological tests as described by Menconi et al, and sporulated (Menconi et al., 2013) . The biological detoxification of AFB1 was determined in an *in vitro* digestion model as described below.

### **Bile salts tolerance**

The method of Gilliland et al., with some modifications, was used to determine bile salt tolerance (Gilliland et al., 1984). TSB containing 0 %, 0.037 %, 0.075 %, 0.150 %, and 0.300 % of bile salts No. 3 (Catalog No. 213010, Becton Dickinson and Co., Sparks, MD 21152 USA) was inoculated with  $10^7$  CFU/mL of each potential probiotic strain, after being centrifuged at 5000 r/min for 15 min and washed three times from their 24 h growth cultures. Samples were incubated for 24 h at 37°C with shaking at 100 r/min. Growth in control (no bile salts) and test cultures was evaluated at 2, 4, and 24 h by streaking samples on trypticase soy agar (TSA) (catalog No. 211822, Becton Dickinson, Sparks, MD) for presence or absence of growth.

### **Resistance in conditions of the intestinal tract evaluation: pH, temperature, and NaCl**

A basal TSB medium was used in these series of *in vitro* studies. A 24 h culture of each isolate was used as the inoculum whereby the cells were spun down and re-suspended in 0.9% sterile saline. Then, 100  $\mu$ L of the suspension was inoculated into 10 mL of TSB of each test tube. Two incubation time points, *i.e.* 2 and 4h, were evaluated for each of the variables (pH, temperature, and NaCl). The rationale for these two points was mainly based on the transit time of food matter in the gastrointestinal tract of poultry. The temperatures tested were 15 and 45°C.

The concentrations of NaCl tested were 3.5% and 6.5% (w/v). The isolates were tested for growth at pH 2 and 3. The tubes were incubated with reciprocal shaking, at the specific test temperatures or 37°C for the tests on pH and concentrations of NaCl. At the time points evaluated, each sample was streaked on TSA for presence or absence of growth, to confirm livability of the strains. The turbidity of each tube was also noted as an indication of growth or no-growth. Each treatment was tested with triplicate tubes.

### **Antibiotic resistance**

Selected colonies on TSA plates were inoculated and cultured for 24 h in TSB at 37°C. Strains were then sent to a Veterinary Diagnostic Laboratory (University of Arkansas, Division of Agriculture, Fayetteville, AR, 72703, USA) for antibiotic sensitivity analysis by using Kirby-Bauer methodology. The diameter of the inhibition zones and the interpretative zone sizes were reported. Twelve antibiotics were tested, and their concentrations were reported as shown on Table 1. The results were expressed in terms of resistant, and susceptible.

### **Sporulation procedure**

In an effort to grow high numbers of viable spores, a solid-state fermentation (SSF) media developed by Zhao *et al.*, was selected and modified for use in these experiments (Zhao *et al.*, 2008). Briefly, a liquid media component was added to a mixture of 70% rice straw and 30% wheat bran at a rate of 40% by weight. The SSF media was added to a 250 mL Erlenmeyer flask and sterilized by autoclaving for 30 min at 121°C. Candidate isolates were grown individually overnight at 37°C in TSB, then 2 mL of a candidate culture were added to the prepared SSF media. The inoculated flasks were incubated for 24 h at 37°C then incubated for another 72 h at 30°C. The cultures were removed from their flasks, placed onto Petri dishes, and then dried at 60°C. Following this, the cultures were aseptically ground into a fine powder to generate stable

spores ( $\sim 10^{11}$  spores/g). Spores were mixed into the feed using a rotary mixer for 15 minutes. Samples of feed containing the DFM candidates culture were taken and a 1:10 dilution was made with saline. All samples were subject to 100°C for 10 minutes. These samples of ten-fold dilutions were plate on TSA and incubated at 37°C for 24 h to count the number of spores per g of feed.

### **Determination of biological detoxification of AFB1 in an *in vitro* digestion model**

Freshly prepared, unmedicated corn-soy based starter feed was used for all *in vitro* trials. DFM candidates were incorporated into 1 of 3 experimental feed groups: 1) Negative control group, with unmedicated starter broiler feed without AFB1; 2) Positive control group, with negative control feed contaminated with 0.01 % AFB1; 3) DFM treated group, with positive control feed supplemented with  $10^9$  spores/g. *In vitro* digestion of the three diets with or without DFM supplementation was performed by triplicate according to previously published methods, with minor modifications (Bedford and Classen, 1993). All *in vitro* digestion steps were carried out at 40°C to simulate avian body temperature, by using a water-jacketed incubator (Forma Scientific Inc., Marietta, OH, USA) customized with bars that rotated the tubes horizontally at 19 r/min. To mimic crop digestion, 50 g each diet and 100 mL of 0.03 mol/L HCl were placed in 50 mL polypropylene centrifuge tubes and mixed vigorously. The pH was measured (range from 5.19 to 5.22) and the tubes were incubated for 30 min. Next, to mimic proventricular digestion, 150000 IU pepsin (Sigma-Aldrich Canada Ltd Oakville, Ont., Canada) and 25 mL of 1.5 mol/L HCl were added to each of the tubes. Values of pH were measured (range from 1.37 to 1.96) and the mixtures were then incubated for a further 45 minutes. Following this, 341.5 mg of 8'pancreatin (Sigma-Aldrich Canada Ltd) was added in 32.5 mL of 1.0 mol/L NaHCO<sub>3</sub>, and the pH was adjusted to between 6.3 and 6.7 with 1.0 mol/L NaHCO<sub>3</sub>. Volumes were equalized in the

tubes by adding distilled water, and the samples were incubated for a further 2h. After removal of solids and awns, the samples were first centrifuged at 4100 r/min for 5 min. After digestion time (3:15h), supernatants and digesta (by triplicate) were collected for AFB1 analysis by high-performance liquid chromatography with fluorescence detection (HPLC-FLD) method by using a Romer Derivatization Unit (Romer Labs, Inc., MO 63084-1156 USA).

### **Statistical Analysis**

Data of the determination of biological detoxification of AFB1 by HPLC-FLD of the DFM candidates in an *in vitro* digestion model were subjected to ANOVA as a completely randomized design by using the GLM procedure of SAS/STAT<sup>®</sup> 9.2. Data were expressed as mean  $\pm$  SE. Significant differences among the means were determined by using Duncan's multiple-range test at  $P < 0.05$ .

### **E. RESULTS**

Table 2 shows the identification of *Bacillus* spp. isolates by bioMerieux API 50 CHB and 16S rRNA sequence analysis. The three isolates were characterized as *Bacillus subtilis* (*B. subtilis*)/*Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) by the bioMerieux API identification kit. However, further sequence analysis of 16S rRNA, which is the predominant molecular technology currently available for microbial identification revealed *B. amyloliquefaciens* for candidate 1, *Bacillus megaterium* (*B. megaterium*) for candidate 2, and *B. subtilis* for candidate 3 (Table 2).

The results of the bile salt tolerance of the *Bacillus* spp. isolates after 2, 4, and 24 hours of incubation are summarized in Table 3. All the three DFM candidates were able to grow when cultured at 0.037% bile salts concentration at 2h, 4h, and 24h of incubation. The results of the effect of pH, temperature, and NaCl on the three DFM candidates are summarized in Table 4.

Vegetative cells were evaluated for conditions similar to those found in the stomach. All three candidates were able to survive at pH 2 and pH 3 for 2h. Furthermore, vegetative cells grew at 15°C and 45°C at both times of incubation of 2h and 4h and were also able to tolerate up to 6.5 % of NaCl (Table 4).

The antibiotic resistance and susceptibility of the DFM candidates to twelve antibiotics are summarized in Table 1. All three DFM candidates were sensitive to gentamycin, neomycin, penicillin, ormethoprim, tetracycline, triple sulfa, and spectinomycin, and resistant to bacitracin, erythromycin, clindamycin, ceftiofur and, novobiocin (Table 1).

Table 5 summarizes the determination of biological detoxification of AFB1 by HPLC-FLD of the DFM candidates in an *in vitro* digestion model. In the present study, no significant differences ( $P>0.05$ ) were observed in the concentrations of AFB1 in neither the supernatants nor digesta samples evaluated by HPLC-FLD between positive control or DFM treated groups.

## **F. DISCUSSION**

Antibiotics as growth promoters in livestock have been in practice for over five decades. However, rising socio-political concerns with their use has prompted a quest for alternative methods of disease intervention and optimization of growth promotion in commercial poultry farming. The use of DFM as an alternative approach has gained momentum in recent years (Wolfenden et al., 2010; Wolfenden et al., 2011; Shivaramaiah et al., 2011; Tellez et al., 2012; Menconi et al., 2013; Latorre et al., 2014a; Latorre et al., 2014b). The advantages of application, pathogen reduction, immunomodulation, performance enhancement and synthesis of antimicrobials and enzymes have given probiotics and DFM a clear edge over antibiotics making their use highly sustainable years (Wolfenden et al., 2010; Wolfenden et al., 2011; Shivaramaiah et al., 2011; Tellez et al., 2012; Menconi et al., 2013; Latorre et al., 2014a; Latorre et al., 2014b).

Conversely, biological degradation of aflatoxins occurs in nature since aflatoxins are chemically stable but do not appear to accumulate in natural environments (Ciegler et al., 1966). Several investigators have demonstrated that microorganisms in the environment can be chosen as sources for biological degradation of aflatoxins (Farzaneh et al., 2012; Gao et al., 2011) .

Earlier research conducted in our laboratory focused on isolation of sixty-nine GRAS *Bacillus* spp. isolates with consistent *in vitro* anti- *Salmonella* spp., *Clostridium* spp., and *Campylobacter* spp. activity years (Wolfenden et al., 2010; Wolfenden et al., 2011; Shivaramaiah et al., 2011; Tellez et al., 2012; Menconi et al., 2013; Latorre et al., 2014a; Latorre et al., 2014b). In the present study, three out of those sixty-nine DFM candidates previously evaluated, in addition showed ability to biodegrade AFB1 *in vitro*, based on growth as well as reduction of fluorescence and area of clearance around each colony (data not shown). Analysis of 16S DNA identified the strains as *B. amyloliquefaciens*, *B. megaterium* and *B. subtilis*; all three were considered GRAS organisms. Furthermore, their physiological properties, tolerance to acidic conditions and high osmotic pressure and relative tolerance to bile salts make them suitable candidates as DFM. In the present study, *in vitro* digestion time was not enough to confirm biodegradation of AFB1. Further studies to evaluate the possible biodegradation effects of the *Bacillus*-DFM, when continuously administered in broiler chickens feed contaminated with AFB1, are in progress.

#### **Conflict of interest statement**

We declare that we have no conflict of interest.

#### **Funding support information**

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**Table 1** Antibiotic sensitivity test results for *Bacillus* spp. isolates.

<b>Antibiotics</b>	<b>Concentration</b>	<b>Candidate 1</b>	<b>Candidate 2</b>	<b>Candidate 3</b>
Bacitracin	10 IUI/IE/U	R	R	R
Erythromycin	15.00 µg	R	R	R
Gentamycin	10.00 µg	S	S	S
Clindamycin	2.00 µg	R	R	R
Ceftiofur	30.00 µg	R	R	R
Neomycin	30.00 µg	S	S	S
Novobiocin	5.00 µg	R	R	R
Penicillin	10 IUI/IE/U	S	S	S
Ormethoprim	1.25 µg	S	S	S
Tetracycline	30.00 µg	S	S	S
Triple sulfa	1.00 mg	S	S	S
Spectinomycin	100.00 µg	S	S	S

R = resistant; S = susceptible.

**Table 2** Identification of *Bacillus* spp. isolates by bioMerieux API 50 CHB and 16S rRNA sequence analysis

<i>Bacillus</i> isolates	API 50 CHB identification (%)	16 S identification (%)
<b>Candidate 1</b>	<i>B. subtilis/amyloliquefaciens</i> (98.2)	<i>B. amyloliquefaciens</i> (96)
<b>Candidate 2</b>	<i>B. subtilis/amyloliquefaciens</i> (96.6)	<i>B. megaterium</i> (99.57)
<b>Candidate 3</b>	<i>B. subtilis/amyloliquefaciens</i> (99.7)	<i>B. subtilis</i> (99.52)

**Table 3** Bile salt tolerance of *Bacillus* spp. isolates after 2, 4, and 24 h of incubation in TBS medium.

<i>Bacillus</i> isolates	0.000%			0.037%			0.075%			0.150%			0.300%		
	2h	4h	24h												
<b>Candidate 1</b>	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
<b>Candidate 2</b>	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

Symbols: +, tolerant; -, non- tolerant.

**Table 4** Effect of pH, temperature, and NaCl on the *Bacillus* spp. isolates.

<i>Bacillus</i> isolates	pH of 2		pH of 3		15°C		45°C		3.5 % NaCl		6.5 % NaCl	
	2h	4h	2h	4h	2h	4h	2h	4h	2h	4h	2h	4h
<b>Candidate 1</b>	+	-	+	-	+	+	+	+	+	+	+	+
<b>Candidate 2</b>	+	-	+	-	+	+	+	+	+	+	+	+
<b>Candidate 3</b>	+	-	+	-	+	+	+	+	+	+	+	+

Symbols: +, tolerant; -, non- tolerant.

**Table 5** Determination of biological detoxification of AFB1 by HPLC-FLD of DFM candidates in an *in vitro* digestion model.

<b>Groups</b>	<b>AFB1 in feed before digestion (ppb)</b>	<b>AFB1 in solid feed after <i>in vitro</i> digestion (ppb)</b>	<b>AFB1 in supernatant after <i>in vitro</i> digestion (ppb)</b>
<b>Negative control</b>	< 1.1	< 1.1	< 1.1
<b>Positive control</b>	750.9	352.60 ± 22.85	40.60 ± 3.49
<b>DFM treated</b>	757.6	349.97 ± 11.52	37.23 ± 2.94

DFM candidates were incorporated into 1 of 3 experimental feed groups: 1) Negative control group, with unmedicated starter broiler feed without AFB1; 2) Positive control group, with negative control feed contaminated with 0.01 % AFB1; 3) DFM treated group, with positive control feed supplemented with 10<sup>9</sup> spores/g.

27 Data expressed as mean ± SE (*P*>0.05).

#### **IV. CHAPTER II**

##### **A. Leaky gut and mycotoxins: Aflatoxin B1 does not increase gut permeability in broiler chickens**

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## B. ABSTRACT

Previous studies conducted in our laboratory have demonstrated that intestinal barrier function can be adversely affected by diet ingredients or feed restriction, resulting in increased intestinal inflammation-associated permeability. Two experiments were conducted in broilers to evaluate the effect of 3 concentrations of Aflatoxin B1 (AFB1; 2, 1.5 or 1 ppm) on gastrointestinal leakage and liver bacterial translocation (BT). In Exp 1, 240 day-of-hatch male broilers were allocated in two groups, each group had six replicates of 20 chickens (n = 120/group): Control feed or feed + 2 ppm AFB1. In Exp 2, 240 day-of-hatch male broilers were allocated in three groups, each group had 5 replicates of 16 chickens (n = 80/group): Control feed; feed + 1 ppm AFB1; or feed + 1.5 ppm AFB1. In both experiments, chickens were fed starter (d1-d7) and grower diets (d8-d21) *ad libitum* and performance parameters were evaluated every week. At day 21, all chicks received an oral gavage dose of FITC-d (4.16 mg/kg) 2.5h before collecting blood samples to evaluate gastrointestinal leakage of FITC-d. In Exp 2 a hematologic analysis was also performed. Liver sections were aseptically collected and cultured using TSA plates to determine BT. Cecal contents were collected to determine total cfu/g of Gram-negative bacteria; lactic acid bacteria (LAB) or anaerobes by plating on selective media. In Exp 2, liver, spleen and bursa of Fabricius were removed to determine organ weight ratio, and also intestinal samples were obtained for morphometric analysis. Performance parameters, organ weight ratio and morphometric measurements were significantly different between control and AFB1 groups in both experiments. Gut leakage of FITC-d was not affected by the three concentrations of AFB1 evaluated ( $P > 0.05$ ). Interestingly, a significant reduction in BT was observed in chickens that received 2 and 1 ppm AFB1. An increase ( $P < 0.05$ ) in total aerobic bacteria, total Gram negatives, and total LAB were observed in chickens fed 2 and 1.5 ppm of

AFB1 when compared with control and 1 ppm chickens. The integrity of gut epithelial barrier was not compromised after exposure to the mycotoxin.

**Key words:** aflatoxin B1, bacterial translocation, broilers, gut leakage, performance

## C. INTRODUCTION

In the winter of 1959, the British cargo ship Rosetti, unloaded a shipment of peanut meal from Brazil to England, which was utilized as a protein supplement in the diets of poultry and other domestic animals. By the summer of 1960, an outbreak of an unknown disease killed several species of poultry including turkeys, ducklings and pheasants. In all, 500 cases were reported involving the deaths of more than 100,000 turkeys. This was the first report of Turkey “X” Disease (Blount, 1961; Siller and Ostler, 1961). Exhaustive research led to the discovery of aflatoxins, secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, as the etiological agents and the development of mycotoxicology (Nesbitt *et al.*, 1962; Spensley, 1963; Cole, 1986). More recent studies demonstrated that aflatoxins are potent carcinogenic compounds (McLean and Dutton, 1995; Fox *et al.*, 2010; Rawal *et al.*, 2010; Rawal and Coulombe, 2011; Yunus *et al.*, 2011b; Zhang *et al.*, 2014). About 14 different types of aflatoxins are produced in nature (Ledoux *et al.*, 1999; Yunus *et al.*, 2011b), but aflatoxin B1 (AFB1) produced by *Aspergillus flavus* and *Aspergillus parasiticus* is considered the most toxic (Andrade *et al.*, 2013; Greco *et al.*, 2014). In spite of 55 years of continuous research on aflatoxins, several areas of aflatoxicosis remain yet to be investigated. It is particularly interesting that studies on poultry aflatoxicosis have not kept pace with the research in mammals, and there still exists an incomplete description of aflatoxicosis in avian species, especially when searching for scientific publications related to the effect(s) of aflatoxins on the gastrointestinal tract (GIT).

The GIT is the first organ coming into contact with mycotoxins from the diet and should be expected to be affected by AFB1 with greater potency as compared to other organs. Nevertheless, literature regarding the effects of AFB1 on the GIT is particularly confusing. Few

researchers have looked at morphometric changes following dietary administration of aflatoxins in chickens, turkeys, and ducks, but results from those studies contradict each other, particularly when looking at villi high and villi to crypt ratio (Warren and Hamilton, 1980; Xu *et al.*, 2003; Diaz *et al.*, 2008; Applegate *et al.*, 2009; Yunus *et al.*, 2011a,b; Smith *et al.*, 2012; Zhang *et al.*, 2014). Similarly, contradictory results arise from the effects of AFB1 on digestibility of amino acids, energy utilization and absorption of macronutrients (Ruff and Wyatt, 1976; Fan *et al.*, 1997; Nelson *et al.*, 1982; Verma *et al.*, 2002, 2007; Kermanshahi *et al.*, 2007; Applegate *et al.*, 2009; Yunus *et al.*, 2010; Smith *et al.*, 2012).

Aflatoxins are absorbed very quickly into the blood from the GIT, followed by an extensive transformation into metabolites primarily in the liver (Ramos and Hernandez, 1996) (Ortatatli and Oğuz, 2001; Rawal and Coulombe, 2011). Contrary to the studies on mucosal damage and nutrient absorption caused by AFB1, there is an universal agreement that beside the carcinogenic and hepatotoxic effects on the liver, dietary aflatoxins reduce weight gain, feed intake, increase feed conversion ratio and are immunosuppressive (Huff *et al.*, 1986; Kubena *et al.*, 1993; Ledoux *et al.*, 1999).

Today, only a few reports can be found in databanks in which the issue of barrier function and intestinal permeability has been reported. From recent studies by Yunus *et al.* (2011a) in broilers, it was suggested that the absorptive surface of the small intestine declines during a chronic exposure to low levels of AFB1. However, in that study, broilers compensated for the reduced absorptive surface by increasing the length of the small intestine (Yunus *et al.*, 2011a). In a second study, transepithelial electrical resistance (TEER), used as an important indicator of barrier function of intestinal epithelial cells (IEC), showed that AFB1 was only moderately affected during acute exposure to the toxin (Yunus *et al.*, 2011b). To our knowledge,

the only study of the effect of AFB1 on possible damage to tight junctions (TJ) was performed by Caloni *et al.*, (2012) who demonstrated that AFB1 does not affect the integrity of tight junction proteins or barrier damage *in vitro*.

We have previously shown that intestinal barrier function can be adversely affected by poorly digested diets, feed restriction, or dexamethasone resulting in increased intestinal inflammation-associated permeability in poultry (Tellez *et al.*, 2014,2015; Vicuña *et al.*, 2015a,b). The purpose of the present investigation was to evaluate the effect of three doses of aflatoxin B1 on growth, physiological parameters, and gut permeability in broiler chickens.

#### **D. MATERIALS AND METHODS**

##### **Animal source, diets, and experimental design**

Two experiments were conducted several weeks apart using two hundred and forty 1-d-old male broiler chicks (Cobb-Vantress, Silom Springs, AR) raised in floor pens. Unmedicated corn-soybean-based broiler starter and medicated (with coccidiostat) corn-soybean-based broiler grower diets were prepared according to recommendations (Cobb-Vantress Inc., 2012). Experiments were conducted to evaluate the effect of 3 concentrations of AFB1 (2 ppm in experiment 1; and 1.5 ppm or 1 ppm in experiment 2) on systemic fluorescein isothiocyanate-dextran (FITC-d; 3–5 kDa) levels and liver bacterial translocation (BT) as indicators of increased gut epithelial leakage. AFB1 was provided by Dr. George E. Rottinghaus, Veterinary Medical Diagnostic Laboratory, University of Missouri, Columbia, MO 65211. AFB1 was produced through the fermentation of rice and the aflatoxin content was measured by spectrophotometric analysis. The aflatoxin within the rice powder consisted of 74.62% AFB1, 22.38% AFG1, 2.48% AFB2, and 0.49% AFG2, based on total aflatoxin in the rice powder. Diets containing AFB1 were analyzed and the presence of parent AF was confirmed by high-performance liquid

chromatography with fluorescence detection (HPLC-FLD) method by using a Romer Derivatization Unit (Romer Labs, Inc., MO 63084-1156, USA). AFB1 was added to the diets and mixed thoroughly in a graded sequence to specified concentrations. The birds were given diets with or without supplemental AFB1 and water *ad libitum*. All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas. In Exp 1, broilers were allocated randomly to two groups, each group had six replicates of 20 chickens (n = 120/group): Control feed or feed + 2 ppm AFB1. In Exp 2, broilers were allocated randomly to three groups, each group had 5 replicates of 16 chickens (n = 80/group): Control feed; feed + 1 ppm AFB1; or feed + 1.5 ppm AFB1. In both experiments, chickens were fed starter (d1-d7) and grower diet (d8-d21) *ad libitum* until the end of the experiment at day 21. In each experiment, each pen was used as a replicate and also as an experimental unit per treatment to evaluate body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR). These growth performance parameters were obtained every week. At the end of experiment 2, blood samples were collected from the wing vein into tubes with heparin as anticoagulant for differential cell counts. In both experiments, 21d old chickens received an oral gavage dose of FITC-d (4.16 mg/kg) 2.5h before collecting blood samples to evaluate passage of FITC-d. Chickens were humanely killed by CO<sub>2</sub> asphyxiation. Blood was collected from the femoral vein to obtain serum for FITC-d determination (as described below) and serum clinical chemistry (in experiment 2 only) with a Corning clinical chemistry analyzer (Chiron Corporation, San Jose, CA). Liver sections (n=12chickens/treatment) were aseptically collected to determine BT, and cecal contents were collected to determine total cfu/g of Gram-negative bacteria; lactic acid bacteria (LAB) or anaerobes by plating on a selective media as describe below.

### **Determination of hematological parameters**

Differential counts of blood samples collected from experiment 2 were determined using a Cell-Dyne 3500 System (Abbott Laboratories, Chicago, IL) that had been standardized for differential counts of poultry blood cells. Hematologic measurements of heparin anticoagulated blood included total numbers of white blood cells (WBC), heterophils, lymphocytes, monocytes, eosinophils, and basophils. Heterophil/lymphocyte ratios (H/L), an indicator of stress in birds (Gross and Siegel, 1983), was calculated by dividing the number of heterophils in 1 mL of peripheral blood by the number of lymphocytes. Total counts of red blood cells, Hemoglobin (HGB), Hematocrit (HCT) %, Mean Corpuscular Volume (MVC), and Mean Corpuscular Hemoglobin (MCH) were also determined. Additionally, in experiment 2, liver, spleen and bursa of Fabricius were removed and cleaned of adherent tissues. The weight of these organs was measured and expressed as percentage of body weight ( $\text{organs weight/final BW} \times 100$ ).

### **Serum determination of FITC-d**

Blood samples were kept at room temperature for 3 h and centrifuged ( $1,000 \times g$  for 15 min) to separate the serum from the red blood cells. FITC-d levels of undiluted serum were measured at excitation wavelength of 485 nm and emission wavelength of 528 nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., Vermont, USA). Fluorescence measured was then compared to a standard curve with known FITC-d concentrations. Gut leakage for each bird was reported as  $\mu\text{g}$  of FITC-d/mL of serum (Vicuña *et al.*, 2015a,b).

### **Bacterial translocation**

The number of birds used was based on published studies in which similar variables were measured (Latorre *et al.*, 2014; Tellez *et al.*, 2015). Briefly, the right half of the liver was removed from each chicken, collected in sterile bags, homogenized, weighed and 1:4 wt/vol

dilutions were made with sterile 0.9% saline. Ten-fold dilutions of each sample, from each group were made in a sterile 96 well Bacti flat bottom plate and the diluted samples were plated on tryptic soy agar plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD).

### **Determination of microbial level in ceca**

Both ceca were aseptically removed, and placed into sterile bags, and homogenized. Samples were weighed, and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Ten-fold dilutions of each sample, from each group were made in a sterile 96 well Bacti flat bottom plate and the diluted samples were plated on four different culture media; to evaluate total number of LAB in deMan Rogosa Sharpe (Difco™ Lactobacilli MRS Agar VWR Cat. No. 90004-084 Suwanee, GA 30024); total recovered Gram-negative bacteria in MacConkey; total anaerobes in tryptic soy agar with sodium thioglycolate plates (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD); total yeast in Sabouraud Glucose Agar Base with antibiotics, tetracycline, 100 mg and sodium benzyl penicillin 100 mg (HiMedia Laboratories Pvt. Ltd. Mumbai-400086, India).

### **Histology and morphometric analysis of intestine**

Intestinal sections from duodenum (~1-cm section was collected from the middle of the descending duodenum), and ileum (0.5-cm section was obtained from the mid-ileum at Meckel's diverticulum) were fixed in 10% neutral buffered formalin and embedded in paraffin, sectioned (5 µm thick), set on a glass slide, and stained with hematoxylin and eosin (H&E), and then examined by light microscopy. Photomicrographs of randomly selected fields of each intestinal sample were acquired using a microscope equipped with a Leica DFC450C camera and Leica v.3.8. Software (Leica Application Suite) and used for morphometric analysis. ImageJ 1.47v software (<http://rsb.info.nih.gov/ij/>) was used for the morphometric measurements of villus height, villus width, and crypt depth. Under a magnification of 20×, ten villi per bird per section

were measured, with a total of 5 birds per group. Villus height was measured from the top of the villus to the upper part of the lamina propria. Crypt depth was measured from the base upwards to the region of transition between the crypt and villus (Aptekmann *et al.*, 2001). Villus width was measured at the widest area of each villus, whereas villus: crypt ratio was determined dividing villus height into crypt depth values. Villus surface area was calculated using the formula  $(2\pi)(VW/2)(VL)$ , where VW = villus width and VL = villus height (Sakamoto *et al.*, 2000).

### **Statistical analysis**

All data were subjected to analysis of variance as an entirely randomized design using the General Linear Models procedure of SAS (SAS Institute, 2002). Data were expressed as mean  $\pm$  standard error. Significant differences among the means were determined by using Duncan's multiple-range test at  $P < 0.05$ .

## **E. RESULTS**

### **Performance parameters**

Body weight of chickens fed 2 ppm of AFB1 was not affected in the first week, however BW was significantly ( $P < 0.05$ ) reduced by 18% and 35% during the second and third week of age, respectively, when compared with Controls, (Table 1). Body weight gain and feed intake were also affected by AFB1 consumption with a reduction of 20% for both variables during the second week and 37% and 49%, respectively, in the third week. Feed conversion ratio only showed a significant difference in the third week with an improvement in the AFB1 group when compared with Controls (Table 1). Administration of 1 and 1.5 ppm of AFB1 also decreased BW by 8% and 11% during the second week and 16% and 26% in the third week, respectively compared with Controls. This reduction was proportionally similar in BWG being 10% and 13%

lower for 1 and 1.5 ppm of AFB1 during the second week; and 17% and 28% for 1 and 1.5 ppm during the third week. Feed intake was not affected by AFB1 consumption during the first two weeks; however, there was a reduction of 15% and 28% in feed intake in chickens that consumed 1 and 1.5 ppm of AFB1, respectively, during the last week (Table 1). Feed conversion ratio varied accordingly in the three diet groups during the whole experiment except the second week where control group had a more efficient ratio compared to the AFB1 groups (Table 1). In experiment 2, the liver-to-body weight ratio was significantly increased in chickens that received 1.5 ppm when compared with Control (Table 2). However, spleen-to-body weight ratio was increased in both groups of chickens that received 1 or 1.5 ppm of AFB1 when compared with Controls. Bursa-to-body weight ratio was increased only in chickens that received 1 ppm (Table 2).

#### **Total bacterial counts in cecum**

In experiment 1, chicks receiving 2 ppm of AFB1 had an increase in the number of total Gram-negative bacteria and total LAB, but the total numbers of aerobes were similar between chickens that received 2 ppm of AFB1 and control chickens (Table 3). In experiment 2, the total number of aerobic bacteria and total Gram negatives were higher in 1.5 ppm AFB1 group. Conversely, the number of total LAB was reduced in chickens fed with 1 ppm AFB1. No difference was observed in total yeast count between groups in neither of both experiments (Table 3).

#### **Hematology**

In experiment 2, a significant heterophilia with a marked lymphopenia was observed in both groups that received AFB1 (Table 4). Consequently, an increase in the heterophils-to-lymphocyte ratio was also observed in those groups when compared with Controls. No

significant differences were found in the numbers of monocytes, eosinophils, or basophils (data not shown). Hemoglobin, MVC, and MCH were significantly decreased in chickens that consumed 1.5 ppm of AFB1 when compared with Controls. These values were not affected in chickens that received 1 ppm when compared with Controls (Table 4).

### **Bacterial translocation and FITC-d leakage**

Chickens receiving a diet with 2 ppm of AFB1 had a significant reduction in BT to the liver when compared to control chickens (Table 5). Interestingly, there were no differences in serum levels of FITC-d levels between control and treated chickens. On the other hand, in experiment 2, chicks fed 1.5 ppm AFB1 did not show significant differences in BT when compared with control chickens, but no bacteria recovery was observed from livers of chickens fed with 1 ppm AFB1. Nevertheless, similar to experiment 1, no significant differences were observed in the levels of serum FITC-d between chicks that received 1 or 1.5 ppm of AFB1 and control chickens (Table 5).

### **Morphometric analysis**

Villus length in both duodenum and ileum sections was significantly increased in a dose-related fashion in chickens that received 1 and 1.5 ppm of AFB1 when compared with controls (Table 6). However, a significant reduction in duodenum crypt depth was observed in chickens that received 1 and 1.5 ppm of AFB1 when compared with control chickens. On the other hand, similar changes in ileum crypt depth were found in chickens that received 1.5 ppm of AFB1 when compared with control or 1 ppm-treated chickens. Changes in duodenum villus height-to-crypt depth ratio were inconsistent between doses of AFB1 in this study.

In the ileum, this relationship was increased in chickens that received 1 ppm, followed by chicks that received 1.5 ppm of AFB1 and control chickens had the lower villus height/crypt

depth ratio. The surface area of the duodenum was significantly higher in chicks that received 1.5 ppm of AFB1, but no changes in ileum surface area were observed between the three groups (Table 6).

## **F. DISCUSSION**

Aflatoxins have several effects in poultry, including poor performance, liver pathology, immunosuppression, and changes in relative organ weights (Huff *et al.*, 1986; Kubena *et al.*, 1993; Kubena *et al.*, 1997; Kubena *et al.*, 2001). Our results were consistent with these previous studies demonstrating dose-related effects on reduction of body weight, body weight gain, feed intake and feed conversion as well as increase relative weights of liver, spleen and bursa of Fabricius.

In spite of the indicated antimicrobial potential of AFB1, we found few reports regarding the effects of the toxin on gut microbial populations. Kubena *et al.* 2001 reported a significant increase in total volatile fatty acids at 5 days of age in chickens that received 2.5 and 7.5 ppm of AFB1, suggesting changes in LAB populations (Mohran *et al.*, 1984; Suti'c and Banina, 1989). In other studies, *Lactobacillus spp.* have been noted to change under the influence of AFB1, but these changes were not associated with any beneficial effects of AFB1 on intestinal microbial population (Peltonen *et al.*, 2001).

In the present study, AFB1 significantly increased the total number of Gram-negative bacteria in chickens fed with 2 and 1.5 ppm and had a not significantly increased in chickens fed with 1 ppm, and a similar trend was observed in the total number of LAB for chickens receiving 2 and 1.5 ppm of AFB1. However, chickens that received 1 ppm showed a significant reduction of total LAB but higher total number of aerobic bacteria when compared with control chickens. Interestingly, little information about the outcomes of AFB1 on gut microbiome is available. In

one study, Kubena et al. 2001 reported that 2.5 ppm of AFB1 increased the production of total volatile fatty acids in broilers, which suggest higher number of total LAB populations. In the present study, no differences were observed in total yeast counts between groups in neither experiment. We were unable to find any previously published report for comparison. Perhaps, such inconsistent results may be a reason of the lack of publications reporting yeast evaluation. Interestingly, it has been showed that fermentation patterns of *Saccharomyces cerevisiae* also change under the influence of AFB1 (Reiss, 1973). AFB1 has also been reported to change fermentation patterns with increased gas production, due to fermentation of other carbohydrates of LAB, that negatively affect the cheese industry (Suti'c and Banina, 1989; Peltonen *et al.*, 2001; Georgianna and Payne, 2009). Several investigators have reported that aflatoxins cause heterophilia, lymphopenia, and hemolytic anemia in poultry (Huff *et al.*, 1986; McLean and Dutton, 1995; Oğuz *et al.*, 2000; Yousef *et al.*, 2003; Yunus *et al.*, 2011b). In experiment 2, a marked increase in the heterophils occurred while the lymphocytes were reduced. Consequently, an increase in the heterophils-to-lymphocyte ratio was also observed in those groups when compared with control chickens. A similar response of circulating leucocytes was also found when a physiological stress was applied to chickens (Gross and Siegel, 1983). In aflatoxicosis, the spleen is enlarged due to the hemolytic anemia (Tung *et al.*, 1975) and some reports indicate that the spleen of chickens is almost doubled in size (Smith and Hamilton, 1970). In experiment 2, spleens of chickens that received 1 and 1.5 ppm were significantly larger when compared with control. The elevated white blood cell counts caused by both doses of AFB1 also support the clinical presentation of hemolytic anemia. Additionally, hemoglobin, MVC, and MCH were significantly decreased in chickens that consumed 1.5 ppm of AFB1 when compared with control chickens, confirming that aflatoxicosis causes a hemolytic anemia in chickens as has

been previously reported (Tung *et al.*, 1975; Smith *et al.*, 1976; Huff *et al.*, 1986; Yousef *et al.*, 2003; Tessari *et al.*, 2010).

We have previously shown that intestinal inflammation can be induced by diet ingredients or stress, affecting intestinal permeability (Tellez *et al.*, 2014, 2015; Vicuña *et al.*, 2015a,b). As the largest barrier in the body, intestinal epithelial cells are responsible for absorption of water and nutrients, but they also prevent the entry of antigens into the blood (Salminen and Isolauri, 2006; Salzman, 2011; Elson and Cong, 2012). Contrary to our initial hypothesis, 2 ppm of AFB1 did not increase intestinal permeability, as was evidenced by a significant reduction in BT or similar levels of serum FITC-d when compared with control chickens. It is possible that the inflammation of the liver that is characterized by infiltration of heterophils and other inflammatory cells may handle cleaning any bacterial leakage that arrives from the portal system to the liver. Those results encouraged us to repeat and extend the experiment with lower doses of AFB1, and by comparing the morphometric changes between control and treated groups. Our findings from experiment 2 showed 1.5 ppm AFB1 fed to chickens caused a not significant reduction in BT when compared with control chickens, but no bacteria were recovered from livers of chickens fed with 1 ppm AFB1. Also, similar to experiment 1, no significant differences were observed in the levels of serum FITC-d between chicks that received 1 or 1.5 ppm of AFB1 and control chickens. Increased intestinal leakage is also associated with BT in the portal circulation (Ilan, 2012; Seki and Schnabl, 2012). Likewise, FITC-d is a bulky molecule (3-5 kDa) which is not absorbed under normal conditions. Nevertheless, if tight junctions between epithelial cells are altered, FITC-d can be detected in serum, indicating damage to the tight junctions following FITC-d gavage administration (Yan *et al.*, 2009). It has been reported that AFB1 does not destroy tight junctions (Caloni *et al.*, 2012), it

has only minor effects on the gut-associated lymphoid tissue (GALT) (Watzl *et al.*, 1999), confirming that AFB1 does not induce inflammation in the GIT. Literature reports on the effects of AFB1 on histology of GIT is limited and not conclusive (Applegate *et al.*, 2009; Awad *et al.*, 2009; Yunus *et al.*, 2010; Yunus *et al.*, 2011b; Zhang *et al.*, 2014). However, it is important to mention that the few studies that have evaluated the effect of AFB1 on intestinal histology, are reports using different concentrations of AFB1, different avian species, different ages, as well as time of AFB1 administration. Interpretation of our morphometric results was also inconclusive. Nevertheless, the GIT is highlighted as a dynamic organ that is able to adapt to a chronic AFB1 as has been demonstrated by several scientists (Ruff and Wyatt, 1976; Fan *et al.*, 1997; Nelson *et al.*, 1982; Kermanshahi *et al.*, 2007; Applegate *et al.* 2009; Yunus *et al.*, 2010; Smith *et al.*, 2012). In summary, the results of the present study suggest that AFB1 does not increase gut leakage as is evidenced by the lack of increase permeability of FITC-d in the serum. On the other hand, further studies are needed to clarify the bacterial translocation and morphometric results with AFB1.

### **Conflict of Interest Statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Table 1** Effect of dietary administration of 2, 1.5 and 1 ppm of aflatoxin B1 on body weight (BW), body weight gain (BWG), feed intake and feed conversion ratio at 7, 14 and 21 days in broiler chickens or experiments 1 and 2.

Parameters	Experiment 1		Experiment 2		
	Control	2 ppm AFB1	Control	1 ppm AFB1	1.5 ppm AFB1
<b>BW, g/broiler</b>					
d 7	144.79 ± 1.85 <sup>a</sup>	142.05 ± 1.04 <sup>a</sup>	136.82 ± 2.87 <sup>a</sup>	134.92 ± 2.44 <sup>a</sup>	133.34 ± 2.74 <sup>a</sup>
d 14	385.88 ± 5.02 <sup>a</sup>	315.42 ± 5.40 <sup>b</sup>	337.03 ± 9.38 <sup>a</sup>	309.76 ± 2.21 <sup>b</sup>	298.95 ± 5.03 <sup>b</sup>
d 21	771.55 ± 8.61 <sup>a</sup>	502.28 ± 7.90 <sup>b</sup>	690.45 ± 19.36 <sup>a</sup>	581.99 ± 8.54 <sup>b</sup>	511.03 ± 11.47 <sup>c</sup>
<b>BWG, g/broiler</b>					
d 0 – 7	97.83 ± 1.71 <sup>a</sup>	95.07 ± 1.03 <sup>a</sup>	93.24 ± 2.71 <sup>a</sup>	90.40 ± 2.9 <sup>a</sup>	88.62 ± 2.89 <sup>a</sup>
d 7 – 14	338.88 ± 4.85 <sup>a</sup>	268.45 ± 5.07 <sup>b</sup>	293.48 ± 9.20 <sup>a</sup>	265.22 ± 2.11 <sup>b</sup>	254.13 ± 4.74 <sup>b</sup>
d 14 – 21	724.60 ± 8.46 <sup>a</sup>	455.30 ± 7.92 <sup>b</sup>	646.65 ± 18.94 <sup>a</sup>	537.47 ± 8.37 <sup>b</sup>	466.22 ± 11.19 <sup>c</sup>
<b>Feed intake, g/broiler</b>					
d 0 – 7	132.1 ± 1.92 <sup>a</sup>	127.44 ± 1.62 <sup>a</sup>	131.35 ± 3.17 <sup>a</sup>	128.34 ± 2.94 <sup>a</sup>	126.42 ± 3.44 <sup>a</sup>
d 7 – 14	505.65 ± 5.86 <sup>a</sup>	405.94 ± 6.12 <sup>b</sup>	405.49 ± 13.15 <sup>a</sup>	406.08 ± 6.40 <sup>a</sup>	399.36 ± 14.80 <sup>a</sup>
d 14 – 21	966.15 ± 17.74 <sup>a</sup>	489.09 ± 16.53 <sup>b</sup>	790.56 ± 40.09 <sup>a</sup>	670.32 ± 17.08 <sup>b</sup>	570.14 ± 53.87 <sup>c</sup>
<b>Feed conversion ratio</b>					
d 0 – 7	1.35 ± 0.01 <sup>a</sup>	1.34 ± 0.01 <sup>a</sup>	1.41 ± 0.02 <sup>a</sup>	1.42 ± 0.01 <sup>a</sup>	1.43 ± 0.02 <sup>a</sup>
d 7 – 14	1.49 ± 0.02 <sup>a</sup>	1.51 ± 0.01 <sup>a</sup>	1.39 ± 0.06 <sup>b</sup>	1.53 ± 0.01 <sup>a</sup>	1.57 ± 0.03 <sup>a</sup>
d 14 – 21	1.33 ± 0.02 <sup>a</sup>	1.08 ± 0.04 <sup>b</sup>	1.23 ± 0.09 <sup>a</sup>	1.25 ± 0.02 <sup>a</sup>	1.22 ± 0.09 <sup>a</sup>

<sup>a-c</sup> Superscripts within rows indicate significant ( P < 0.05) difference within each experiment

**Table 2** Effect of 1 and 1.5 ppm of aflatoxin B1 on organ-to-body weight ratios for liver, spleen and bursa of Fabricius in 21-day-old broiler chickens. Experiment 2.

<b>Treatment</b>	<b>Liver ratio (%)</b>	<b>Spleen ratio (%)</b>	<b>Bursa of Fabricius ratio (%)</b>
Control	3.24 ± 0.09 <sup>b</sup>	0.11 ± 0.01 <sup>b</sup>	0.15 ± 0.01 <sup>b</sup>
1 ppm AFB1	3.60 ± 0.19 <sup>ab</sup>	0.16 ± 0.02 <sup>a</sup>	0.20 ± 0.02 <sup>a</sup>
1.5 ppm AFB1	4.23 ± 0.34 <sup>a</sup>	0.15 ± 0.01 <sup>a</sup>	0.18 ± 0.02 <sup>ab</sup>

Mean ± SE from 10 chickens.

<sup>a-b</sup> Superscripts within columns indicate significant difference at  $P < 0.05$ .

**Table 3** Effect of 2 ppm of aflatoxin B1 (experiment 1) or 1 ppm and 1.5 ppm of aflatoxin B1 (experiment 2) on total bacterial and yeast counts from cecum samples in broiler chickens at 21 days.

Diet	Ceca			Log10 cfu
	/g of tissue			
	Total aerobic bacteria	Total Gram negative bacteria	Total lactic acid bacteria	Total yeast
<b>Experiment 1</b>				
Control	6.41 ± 0.19 <sup>a</sup>	6.08 ± 0.22 <sup>b</sup>	5.75 ± 0.21 <sup>b</sup>	3.13 ± 0.20 <sup>a</sup>
2 ppm AFB1	6.83 ± 0.29 <sup>a</sup>	7.00 ± 0.21 <sup>a</sup>	6.56 ± 0.13 <sup>a</sup>	3.33 ± 0.07 <sup>a</sup>
<b>Experiment 2</b>				
Control	6.98 ± 0.23 <sup>b</sup>	6.51 ± 0.37 <sup>b</sup>	6.91 ± 0.14 <sup>a</sup>	2.74 ± 0.33 <sup>a</sup>
1 ppm AFB1	7.25 ± 0.22 <sup>b</sup>	7.04 ± 0.24 <sup>ab</sup>	6.33 ± 0.15 <sup>b</sup>	3.36 ± 0.18 <sup>a</sup>
1.5 ppm AFB1	7.82 ± 0.17 <sup>a</sup>	7.66 ± 0.15 <sup>a</sup>	7.22 ± 0.16 <sup>a</sup>	2.86 ± 0.33 <sup>a</sup>

Data is expressed as mean ± SE from 12 chickens.

<sup>a-b</sup> Superscripts within columns indicate significant difference at P < 0.05.

**Table 4** Effect of 1 and 1.5 ppm of aflatoxin B1 on blood parameters and serum chemistry in broiler chickens at 21 days. Experiment 2.

Hematological parameters	Treatments		
	Control	1 ppm AFB1	1.5 ppm AFB1
White Blood Cells	30.02 ± 4.57 <sup>a</sup>	27.89 ± 2.50 <sup>a</sup>	37.20 ± 4.23 <sup>a</sup>
Heterophils	13.21 ± 1.38 <sup>b</sup>	26.39 ± 2.04 <sup>a</sup>	28.62 ± 2.70 <sup>a</sup>
Lymphocytes	77.15 ± 2.07 <sup>a</sup>	62.58 ± 3.31 <sup>b</sup>	58.08 ± 2.11 <sup>b</sup>
Heterophils lymph. ratio (HLR)	0.18 ± 0.02 <sup>b</sup>	0.45 ± 0.05 <sup>a</sup>	0.51 ± 0.06 <sup>a</sup>
Red blood cells	1.81 ± 0.09 <sup>a</sup>	1.70 ± 0.04 <sup>a</sup>	1.68 ± 0.06 <sup>a</sup>
Hemoglobin (HGB)	5.98 ± 0.17 <sup>a</sup>	5.56 ± 0.18 <sup>a</sup>	4.90 ± 0.13 <sup>b</sup>
Hematocrit (HCT) %	44.95 ± 2.41 <sup>a</sup>	42.07 ± 1.33 <sup>a</sup>	39.23 ± 1.29 <sup>a</sup>
Mean corpuscular Volume (MVC)	248.1 ± 2.83 <sup>a</sup>	247.0 ± 3.23 <sup>a</sup>	234.4 ± 3.19 <sup>b</sup>
Mean corpuscular hemoglobin (MCH)	33.58 ± 1.15 <sup>a</sup>	32.63 ± 0.56 <sup>ab</sup>	29.42 ± 0.81 <sup>b</sup>

Mean ± SE from 10 chickens.

<sup>a-b</sup> Superscripts within rows indicate significant difference at P < 0.05.

**Table 5** Effect of 2 ppm of aflatoxin B1 (experiment 1) or 1 ppm and 1.5 ppm of aflatoxin B1 (experiment 2) on liver bacterial translocation and serum FITC-d levels in broiler chickens at 21 days.

<b>Diet</b>	<b>Liver bacterial translocation<sup>1</sup> log<sub>10</sub> cfu /g of tissue</b>	<b>FITC-d<sup>2</sup> µg/mL of serum</b>
<b>Experiment 1</b>		
Control	2.77 ± 0.50 <sup>a</sup>	0.34 ± 0.01 <sup>a</sup>
2 ppm AFB <sub>1</sub>	1.13 ± 0.49 <sup>b</sup>	0.39 ± 0.05 <sup>a</sup>
<b>Experiment 2</b>		
Control	1.51 ± 0.46 <sup>a</sup>	0.34 ± 0.02 <sup>a</sup>
1 ppm AFB <sub>1</sub>	0.00 ± 0.00 <sup>b</sup>	0.31 ± 0.02 <sup>a</sup>
1.5 ppm AFB <sub>1</sub>	1.30 ± 0.47 <sup>a</sup>	0.31 ± 0.01 <sup>a</sup>

<sup>1</sup> Data is expressed as mean ± SE, n=12 birds/treatment.

<sup>2</sup> Data is expressed as mean ± SE, n=20 birds/treatment.

<sup>a-b</sup> Superscripts within columns indicate significant difference at P < 0.05.

**Table 6** Morphometric analysis of duodenum and ileum samples from broiler chickens at 21 days. Experiment 2.

Parameters	Duodenum			Ileum		
	Control	1 ppm AFB1	1.5 ppm AFB1	Control	1 ppm AFB1	1.5 ppm AFB1
Villus height ( $\mu\text{m}$ )	382.41 $\pm$ 5.03 <sup>c</sup>	398.40 $\pm$ 2.01 <sup>b</sup>	437.00 $\pm$ 7.50 <sup>a</sup>	164.32 $\pm$ 3.75 <sup>c</sup>	175.42 $\pm$ 3.13 <sup>b</sup>	199.78 $\pm$ 3.42 <sup>a</sup>
Villus width ( $\mu\text{m}$ )	45.22 $\pm$ 1.66 <sup>a</sup>	45.83 $\pm$ 1.38 <sup>a</sup>	47.74 $\pm$ 1.50 <sup>a</sup>	38.93 $\pm$ 0.68 <sup>a</sup>	37.69 $\pm$ 1.33 <sup>a</sup>	32.57 $\pm$ 0.78 <sup>b</sup>
Crypt depth ( $\mu\text{m}$ )	31.83 $\pm$ 1.03 <sup>a</sup>	26.01 $\pm$ 0.79 <sup>b</sup>	24.35 $\pm$ 0.15 <sup>b</sup>	23.24 $\pm$ 0.49 <sup>b</sup>	21.51 $\pm$ 0.69 <sup>b</sup>	26.67 $\pm$ 0.67 <sup>a</sup>
Villus height/crypt depth ratio	12.45 $\pm$ 0.33 <sup>c</sup>	16.03 $\pm$ 0.48 <sup>b</sup>	18.06 $\pm$ 0.39 <sup>a</sup>	7.11 $\pm$ 0.12 <sup>c</sup>	8.49 $\pm$ 0.24 <sup>a</sup>	7.58 $\pm$ 0.10 <sup>b</sup>
Villus surface area ( $\text{mm}^2$ ) <sup>1</sup>	0.054 $\pm$ 0.019 <sup>b</sup>	0.057 $\pm$ 0.001 <sup>b</sup>	0.066 $\pm$ 0.002 <sup>a</sup>	0.020 $\pm$ 0.005 <sup>a</sup>	0.021 $\pm$ 0.009 <sup>a</sup>	0.020 $\pm$ 0.007 <sup>a</sup>

<sup>a-c</sup> Superscripts within rows within intestinal section indicate significant difference at  $P < 0.05$ .

<sup>1</sup> Surface was calculated as:  $[2\pi \times (\text{villus width}/2) \times (\text{villus height})]$  (Sakamoto *et al.*, 2000).

**V. CHAPTER III**

**A. Evaluation of *Bacillus spp.* as direct fed microbial (DFM) candidates for aflatoxin B1 biodegradation in broiler chickens**

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## B. ABSTRACT

The limits of physical and chemical methods to decrease aflatoxins in feed ingredients, stimulated the search on biological approaches of degradation. Recently, we identified three *Bacillus spp.* candidates that showed *in vitro* activity to biodegrade aflatoxin B1 (AFB1). The aim of this study was to evaluate the biodegradation potential of previously selected *Bacillus spp.* provided as a direct-fed microbial candidate (DFM), in broiler chickens consuming feed containing different concentrations of AFB1. In the present study, three independent experiments were conducted. In Exp 1, broilers were allocated randomly to four groups: Control feed; feed + DFM; feed + 2 ppm AFB1; feed + DFM + 2 ppm AFB1. Each group had six replicates of 20 chickens (n = 120/group). In Exp 2, broilers were allocated randomly to six groups: Control feed; feed + DFM; feed + 1 ppm AFB1; feed + DFM + 1 ppm AFB1; feed + 1.5 ppm AFB1; feed + DFM + 1.5 ppm AFB1. Each group had 5 replicates of 16 chickens (n = 80/group). In Exp 3, broilers were allocated randomly to six groups: Control feed; feed + DFM; feed + 50 ppb AFB1; feed + DFM + 50 ppb AFB1; feed + 500 ppb AFB1; feed + DFM + 500 ppb AFB1, each group had 5 replicates of 10 chickens (n = 80/group). In all experiments, chickens were fed starter (d1-d7) or grower diet (d8-d21) *ad libitum* until the end of the experiment at day 21. All broilers were individually weighed and body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were obtained weekly. The results of the present study show clear evidence, that not all DFM are the same, and in this case, administration of  $10^6$  spores/gram of feed showed no beneficial performance effects in two out of three independent experiments. In addition, even though the individual isolates incorporated in the DFM showed some *in vitro* activity to biodegrade AFB1, when administered in the diets at 5 different

concentrations of AFB1, no significant performance differences were observed when compared with their respective control diets.

**Key words:** Aflatoxin B1, *Bacillus*, DFM, biodegradation, broiler feed

## C. INTRODUCTION

During the summer of 1961, over 100,000 turkeys and other avian species died of a mysterious disease that was named “Turkey X Disease” by veterinarians, because some of the first signs were neurological symptoms, coma, and death, which resembled a viral disease of unknown etiology at the time (Blount, 1961). Exhaustive research led to the discovery of aflatoxins, secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, as the etiological agents and the development of mycotoxicology (Spensley, 1963; Nesbitt et al., 1962; Cole, 1986). More recent studies demonstrated that aflatoxins are potent carcinogenic compounds (McLean and Dutton, 1995; Yunus et al., 2011; Zhang et al., 2014; Rawal and Coulombe, 2011; Fox et al., 2010). About 14 different types of aflatoxins are produced in nature (Ledoux et al., 1999; Yunus et al., 2011), but aflatoxin B1 (AFB1) produced by *A. flavus* is considered the most toxic (Andrade et al., 2013; Greco et al., 2014). For over 55 years, aflatoxicosis has had a substantial health and economic impact for humans and domestic animals (Smith et al., 1976; Blount, 1961; Siller and Ostler, 1961; Greco et al., 2014; Warburton and Williams, 2014). The liver is the organ that is most severely affected by aflatoxins, and several investigators have demonstrated the detrimental effects on performance parameters, immunosuppression, and hemolytic anemia in poultry (Tung et al., 1975; Huff et al., 1986; Rawal et al., 2010; Kubena et al., 1993). Hence, control of aflatoxins is critical, because their incidence in feeds is a threat for the health and economics of humans and domestic animals. In addition to post-harvest preventive measures, appropriate detoxification methods have been developed for inactivating aflatoxins from contaminated grains, since aflatoxins are also produced during pre-harvest stages. Hence, to reduce the effect of aflatoxins, physical and chemical methods have been explored (Greco et al., 2014). However, these methods have

multiple limitations for practical use (Basappa and Shantha, 1996). Such restrictions have motivated several investigators to evaluate biological methods of degradation of aflatoxins (Ciegler et al., 1966; Farzaneh et al., 2012; Gao et al., 2011). Detoxification by microbiological means has also been evaluated with regard to potential microorganisms and their enzymes that can degrade aflatoxins to less toxic or innocuous end products. Some strains of *Bacillus spp.* are identified as generally recognized as safe (GRAS) organisms with probiotic properties in humans and animals. Recently, we selected three *Bacillus spp.* candidates that showed *in vitro* activity to biodegrade AFB1 based on growth, reduction of fluorescence and area of clearance around each bacterial colony (Galarza-Seeber et al., 2015). Selected *Bacillus* strains showed an inhibitory halo clearly visible under daylight after 48 h of evaluation. Analysis of 16S rRNA identified the strains as *B. amyloliquefaciens*, *B. megaterium* and *B. subtilis* (Galarza-Seeber et al., 2015). The aim of this study was to evaluate the biodegradation potential of previously selected *Bacillus spp.* provided as a direct-fed microbial candidate (DFM), assuming that continuous feed delivery of  $10^6$  spores/gram of feed during 21 days, could have some effect in reducing the detrimental performance effects of AFB1 in broiler chickens.

#### **D. MATERIALS AND METHODS**

##### **Animal source and diets**

In the present study, three independent experiments were conducted several weeks apart, using 1-d-old male broiler chicks (Cobb-Vantress, Silom Springs, AR) raised in floor pens. Unmedicated mash corn-soybean-based broiler starter and medicated (with coccidiostat) mash corn-soybean-based broiler grower diets (Table 1) were prepared according to NRC specifications (National Research Council, 1994). Experiments were conducted to evaluate the effect of selective *Bacillus spp.* DFM candidates on five concentrations of Aflatoxin B1 (AFB1);

2 ppm in experiment 1; 1.5 ppm or 1 ppm in experiment 2; and 500 ppb or 50 ppb in experiment 3) on performance parameters. The *Bacillus*-DFM candidate with or without AFB1 was added to the experimental diets and mixed thoroughly in a graded sequence to specified concentrations. The birds were given diets and water *ad libitum*. All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas.

### **Experimental design**

In Exp 1, broilers were allocated randomly to four groups: Control feed; feed + DFM; feed + 2 ppm AFB1; feed + DFM + 2 ppm AFB1, each group had six replicates of 20 chickens (n = 120/group).

In Exp 2, broilers were allocated randomly to six groups: Control feed; feed + DFM; feed + 1 ppm AFB1; feed + DFM + 1 ppm AFB1; feed + 1.5 ppm AFB1; feed + DFM + 1.5 ppm AFB1, each group had 5 replicates of 16 chickens (n = 80/group).

In Exp 3, broilers were allocated randomly to six groups: Control feed; feed + DFM; feed + 50 ppb AFB1; feed + DFM + 50 ppb AFB1; feed + 500 ppb AFB1; feed + DFM + 500 ppb AFB1, each group had 8 replicates of 10 chickens (n = 80/group).

In all experiments, chickens were fed starter (d1-d7) or grower diets (d8-d21) *ad libitum* until the end of the experiment at day 21. Replicates in each experiment were used as experimental units for growth performance parameters. All broilers were individually weighed and body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were obtained every week.

### **Bacillus Direct-Fed Microbial candidate**

In the present study, three isolates previously identified by sequence analysis of 16S rRNA as *B. amyloliquefaciens* for strain 1; *B. megaterium* for strain 2; and *B. subtilis* for strain 3 were combined in equal amounts, and evaluated in all the experiments. Isolation, characterization, identification, *in vitro* evaluation of biodegradation of aflatoxin B1, and sporulation procedures are described in our previous publication (Galarza-Seeber et al., 2015). In all diets for all the experiments, the *Bacillus*-DFM candidate was added at a concentration of  $10^6$  spores/gram of feed.

### **Statistical Analysis**

Data were subjected to ANOVA as complete randomized design using the GLM procedure of SAS (SAS Institute, 2002). Data are expressed as mean  $\pm$  standard error. Significant differences among means were determined by using Duncan's multiple-range test at  $P < 0.05$ .

## **E. RESULTS AND DISCUSSION**

The most important aflatoxin in terms of toxic potency and occurrence is AFB1 and poultry are particularly sensitive to the toxic and carcinogenic action of AFB1, leading to significant economic losses to the poultry industry every year (Warburton and Williams, 2014; Rawal et al., 2010). A number of recent studies have demonstrated that probiotic bacteria offers protection against AFB1 in humans and animals through binding of AFB1 by cell wall constituents of lactic acid bacteria and yeast (Rawal et al., 2010; Slizewska et al., 2010; El-Nezami et al., 1998; Peltonen et al., 2001; Hernandez-Mendoza et al., 2009). On the other hand, other studies have shown that some strains of *B. subtilis* have the robust ability to detoxify aflatoxins and ameliorate the damage of the liver and kidney of poultry and fish (Ma et al., 2012; Farzaneh et al., 2012). Some of these strains, in addition to detoxifying aflatoxins, have also

shown antimicrobial activities against *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* (Ciegler et al., 1966; Gao et al., 2011), suggesting a prominent potential in industrial applications, not only as probiotics but also as a biological means to detoxify aflatoxins. The use of spore former probiotics used as DFM have an appealing advantage over lactic acid bacteria since they are the toughest form of life on earth (Vreeland et al., 2000). In recent years, DFM have also become an alternative to antibiotic growth promoters showing clear advantages in terms of health and performance by several investigators (Hong et al., 2005; Huang et al., 2010; Duc et al., 2003; Shivaramaiah et al., 2011; Latorre et al., 2014; Wolfenden et al., 2011; Tellez et al. 2014). Nevertheless, in spite of all this evidence, the results of the present study were not as good as expected. Table 2 summarizes the evaluation of a selected *Bacillus spp.* DFM candidate on performance parameters in chickens with or without 2 ppm of aflatoxin B1 in Exp 1. The addition of the DFM in the diet showed a significant increase in BW and BWG as compared to the chickens that received the control diet ( $P < 0.05$ ). However, the inclusion of the DFM had no effect when added to the group that received 2 ppm AFB1. In the present study, the addition of 2 ppm AFB1 reduced BW approximately 35% regardless of DFM supplementation. FI and FCR were also severely affected by the administration of 2 ppm AFB1, regardless of the use of DFM (Table 2), and this was the reason to evaluate the *Bacillus*-DFM effect with lower concentrations of AFB1 used in Exp 2, which results are summarized in Table 3. In contrast to our first experiment, chickens that received the DFM did not improve BW or BWG when compared with control chickens. In this experiment, a clear dose response relationship was observed severely affecting performance of the chickens. Broilers that received 1 ppm of AFB1 had a reduction of BW of ~ 16% when compared with control chickens, whereas chickens that received 1.5 ppm had a reduction of ~ 26%, regardless of administration of DFM

(Table 3). To evaluate lower and more realistic doses of AFB1, experiment 3 was conducted and results are summarized in Table 4. Once again, the use of the mixture of the three selected *Bacillus spp.* as a DFM candidate had caused no significant improvements in performance when compared with control chickens. In this experiment, it was also clear that the use of 500 ppb for three weeks, had no negative effects on BW or BWG, and the only beneficial difference observed by the addition of the *Bacillus*-DFM in the diet mixed with 50 ppb was on FCR (Table 4). The results of the present study, provide clear evidence, that not all DFM are the same, and in this case, administration of  $10^6$  spores/gram of feed showed no beneficial performance effects in two out of three independent experiments. In addition, even though the individual isolates incorporated in the DFM candidate showed some *in vitro* activity to biodegrade AFB1, when administered in the diets at 5 different concentrations of AFB1, no significant performance differences were observed when compared with their respective control diets.

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**Table 1** Ingredient composition and nutrient content of broiler chicken corn-soybean based diets used in all experiments on as-is basis with or without different concentrations of aflatoxin B1.

Item	Amount per kg of starter diet	Amount per kg of grower diet
<b>Ingredients (%)</b>		
Corn	59.72	64.40
Soybean meal	33.87	29.04
Poultry fat	2.29	2.67
Dicalcium phosphate	1.85	1.73
Calcium carbonate <sup>1</sup>	0.92	0.88
Salt	0.37	0.37
DL-Methionine	0.30	0.26
Vitamin premix <sup>2</sup>	0.10	0.10
L-Lysine HCl	0.15	0.14
Choline chloride 60%	0.20	0.20
Mineral premix <sup>3</sup>	0.10	0.10
Threonine	0.11	0.09
Antioxidant <sup>4</sup>	0.02	0.02
Total	100	100
<b>Calculated analysis</b>		
ME, kcal/ kg	3,035	3,108
CP, %	21.00	19.00
Dig Lys, %	1.18	1.05
Dig Met, %	0.45	0.42
Dig Met + Cys, %	0.88	0.80
Dig Thr, %	0.77	0.69
Dig Trp, %	0.18	0.17
Total calcium, %	0.90	0.84
Available phosphorus, %	0.45	0.42
Sodium, %	0.16	0.16

<sup>1</sup>Inclusion of 10<sup>6</sup> spores/g of feed mixed with calcium carbonate.

<sup>2</sup>Vitamin premix supplied the following per 1,000 kg: vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO 64850).

<sup>3</sup>Mineral premix supplied the following per 1,000 kg: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10 to 15 g; iodine, 0.7 g; selenium, 0.2 g; and cobalt, 0.2 g (Nutra Blend LLC, Neosho, MO 64850).

<sup>4</sup>Ethoxyquin.

**Table 2** Evaluation of a selected *Bacillus spp* direct-fed microbial candidates on body weight (BW), body weight gain (BWG), feed intake, and feed conversion ratio (FCR), in broiler chickens with or without 2 ppm of aflatoxin B1 (AFB1). Experiment 1.

Parameters	Control	DFM	AFB1	AFB1 + DFM
<b>BW, g/broiler</b>				
d 7	144 ± 1.85 <sup>a</sup>	147 ± 2.05 <sup>a</sup>	142 ± 1.04 <sup>a</sup>	143 ± 1.44 <sup>a</sup>
d 14	384 ± 5.02 <sup>a</sup>	396 ± 5.00 <sup>a</sup>	315 ± 5.40 <sup>b</sup>	315 ± 6.66 <sup>b</sup>
d 21	771 ± 8.61 <sup>b</sup>	800 ± 11.10 <sup>a</sup>	502 ± 7.90 <sup>c</sup>	509 ± 10.30 <sup>c</sup>
<b>BWG, g/broiler</b>				
d 0 – 7	99 ± 1.71 <sup>ab</sup>	100 ± 1.81 <sup>a</sup>	95 ± 1.03 <sup>b</sup>	97 ± 1.08 <sup>ab</sup>
d 7 – 14	338 ± 4.85 <sup>a</sup>	350 ± 4.76 <sup>a</sup>	268 ± 5.07 <sup>b</sup>	269 ± 4.82 <sup>b</sup>
d 14 – 21	723 ± 8.46 <sup>b</sup>	754 ± 10.81 <sup>a</sup>	455 ± 7.92 <sup>c</sup>	462 ± 10.01 <sup>c</sup>
<b>Feed Intake, g/broiler</b>				
d 0 – 7	131 ± 1.92 <sup>ab</sup>	133 ± 1.71 <sup>a</sup>	127 ± 1.62 <sup>b</sup>	128 ± 1.64 <sup>ab</sup>
d 7 – 14	505 ± 5.86 <sup>a</sup>	510 ± 5.14 <sup>a</sup>	406 ± 6.12 <sup>b</sup>	401 ± 6.12 <sup>b</sup>
d 14 – 21	965 ± 17.74 <sup>a</sup>	988 ± 21.17 <sup>a</sup>	490 ± 16.53 <sup>b</sup>	503 ± 24.85 <sup>b</sup>
<b>Feed Conversion Ratio</b>				
d 0 – 7	1.34 ± 0.01 <sup>a</sup>	1.33 ± 0.02 <sup>a</sup>	1.34 ± 0.01 <sup>a</sup>	1.33 ± 0.01 <sup>a</sup>
d 7 – 14	1.48 ± 0.02 <sup>ab</sup>	1.46 ± 0.01 <sup>b</sup>	1.51 ± 0.01 <sup>a</sup>	1.49 ± 0.02 <sup>ab</sup>
d 14 – 21	1.32 ± 0.02 <sup>a</sup>	1.31 ± 0.01 <sup>a</sup>	1.08 ± 0.04 <sup>b</sup>	1.08 ± 0.03 <sup>b</sup>

<sup>abc</sup> Superscripts within rows indicate significant difference at  $p < 0.05$ .

**Table 3** Effect of dietary administration of 1.5 or 1 ppm of aflatoxin B1 (AFB1) on body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) at 7, 14 and 21 days-old broiler chickens. Experiment 2.

Parameters	Control	DFM	1 ppm AFB1	1 ppm AFB1 + DFM	1.5 ppm AFB1	1.5 ppm AFB1 + DFM
<b>BW, g/broiler</b>						
d 7	136 ± 2.87 <sup>a</sup>	136 ± 1.15 <sup>a</sup>	135 ± 2.44 <sup>a</sup>	137 ± 2.59 <sup>a</sup>	133 ± 2.74 <sup>a</sup>	136 ± 2.80 <sup>a</sup>
d 14	337 ± 9.38 <sup>a</sup>	338 ± 3.70 <sup>a</sup>	310 ± 2.21 <sup>bc</sup>	316 ± 3.57 <sup>b</sup>	299 ± 5.03 <sup>c</sup>	306 ± 4.71 <sup>bc</sup>
d 21	691 ± 19.36 <sup>a</sup>	675 ± 7.05 <sup>a</sup>	582 ± 8.54 <sup>b</sup>	576 ± 6.13 <sup>b</sup>	511 ± 11.47 <sup>c</sup>	530 ± 12.54 <sup>c</sup>
<b>BWG, g/broiler</b>						
d 0 – 7	93 ± 2.71 <sup>a</sup>	92 ± 1.26 <sup>a</sup>	90 ± 2.19 <sup>a</sup>	94 ± 2.52 <sup>a</sup>	89 ± 2.89 <sup>a</sup>	91 ± 2.55 <sup>a</sup>
d 7 – 14	294 ± 9.20 <sup>a</sup>	296 ± 2.83 <sup>a</sup>	265 ± 2.11 <sup>bc</sup>	272 ± 3.48 <sup>b</sup>	254 ± 4.74 <sup>c</sup>	261 ± 4.55 <sup>bc</sup>
d 14 - 21	647 ± 18.94 <sup>a</sup>	631 ± 7.27 <sup>a</sup>	537 ± 8.37 <sup>b</sup>	532 ± 6.16 <sup>b</sup>	467 ± 11.19 <sup>c</sup>	485 ± 12.66 <sup>c</sup>
<b>Feed Intake, g/broiler</b>						
d 0 – 7	131 ± 3.17 <sup>a</sup>	129 ± 2.65 <sup>a</sup>	128 ± 2.94 <sup>a</sup>	132 ± 1.85 <sup>a</sup>	126 ± 3.44 <sup>a</sup>	133 ± 2.77 <sup>a</sup>
d 7 – 14	405 ± 13.15 <sup>b</sup>	437 ± 6.52 <sup>a</sup>	406 ± 6.40 <sup>b</sup>	412 ± 3.78 <sup>ab</sup>	399 ± 14.80 <sup>b</sup>	393 ± 8.89 <sup>b</sup>
d 14 - 21	791 ± 40.09 <sup>a</sup>	825 ± 16.65 <sup>a</sup>	671 ± 17.08 <sup>b</sup>	669 ± 5.23 <sup>b</sup>	570 ± 53.87 <sup>c</sup>	569 ± 22.36 <sup>c</sup>
<b>Feed Conversion Ratio</b>						
d 0 – 7	1.41 ± 0.02 <sup>a</sup>	1.40 ± 0.02 <sup>a</sup>	1.42 ± 0.01 <sup>a</sup>	1.41 ± 0.02 <sup>a</sup>	1.43 ± 0.02 <sup>a</sup>	1.46 ± 0.04 <sup>a</sup>
d 7 – 14	1.39 ± 0.06 <sup>b</sup>	1.48 ± 0.01 <sup>a</sup>	1.53 ± 0.01 <sup>a</sup>	1.51 ± 0.01 <sup>a</sup>	1.57 ± 0.03 <sup>a</sup>	1.50 ± 0.03 <sup>a</sup>
d 14 - 21	1.23 ± 0.09 <sup>a</sup>	1.31 ± 0.02 <sup>a</sup>	1.25 ± 0.02 <sup>a</sup>	1.26 ± 0.01 <sup>a</sup>	1.22 ± 0.09 <sup>a</sup>	1.17 ± 0.03 <sup>a</sup>

<sup>abc</sup> Superscripts with in rows indicate significant difference between treatments (P<0.05)

**Table 4** Effect of dietary administration of 50 or 500 ppb of aflatoxin B1 (AFB1) on body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) at 7, 14 and 21 days-old broiler chickens. Experiment 3.

Parameters	Control	DFM	50 ppb AFB1	50 ppb AFB1 + DFM	500 ppb AFB1	500 ppb AFB1 + DFM
<b>BW, g/broiler</b>						
d 7	150 ± 3.16 <sup>a</sup>	146 ± 2.25 <sup>a</sup>	149 ± 2.46 <sup>a</sup>	147 ± 2.06 <sup>a</sup>	147 ± 0.66 <sup>a</sup>	152 ± 1.16 <sup>a</sup>
d 14	351 ± 9.85 <sup>abc</sup>	368 ± 10.48 <sup>ab</sup>	336 ± 12.97 <sup>c</sup>	372 ± 7.61 <sup>a</sup>	328 ± 8.72 <sup>c</sup>	339 ± 12.28 <sup>bc</sup>
d 21	747 ± 22.55 <sup>a</sup>	749 ± 19.97 <sup>a</sup>	709 ± 12.37 <sup>a</sup>	746 ± 17.40 <sup>a</sup>	698 ± 17.14 <sup>a</sup>	731 ± 9.5 <sup>a</sup>
<b>BWG, g/broiler</b>						
d 0-7	104 ± 3.17 <sup>ab</sup>	101 ± 2.40 <sup>b</sup>	103 ± 2.18 <sup>ab</sup>	102 ± 0.02 <sup>ab</sup>	103 ± 0.64 <sup>ab</sup>	108 ± 1.06 <sup>a</sup>
d 8-14	304 ± 9.79 <sup>ab</sup>	317 ± 6.71 <sup>a</sup>	290 ± 12.85 <sup>ab</sup>	321 ± 8.71 <sup>a</sup>	282 ± 8.58 <sup>b</sup>	294 ± 12.36 <sup>ab</sup>
d 15-21	700 ± 22.51 <sup>a</sup>	702 ± 20.26 <sup>a</sup>	673 ± 10.87 <sup>a</sup>	698 ± 17.22 <sup>a</sup>	652 ± 16.84 <sup>a</sup>	686 ± 8.92 <sup>a</sup>
<b>Feed intake, g/broiler</b>						
d 0-7	138 ± 2.81 <sup>a</sup>	130 ± 3.67 <sup>ab</sup>	136 ± 1.14 <sup>a</sup>	131 ± 2.31 <sup>ab</sup>	125 ± 4.71 <sup>b</sup>	131 ± 2.86 <sup>ab</sup>
d 8-14	495 ± 6.68 <sup>a</sup>	480 ± 7.79 <sup>a</sup>	486 ± 8.44 <sup>a</sup>	484 ± 11.08 <sup>a</sup>	452 ± 10.32 <sup>b</sup>	470 ± 6.24 <sup>ab</sup>
d 15-21	866 ± 26.12 <sup>a</sup>	846 ± 27.15 <sup>a</sup>	858 ± 25.37 <sup>a</sup>	828 ± 27.92 <sup>a</sup>	728 ± 26.37 <sup>b</sup>	812 ± 22.13 <sup>a</sup>
<b>Feed Conversion Ratio</b>						
d 0-7	1.33 ± 0.04 <sup>a</sup>	1.29 ± 0.05 <sup>a</sup>	1.32 ± 0.02 <sup>a</sup>	1.30 ± 0.02 <sup>a</sup>	1.23 ± 0.04 <sup>a</sup>	1.23 ± 0.02 <sup>a</sup>
d 8-14	1.64 ± 0.05 <sup>ab</sup>	1.52 ± 0.05 <sup>b</sup>	1.70 ± 0.05 <sup>a</sup>	1.51 ± 0.05 <sup>b</sup>	1.61 ± 0.06 <sup>ab</sup>	1.62 ± 0.06 <sup>ab</sup>
d 15-21	1.24 ± 0.02 <sup>ab</sup>	1.20 ± 0.02 <sup>abc</sup>	1.27 ± 0.03 <sup>a</sup>	1.19 ± 0.03 <sup>bc</sup>	1.13 ± 0.02 <sup>c</sup>	1.18 ± 0.03 <sup>bc</sup>

<sup>abc</sup> Superscripts within rows indicate significant difference between treatments (P<0.05)

## VI. CONCLUSIONS

Aflatoxins as well as mycotoxins in general are a common threat not only for poultry industry but also for other production animal species. They are also important contaminants of human food. They can contaminate cereals or other crop products and can also be found as less potent but still active metabolites in meat, milk and eggs. Aflatoxin B1 is considered the most important human carcinogen.

Many different types of treatments have been evaluated for production, and thus feed contamination, or its deleterious effect when consumed by animals or humans. None of these methods seem to be cost effective, making mycotoxins detection one of the major procedures necessary to prevent mycotoxicosis.

In the first chapter of this thesis, three different *Bacillus* colonies shown some inhibitory activity against AFB1 in culture were isolated. However, when these *Bacillus* were evaluated in an *in vitro* digestive model, no reduction of the aflatoxin content was observed.

In the second chapter, it was shown that even if no damage to the tight junctions in the gastrointestinal tract was produced with consumption of AFB1, they produce important negative effects on productive parameters when administered from day of hatch until 21 days of age at concentrations of 1 ppm to 2 ppm of AFB1. There were no significant negative effects of AFB1 when administered at 50 and 500 ppb during 21 consecutive days from day of hatch. Nevertheless, it is well documented that concentrations over 20 ppb of AFB1 can be harmful to poultry.

In the third and last chapter, selected *Bacillus spp.* from the first chapter were administered with the diet in groups consuming regular feed or feed contaminated with different concentrations of AFB1. Groups consuming contaminated feed had significantly lower body

weight and body weight gain when compared with the control group, confirming the negative effect of AFB1 in chickens. On the other hand, groups consuming DFM plus AFB1 had the same negative effects when compared with groups receiving the same concentration of AFB1 alone. These results show that the selected *Bacillus* evaluated in the present study did not have any meaningful detoxification effect on AFB1.

DFM candidates not only failed to have a detoxification effect but also did not have a positive effect in those chickens consuming only DFM when compared with control groups.

Many deleterious effects are produced by AFB1 in chickens but gut leakage was not observed in any of the experiments suggesting that although the intestinal tract is the first organ to come into contact with this toxin, no damage of tight junctions is produced due to AFB1 consumption.

Among a variety of bacteria used as probiotics, some *Bacillus* are described to have beneficial effects on productive parameters when administered with the diet. Nevertheless, it can be seen in the last chapter of this thesis that not all *Bacillus* isolates will have beneficial effects in chickens even when they belong to a species that proved to be effective as probiotic.

The use of bacteria and especially spores as probiotics with biological detoxification function of AFB1 could be an interesting and feasible discovery, however more research is needed and other *Bacillus* or bacteria candidates should be evaluated.

## APPENDIX I



UNIVERSITY OF  
ARKANSAS

Office of Research Compliance

### MEMORANDUM

TO: Guillermo Tellez  
FROM: Craig N. Coon, Chairman  
DATE: May 8, 2015  
SUBJECT: IACUC Approval  
Expiration Date: Jul 31, 2015

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your Protocol: 15051 "Evaluation of Bacillus spp. as direct-fed microbial (DFM) candidates for aflatoxin B1 biodegradation" to begin May 28, 2015

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Jul 31, 2015 you must submit a modification or new protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

APPENDIX II



UNIVERSITY OF  
ARKANSAS

Office of Research Compliance

April 13, 2015

MEMORANDUM

TO: Dr. Billy Hargis

FROM: W. Roy Penney  
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 15018

Protocol Title: "Evaluation of Bacillus spp. as direct-fed microbial (DFM) candidates for aflatoxin B1 biodegradation"

Approved Project Period: Start Date: April 9, 2015  
Expiration Date: April 8, 2018

The Institutional Biosafety Committee (IBC) has approved Protocol 15018, "Evaluation of Bacillus spp. as direct-fed microbial (DFM) candidates for aflatoxin B1 biodegradation" You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.