Research in Veterinary Science 97 (2014) 274–281

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Aflatoxicosis chemoprevention by probiotic *Lactobacillus* and lack of effect on the major histocompatibility complex

Sumit Rawal [a,1], Miranda M. Bauer [b,2], Kristelle M. Mendoza [b], Hani El-Nezami [c], Jeffery R. Hall [a], Ji Eun Kim [a,3], John R. Stevens [d], Kent M. Reed [b] and Roger A. Coulombe, Jr. [a,*]

[a] Graduate Toxicology Program, and Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, UT 84322, USA
[b] Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, USA
[c] School of Biological Sciences, University of Hong Kong, Hong Kong, China
[d] Department of Mathematics and Statistics, Utah State University, Logan, UT 84322, USA

ARTICLE INFO

Article history:
Received 23 January 2014
Accepted 7 June 2014

Keywords:
Gene expression
Aflatoxin
Chemoprevention
Toxicology
Probiotics
Turkeys
Poultry
MHC
BG

ABSTRACT

Turkeys are extremely sensitive to aflatoxin B_1 (AFB_1) which causes decreased growth, immunosuppression and liver necrosis. The purpose of this study was to determine whether probiotic *Lactobacillus*, shown to be protective in animal and clinical studies, would likewise confer protection in turkeys, which were treated for 11 days with either AFB_1 (AFB; 1 ppm in diet), probiotic (PB; 1×10^{11} CFU/ml; oral, daily), probiotic + AFB_1 (PBAFB), or PBS control (CNTL). The AFB_1 induced drop in body and liver weights were restored to normal in CNTL and PBAFB groups. Hepatotoxicity markers were not significantly reduced by probiotic treatment. Major histocompatibility complex (MHC) genes *BG1* and *BG4*, which are differentially expressed in liver and spleens, were not significantly affected by treatments. These data indicate modest protection, but the relatively high dietary AFB_1 treatment, and the extreme sensitivity of this species may reveal limits of probiotic-based protection strategies.

1. Introduction

Aflatoxin B_1 (AFB_1) is a carcinogenic contaminant commonly present in maize and other corn-based animal feeds. The potent effects of this mycotoxin on young turkey poults were first identified as a result of the Turkey X outbreak of 1960, which involved the deaths of more than 100,000 birds and led to the discovery of AFB_1 (Blount, 1961; Spensley, 1963). In poultry, AFB_1 induces many deleterious effects, including reduced body weight gain, decreased organ weights, immunosuppression, hepatic necrosis, and leads to remodeling of the liver, especially fibrosis, biliary hyperplasia, and nodular tissue regeneration (Giambrone et al., 1985a, 1985b, 1985c; Newberne and Butler, 1969; Pandey and Chauhan, 2007; Sims et al., 1970). Even at concentrations below the US FDA action level of 110 ppb, AFB_1 causes poor performance, along with decreased growth rate, body weight, weight gain, egg production, reproductive performance, feed efficiency (Arafa et al., 1981; Pandey and Chauhan, 2007) and overall productivity in commercial birds (Weibking et al., 1994).

Domestic turkeys are one of the most susceptible animals to AFB_1. Their extreme sensitivity to aflatoxicosis is associated with a combination of efficient microsomal activation of AFB_1 to the toxic intermediate, *exo*-AFB_1-8,9-epoxide (AFBO), via cytochrome P450 enzymes, and inefficient detoxification of this intermediate by glutathione-S-transferase (GST) enzymes (Diaz et al., 2010; Klein et al., 2000; Rawal and Coulombe, 2011). For toxicosis to occur, AFB_1 must be bioactivated to the reactive electrophile AFBO, which in turkey liver is catalyzed primarily by hepatic cytochrome P450 CYP1A5 and CYP3A37 (Rawal, 2010; Rawal et al., 2009; Reed et al., 2007; Yip and Coulombe, 2006). AFBO forms adducts with DNA, RNA, and proteins and other critical biomolecules (Bedard and Massey, 2006; Corrier, 1991; Coulombe, 1993; Eaton and Gallagher, 1994; Klein et al., 2000; Rawal et al., 2010). Detrimental effects from these adducts are facilitated in domestic turkeys by a lack of GST-mediated AFBO detoxification activity in the liver (Klein et al., 2000, 2002a, 2002b, 2003).

Several studies have shown that certain species of viable or heat-killed bacteria, including *Lactobacillus* and *Propionibacterium*, are

* Corresponding author. Tel.: +1 435 797 1598; fax: 435 797 2118.
E-mail address: roger@usu.edu (R.A. Coulombe, Jr.).
[1] Current address: Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN 38163, USA.
[2] Current address: National Marrow Donor Program, Be The Match, Minneapolis, MN 55413, USA.
[3] Current address: Avian Research Center, University of British Columbia, 2357 Main Mall, Vancouver, BC, Canada V6T 1Z4.

http://dx.doi.org/10.1016/j.rvsc.2014.06.008
0034-5288/

capable of binding to and thereby detoxifying AFB_1 in many animals including chickens, rats, mice, and in humans (Da-Silva and Oluwafemi, 2009; Deabes et al., 2012; El-Nezami et al., 2000; Gratz et al., 2005, 2006; Lahtinen et al., 2004; Lee et al., 2003). *Lactobacillus sp.* are of particular interest, because of their widespread use in animal feeds in addition to their chemoprotective effects in humans and animals (Commane et al., 2005). Several species of *Lactobacillus* improve daily feed intake of AFB_1 challenged rats, returning measurements to normal levels (Hathout et al., 2011).

While primarily an hepatotoxin and hepatocarcinogen, AFB_1 is also a potent immunotoxin in poultry, suppressing cell-mediated, humoral, and phagocytic functions (Coulombe, 1993; Qureshi et al., 1998, 2000). Embryonic exposure to AFB_1 results in a compromised immune response in hatched chicks through suppression of humoral and cellular immunity (Qureshi et al., 2000). Immunosuppression appears to be at least partly due to upregulation of cytokines, including IL-6 (Yarru et al., 2009). Microarray gene expression profiling of broiler chickens exposed to dietary AFB_1 revealed downregulation of many hepatic genes related to immune function, consistent with the immunosuppressive effects of aflatoxicosis (Yarru et al., 2009).

Knowledge of host gene expression in AFB_1-challenged animals is limited, and there are no published data regarding changes in expression of genes in the major histocompatibility complex (MHC), despite the well-known role of these genes in disease resistance and antibody production. Preliminary RNA-seq data suggest that *BG* genes in the turkey may be responding to AFB_1 exposure (KM Reed, unpublished observations). *BG* genes are part of a polymorphic multi-gene family in the MHC of avian genomes. In the turkey, there are at least six *BG* genes, which are organized in two clusters and tightly linked to the class I and class II loci (Bauer and Reed, 2011; Chaves et al., 2009). Expression data on *BG* genes is limited, and their functions have yet to be fully elucidated, although many are hypothesized (Goto et al., 2009; Kaufman et al., 1991). Early studies describe *BG* molecules as erythrocyte antigens (Koch et al., 1983; Longenecker and Mosmann, 1980), and there is convincing evidence for cell surface expression on erythrocytes as well as in cecal, small intestine, and liver tissues (Miller et al., 1990; Salomonsen et al., 1987, 1991). Given their hypothesized cell signaling and regulatory functions, we were interested in their potential association with aflatoxicosis in a sensitive animal species.

This study was designed to examine the potential protective effect of a *Lactobacillus*-based probiotic mixture (El-Nezami et al., 1998a, 1998b) in the turkey, an extremely sensitive species. Endpoints of aflatoxicosis included liver histopathology, serum profile, and *BG* expression as measured by quantitative real-time polymerase chain reaction (qRT-PCR).

2. Materials and methods

2.1. Probiotic mixture

A probiotic mixture of lyophilized bacteria from Valio Ltd. (Helsinki, Finland) was used in the challenge trial. This mixture contained 2.3×10^{10} CFU/g of *L. rhamnosus* GG, 3.0×10^{10} CFU/g of *L. rhamnosus* LC-705, 3.5×10^{10} CFU/g of *Propionibacterium freunchdenreichii sp.* shermani JS, and 2.9×10^{10} CFU/g of *Bifidobacterium sp.*, along with 58% microcrystalline cellulose, 27% gelatin and magnesium salt. The probiotic solution was prepared by directly suspending bacteria in phosphate buffered saline (PBS, pH 7.4) at a final concentration of 1×10^{11} CFU/ml as described in previous AFB_1 protection studies (Gratz et al., 2006).

2.2. Animals and experimental treatment

Briefly, the experimental design was as follows: after acclimatization for 10 days, 10 birds were randomly placed into one of four groups, AFB_1 (AFB), probiotic (PB), probiotic plus AFB_1 (PBAFB), and control (CNTL). For the first 10 days, the PB and AFB groups received pretreatment with 0.5 ml of probiotic mix (final concentrations of 1×10^{11} CFU/ml PBS by oral gavage, daily) and the CNTL and AFB groups received pretreatment with PBS (0.5 ml by oral gavage, daily). After 10 days, dietary AFB_1 (1 ppm; for groups AFB and PBAFB) commenced in similar fashion to previous experiments (Klein et al., 2002b). The probiotic and dietary AFB_1 treatments continued for another 11 days, and Animals were then euthanized by CO_2 asphyxiation. Blood was collected by cardiac puncture into sterile, no-additive tubes (Becton Dickinson Vacutainer®, Franklin Lakes, NJ) for serum and in heparinized tubes for plasma. The blood was allowed to clot and serum removed within 1 h of collection. Likewise, whole blood was centrifuged and plasma was removed within 1 h of blood collection in heparinized tubes. Samples were analyzed for total protein, *alkaline phosphatase* (ALP) and *gamma glutamyl transpeptidase* (GGT) at Intermountain Health Care Analytical Laboratory (Logan, UT). Livers were rapidly removed and frozen on dry ice. Livers were rapidly removed and portions frozen on dry ice or sections were fixed in neutral buffered 10% formalin for histological analysis or perfused in RNAlater (Ambion Inc., Austin, TX) solution for qRT-PCR analysis. Sections of spleens were also perfused in RNAlater. The serum, plasma, and tissues collected were stored at – 80 °C until analyzed. RNAlater samples were perfused at 4 °C overnight then stored at –20 °C. Turkeys were weighed on days 1, 8, 15, 19, and before euthanasia on day 21, and livers were weighed when sampled.

2.3. Histological analysis

A section of each liver was embedded in paraffin using a Model TP1050 Embedding Station (Leica Microsystems, Deerfield, IL), thin sectioned (RM 2145 Microtome, Leica Microsystems), and stained with hematoxylin and eosin (H&E) (Jung Autostainer XL, Leica Microsystems). Tissues were coded, then fixed to slides for histological analysis for hepatic necrosis and biliary hyperplasia, typical signs of aflatoxicosis in turkeys (Klein et al., 2002b). A numerical score of 1 to 5 for the severity was assigned to each sample as follows: hepatocellular necrosis, based on percent of viewed cells affected, $1 \leq 5\%$, $2 \geq 5$ to 30%, $3 \geq 30$ to 60%, $4 \geq 60$ to 80%, or $5 \geq 80\%$; biliary hyperplasia, 1 = normal, 2 = mild proliferation without parenchymal displacement, 3 = moderate proliferation with some mild parenchymal displacement, 4 = moderate to severe proliferation with moderate parenchymal displacement, or 5 = diffuse proliferation with severe parenchymal displacement (Klein et al., 2002b).

2.4. Statistical analysis

Treatment groups were compared for differences, using appropriate ANOVA models and post-hoc tests as described in Results. LSMEANS and Tukey method were used for analysis of serology data with $P = 0.05$ chosen as statistically significant.

2.5. Quantitative analysis of BG expression

2.5.1. RNA isolation and cDNA synthesis

Total RNA was isolated from liver and spleen samples (9 individuals per group) using Trizol (Ambion Inc.), extracted using phenol/chloroform following the manufacturer's protocol, DNase treated (Turbo DNA-*free*™ kit RNA, Ambion Inc.) and stored at –80 °C. Quality of extracted RNA was determined by visualization on a 1% form-