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Effects of *Curcuma longa* dietary inclusion against *Eimeria* spp. in naturally-infected lambs

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ABSTRACT

Ovine coccidiosis caused by *Eimeria* spp. can negatively impact health and overall productive performance in sheep with mortalities up to 20% in lambs. It is characterized by high production of pro-inflammatory cytokines and oxidative stress that can damage intestinal tissue. Currently, only drugs are used for the treatment of ovine coccidiosis. Nevertheless, anticoccidial resistance and the concern of drug residues in edible tissues and milk have prompted the evaluation of alternatives to prevent and control this disease. Based on preliminary findings, the use of *Curcuma longa* dietary supplementation was evaluated in this trial. Twenty crossbred lambs naturally infected with *Eimeria* spp., aged 28-days-old with an average weight of 12 kg, were divided in five groups. Three groups were treated orally for 14 days with 50 mg/kg, 100 mg/kg, or 200 mg/kg of *C. longa*. A placebo-treated group and untreated controls were included in this trial, too. Stool samples were obtained every other day to determine anticoccidial efficacy. Also, animals were weighed on day 0 and 42. To evaluate the immunomodulatory activity of curcumin, a proinflammatory (IFN-γ) and an immunoregulatory (IL-10) cytokine were measured by ELISA. Lipid peroxidation and nitrite generation were determined by means of the serum malondialdehyde test and the Griess reaction, respectively. *C. longa* anticoccidial activity increased over time in treated groups and reached a 100% efficacy on day 42. Animals treated with 200 mg/kg of this plant gained twice the daily weight recorded for untreated groups. IL-10 levels were higher in supplemented animals, whereas lipid peroxidation and generation of nitrites were significantly lower. Results showed that curcumin administration may reduce oocyst output, weight-loss, inflammation and oxidative stress-related effects caused by *Eimeria* spp. infection in lambs.

1. Introduction

Ovine coccidiosis is a disease caused by *Eimeria* protozoans. It affects the small and large intestine portions of the gastrointestinal tract. Animals become orally infected and they can be simultaneously affected by different species. Currently, 11 species that affect sheep have been recognized with *Eimeria crandallis* and *Eimeria ovinoidalis* being the most pathogenic ones (Andrews, 2013; Chartier and Paraud, 2012). This disease has economic and health impacts, mainly because lambs develop a subclinical disease. Yet, mortality can reach up to 20% in lambs, mainly because they have not yet acquired specific immunity (Catchpole et al., 1993; Kommuru et al., 2014; Veira, 2002). Currently, there is no vaccine available to prevent coccidiosis in sheep. However, in recent years, many studies have shed light on the protective immune responses that the ruminant host develops against *Eimeria*. The parasite is capable of inducing a functional immune response associated with the induction of innate and adaptive immune responses. Macrophages and polymorphonuclear neutrophils (PMN), are the main contributors to the cytokine generation that is observed in ruminant coccidiosis (Hermosilla et al., 2006). Previous studies have also shown that these cells are the main sources of reactive oxygen species (ROS) (Taubert et al., 2009), such as peroxynitrite, hypochlorous acid and hydroxyl radicals (Sild et al., 2011). In turn, there is a

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growing body of evidence that suggests that ROS contribute to several signalling pathways and can produce dysregulated responses eventually cause lethal pro-inflammatory-mediated tissue damage (Aline et al., 2002; Chow et al., 2011; Simons et al., 2012). It is also well known that neutrophils and leukocytes trigger an increased production of reactive nitrogen species in the intestinal mucosa, and cause protein modification and induction of pro-inflammatory cytokines (Catanzaro et al., 2015). Regarding coccidiosis, it has been shown that PMN induce iNOS upon exposure to *Eimeria bovis* sporozoites (Behrendt et al., 2008). Previous reports have demonstrated that challenge infections with *E. bovis*, generate an infiltration of CD4+ and CD8+ T cells in the intestinal tissue (Sühwold et al., 2010). Cytokines such as interleukin-2 (IL-2) and IFN-γ increase during prepatency in the primary infection caused by *E. bovis*; whereas IL-4 is dominant in patency (Taubert et al., 2008). On the other hand, IL-10 displays an immunoregulatory function and prevents tissue damage induced by infection with pathogens that are controlled by CD4+ T under high inocula situations or certain immunoregulatory deficiencies (Jankovic et al., 2010; Barbosa et al., 2015; Magombedze et al., 2015).

Several drugs are licensed for the control of ovine coccidiosis, such as decoquinate, diclazuril and toltrazuril. However, there is growing concern in using them due to drug-residue issues, and strict withdrawal periods that must be observed prior to marketing milk or meat (Andrews, 2013). This has prompted the study of alternative methods to control this parasite while keeping in mind that concerns of potentially toxicity derived from residues in edible animal proteins and environmental impact must be accounted for (Chagas, 2004). Some natural products have shown promising results; for example clinoptilolite (Alcala-Canto et al., 2011), plants that contain condensed tannins, (Burke et al., 2013); and the dried rhizome of *Curcuma longa*, all having anticoccidial effects (Kommuru et al., 2014; Saratsis et al., 2012). In recent decades, there have been several studies detailing the antiparasitic effects of curcumin, especially in *Plasmodium falciparum* (Reddy et al., 2005), *Cryptosporidium parvum* (Shahiduzzaman et al., 2009), *Giardia lamblia* (Said et al., 2012), *Trypanosoma* spp. (Nagajyothi et al., 2012) and *Eimeria tenella*. In this latter scenario, curcumin induces apoptosis by the presence of precipitates on the sporozoite surface that affect its morphology, viability and adhesion ability (Chattopadhyay et al., 2004). The severity of these alterations depend on the dose and delivery time of the *C. longa* extract denominated curcumin (Khalafalla et al., 2011). In spite of the above, information about the dosage and delivery time of curcumin from *C. longa* in lambs is not available. Under certain circumstances, a severe inflammation of the intestinal tissue of infected lambs can occur i.e., ingestion of a high inoculum of *Eimeria* and a dysfunctional immune response. Consequently, a field trial with lambs naturally-infected with *Eimeria* spp. was regarded as a useful setting to assess the anticoccidial and immunomodulatory activities of a powdered preparation of *C. longa,* measuring oocyst counts, lipid peroxidation, nitrite generation, as well as concentrations of a proinflammatory (IFN-γ) and an immunoregulatory cytokine that prevents excessive inflammation (IL-10).

2. Materials and methods

2.1. Study site and animals

The study was conducted at the experimental farm of the Autonomous University of Chapingo, which is located in Texcoco, State of Mexico, according to procedures approved by its Animal Care and Use Committee. This area has a semi-humid weather, with an average annual temperature of 15.9 °C and an annual rainfall of 686 mm.

This trial included 20 crossbred female lambs with a mean body weight (±standard deviation) of 12.345 ± 1–15 kg, naturally infected with *Eimeria* spp. and without a history of anticoccidial treatment. The lambs were approximately 28 days old at the beginning of this study. Animals were fed with a diet that consisted of 60.5% of corn silage, 24.2% alfalfa pellets, 8.5% oatmeal, 5% molasses, 1.5% calcium carbonate, 0.2% of salt and 0.5% of a vitamin and mineral premix, with a content of 13.56% crude protein, 2.68% ether extract, 19.66% fiber and 13.29% ashes, according to the Proximal Chemical Analysis performed in the Animal Nutrition and Biochemistry laboratory of the Veterinary Medicine and Animal Science Faculty of the UNAM. Water was provided ad libitum. Lambs were kept on 8-m^2 concrete floor pens with rough surfaces to prevent slipping and straw bedding. A shovel was used to remove bedding and manure in order to clean the pens. Each animal was screened for the presence of *Eimeria* spp. by faecal oocyst count examination prior to the start of the study. Initially, 30 animals were included. Nevertheless, ten lambs shed less than 100 *Eimeria* oocysts per gram of faeces. In consequence, they were not used in the study

2.2. Curcuma longa *crackers and total curcuminoid quantification*

To reduce costs, a food-grade brand of commercial powdered *C. longa* (Shan Khalis Haldee Turmeric Powder) was used in this study and purchased from a local spice-importing company (Ultra Chem, Estado de Mexico, Mexico). Total curcuminoid concentration of the powdered presentation of *C. longa* was quantitatively determined using a UV spectrophotometric method validated in a previous study (Sharma et al., 2012). In order to carry out the analytical method, the UV spectra of a standard solution of curcumin prepared in methanol (3 μg/ml) was registered. The wavelength of maximum absorbance determined via UV spectrophotometry was 421 nm. A curcumin standard (Sigma–Aldrich, St. Louis, MO) containing 94% of curcuminoids was used. Subsequently, different concentrations of curcumin were prepared to evaluate linearity, range and accuracy of the method. Validation of this method was carried out according to the International Conference on Harmonization (2005). The coefficient of variation was <2.0% and linear correlation coefficient was 0.9982, suggesting a good model fit.

In order to ease *C. longa* intake by the animals, crackers (biscuits) were made with this spice. Each cracker contained wheat flour, water, distilled sugar cane and an artificial pineapple flavoring. For each cracker, *C. longa* powder was incorporated according to the dose used for each animal and for each group. For the purpose of making the *C. longa* powder homogenous, distilled sugar cane was added. To improve palatability, an artificial pineapple food flavouring was stirred into the mixture. Crackers without *C. longa* were made as placebo.

In the interest of determining curcuminoid contents, *C. longa* crackers were oven-dried at 35 °C for 24 h. Each cracker was individually weighed using an analytical balance and crushed to a fine powder. A sample of homogenized powder was weighed and diluted in methanol. For extraction purposes, ultrasound was applied to the samples for 15 min. The extract was then filtered through 45 μm filter membranes (Millipore México). The commercial curcumin standard was prepared in the same way as the samples. Both samples and the standard were introduced into a spectrophotometer (Evolution™ 60, Thermo Scientific), which was set at 421 nm. A calibration curve was created by plotting the peak area ratio of curcumin to internal standard versus the curcumin concentration (Jäger et al., 2014). Crackers prepared with 50 mg, 100 mg or 200 mg of *C. longa* powder were shown to contain 1.248 mg, 2.307 mg or 4.336 mg of curcumin per 100 mg of powder, respectively.