The effects of combining Artemisia annua and Curcuma longa ethanolic extracts in broilers challenged with infective oocysts of Eimeria acervulina and E. maxima

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Parasitology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>PAR-2012-0356</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Research Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>05-Oct-2012</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Almeida, Gustavo; Aarhus University, Agroecology  
Thamsborg, Stig; University of Copenhagen, Danish Centre for Experimental Parasitology  
Madeira, Alda; University of São paulo, Department of Parasitology, Institute of Biomedical Sciences  
Ferreira, Jorge; USDA-ARS, US Salinity Laboratory  
Magalhães, Pedro; University of Campinas, Chemical, Biological and Agricultural Pluridisciplinary Research Centre (CPQBA)  
Demattê Filho, Luiz; Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Department of Economy and Rural Sociology  
Horsted, Klaus; Aarhus University, Department of Agroecology  
Hermansen, John; Aarhus University, Department of Agroecology |
| Key Words: | Broiler, Natural anti-protozoa drugs, coccidiostats, drug combination, plant extract, herbal medicine, coccidiosis |
The effects of combining *Artemisia annua* and *Curcuma longa* ethanolic extracts in broilers challenged with infective oocysts of *Eimeria acervulina* and *E. maxima*

Gustavo F. d. Almeida\(^{a}\); Stig M. Thamsborg\(^{b}\); Alda M. B. N. Madeira\(^{c}\); Jorge F. S. Ferreira\(^{d}\); Pedro M. Magalhães\(^{c}\); Luiz C. Demattê Filho\(^{f}\); Klaus Horsted\(^{a}\); John E. Hermansen\(^{a}\)

\(^{a}\) Department of Agroecology, Faculty of Sciences and Technology, Aarhus University. Research Centre Foulum, Blichers Allé 20, P.O. Box 50 DK-8830 Tjele, Denmark.

\(^{b}\) Danish Centre for Experimental Parasitology, Faculty of Life Sciences, University of Copenhagen, Dyrlægevej 100, DK-1870 Frederiksberg C, Denmark.

\(^{c}\) Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 1374, São Paulo, SP, Brazil.

\(^{d}\) USDA-ARS, US Salinity Laboratory, 450 W. Big Springs Rd., Riverside, CA 92507-4617.

\(^{e}\) Chemical, Biological and Agricultural Pluridisciplinary Research Centre (CPQBA), University of Campinas – UNICAMP, P.O. Box 6171, BR-13081-970 Campinas, SP, Brazil.

\(^{f}\) Department of Economy and Rural Sociology, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Avenida Pádua Dias 11, Piracicaba, SP, Brazil.

\(^{*}\) Corresponding author: Gustavo Fonseca de Almeida; Tel. +45 8715 4757; Fax. +45 8715 4798.

E-mail address: [Gustavo.deAlmeida@agrsci.dk](mailto:Gustavo.deAlmeida@agrsci.dk)

Correspondence address for proofs:

Department of Agroecology, Faculty of Sciences and Technology, Aarhus University. Research Centre Foulum, Blichers Allé 20, P.O. Box 50, DK-8830, Tjele, Denmark.
SUMMARY

Due to an increasing demand for natural products to control parasites in animal production we investigated the effects of combining ethanolic extracts of *Artemisia annua* and *Curcuma longa* supplemented in drinking water to prevent coccidiosis in broilers. Three different dosages of the herbal mixture were compared with a negative control (uninfected), a positive control (infected and untreated), chemical coccidiostats (nicarbazin+narazin and later salinomycin), vaccination, and a product based on oregano. Differences in performance (weight gain, feed intake, and feed conversion rate), mortality, gross intestinal lesions and oocyst excretion were investigated. Broilers supplemented with chemical coccidiostats presented superior performance attributes compared to all other groups. A dose-dependent response for scoring of lesions was observed for the herbal extracts. In average, broilers supplemented with the two highest dosages of the mixture presented intermediate scores, higher than broilers supplemented with coccidiostats but less than broilers supplemented with vaccination, oregano and negative controls. A trend for lower mortality (p=0.08) in the last part of growing period (23-43d) was also observed in broilers supplemented with the highest dosage when compared to broilers supplemented with chemical coccidiostats. In conclusion, the delivery strategy is practical for implementation at farm level but further studies on dose levels and modes of action are needed.

**Keywords**: Broiler; Natural antiprotozoa drugs; coccidiostats; drug combination; plant extract; herbal medicine; coccidiosis.
INTRODUCTION

Coccidiosis is a very important parasitic disease in the broiler industry (Shirley et al., 2005) and it is caused by highly host-specific protozoan parasites belonging to the genus *Eimeria* (Williams, 1999). Parasites multiply in the intestines damaging gut tissues which, in turn, reduces feed intake and absorption of nutrients (Morris et al., 2007). Infections caused simultaneously by *E. acervulina*, *E. maxima*, and *E. tenella* are frequently diagnosed in intensive poultry farming systems (McDougald et al., 1997), with their control usually at a higher priority over the other *Eimeria* species affecting broilers (Shirley et al., 2005). The disease is primarily controlled by medication and a range of limitations have been reported in the past few years (Martin et al., 1997). Underdose of anticoccidials used in the feed of broilers could lead to parasitic resistance (Daugschies et al. 1998) and the extensive use of drugs has led to the development of resistance to coccidostat products (Chapman, 1993), which also could be phased out as feed additives before January 1st 2013 for public health and food safety reasons (COM, 2008).

Research has been carried out to find effective, but non-pathogenic, vaccines (Lillehoj and Trout, 1993) to be used in substitution to anticoccidial products. In addition, the use of plants and extracts as therapeutics might be an option (Akhtara et al., 2012). The substitution of ionophore drugs is unlikely to be matched by a single approach, but a comprehensive combination of strategies could help to reduce the prevalence of coccidiosis in intensive production systems (Pinard-van der Laan et al., 1998).

In organic systems, the use of live attenuated vaccines (Williams, 2002) is a useful option available in the market but these are expensive to produce (Abbas et al., 2012). Cost effective alternatives are demanded for safer and effective strategies to control the disease, and several candidate medicinal plants have been investigated (Naidoo et al., 2008; Wang et al., 2008). New natural ingredients to substitute chemicals products, e.g. plants, extracts and their combinations are believed to play an important role in the near future because they are usually residue free and well accepted by consumers (Orengo et al., 2012). For example, *Artemisia annua* was earlier suggested to control *Eimeria acervulina* in prophylactic supplementation strategies before
coccidial challenges (Allen et al., 1997). However, different levels of inclusion of artemisinin (main bioactive component of *A. annua*) in feed and oral supplementation had no effect against *E. maxima*, suggesting a species-specific mode of action (Arab et al., 2006; Allen et al., 1997).

Another relevant plant with known anti-protozoa properties (Shahiduzzaman et al., 2009) is the spice turmeric *Curcuma longa*, suggested as a potential candidate to treat human malaria (Cui et al., 2007; Reddy et al., 2005). In Pakistan, small farmers supplement turmeric powder as a feed additive for the control of coccidiosis in broilers (Abbas et al., 2012). Allen et al. (1998) reported reduction on intestinal lesions and suppression in oocyst excretion when 1% dietary inclusion was available to broilers challenged with *E. maxima* oocysts. The bioactive ingredient responsible for efficacy against the parasites is curcumin (diferuloylmethane), a phenolic compound with high antioxidant (Subramanian et al., 1994), anti-inflammatory (Huang et al., 1997), anti-tumor properties (Rao et al., 1995), and found in *C. longa* roots in concentrations ranging from 1 to 5% (Conney et al., 1991). Souza and Glória (1998) screened a range of samples from important commercial sites in Brazil and reported an average concentration of 4% w/w curcumin in the powder.

Artemisinin was reported to have a rapid metabolism *in vivo* (Chen et al., 2009) with low stability and fast decomposition following ingestion (Klaiman, 1985). Curcumin has been tested as an attractive partner for artemisinin in combination therapy against malaria as the molecule has a short half-life similar to artemisinin (Nandakumar et al., 2006). With similar pharmacokinetics an optimum protection in a resistance prevention perspective may be achieved (Nosten and White, 2007).

With reported anti-protozoan activity of both artemisinin and curcumin, we hypothesized that the combination of these ingredients available in *A. annua* and *C. longa* herbal extracts could provide synergistic effects and act against dual infections caused by *E. maxima* and *E. acervulina* oocysts in fast growing broilers, similar to chemical coccidiostats.

Regarding strategies for delivering herbal extracts to broilers, 3% dried leaves of *A. annua* was supplemented in the feed three weeks before a challenge with *E. acervulina* and *E.
maxima (Almeida et al., 2012). Feed intake was impaired supposedly due to unpalatable components available in the A. annua leaves even though oocyst excretion was suppressed by 60-70% (Almeida et al., 2012). In another study comparing different strategies of delivery of A. annua to chickens naturally infected with Eimeria spp., supplementation of ethanolic extracts via drinking water, as recommended by an organic farmer, did not impair feed intake but the effect on oocyst excretion was limited (Almeida et al., unpublished data).

On this background, this study was undertaken to evaluate the effect of three different dose levels of herbal extracts of artemisinin:curcumin on avian coccidiosis (intestinal lesions, oocyst excretion and performance attributes). The extracts were administered in the drinking water and compared to appropriate controls, to broilers supplemented with chemical products (representing the conventional production system), vaccinated or supplemented with a product based on oregano, thus representing alternative strategies available in the market to control coccidiosis in organic production systems.

MATERIALS AND METHODS

Experimental design and broilers

The study was carried out at the experimental stable at Korin Agricultural Ltd. (www.korin.com.br) located in the municipality of Ipeúna, Sao Paulo, Brazil (22° 24′ S, 47° 41′ W). On 15th of August 2011, one-day-old male broilers (Cobb 500S, n=1440) previously vaccinated in hatchery against Marek, fowlpox, and Gumboro were randomly allocated to 48 pens distributed in eight treatment combinations with six replicates of 30 broilers totalizing 180 broilers per treatment. Three different dose levels of a combination A. annua and C. longa ethanolic extracts (LOW; MEDIUM and HIGH) supplemented in drinking water were compared with a negative control (uninfected, untreated), a positive control (infected and untreated), treatment with chemical coccidiostats (see next section), vaccination and a product based on oregano. Table 1 describes the treatments, the supplementation strategies, doses, age and duration of treatments. Each pen was 3.0 m² (1.5 x 2 m) with food and water supplied ad libitum.
All pens were cleaned and disinfected two days before the beginning of the experiment and high standards of hygiene were followed during the trial. All practical handling was performed from outside the 48 pens in lateral corridors and the pens were isolated from each other by the use of PVC walls. When it was necessary, technicians were trained in using disposable plastic bags in their shoes to avoid contamination. Broilers were raised on concrete floor with wood shavings until they were 43 days of age, when the experiment was terminated. Broilers were raised in the comfort range temperature and humidity recommended by the strain manual and the experimental protocols evolving broilers were in accordance with international guidelines for animal care and health and with the Brazilian legislation.

Experimental diets, water supply and oral administration of herbal extracts

The feeding program in our study followed the routine of farmers linked to the collaborative broiler company: initial (1-8 days), growth (9-22 days), fattening I (23-29 days), fattening II (30-36 days old) and final (37-43 days). Broilers in the control groups (positive - T1 and negative – T4) were supplemented with basal diet without any other type of supplementation. Nicarbazin + narazin (Maxiban®) 50 ppm was supplemented from 1 to 22 days of age and salinomycin (Coxistac®) 66 ppm supplemented from 22 to 39 days of age in diet for broilers receiving the chemical coccidostats (T3). Livacox vaccine at recommended doses (Merial Ltd. Brazil) was administrated to the broilers in the same day broilers arrived from hatchery by the use of a movable drinker allocated in each replicated pen (T2). Broilers in the group T5 were supplemented with a product based on oregano extract with carvacrol + timol (Regano®) and broilers in T6, T7 and T8 received the herbal mixtures via drinking water since the first day of life.

Eight water tanks (one tank per treatment) with capacity for 160 L were placed in the top of the barn and water was distributed by gravity to each replicated pens (n=6 per treatment) by the use of plastic hoses. Every second day from the beginning of the trial, water from the tanks with the mixture of herbal extracts, also including T5, was replaced. Treatments were prepared fresh daily to avoid any confounding factor related to possible instability or oxidation of the herbal components.
The herbal extracts of *A. annua* and *C. longa* were prepared at CPQBA - University of Campinas - using the same extraction procedure. In summary, in two 1 L Erlenmeyer flasks wrapped with aluminium foil to prevent oxidation and degradation by light, 100 g of dried leaves or 100 g curcuma powder were mixed with 1 L ethanol 70% (700 mL ethanol 97° + 300 mL distilled water). The mixtures were kept in a dark room with controlled temperature (22°C) and homogenized with circular movements twice per day for 20 days. The contents were filtered by using paper filters and transferred to 100mL flasks with dropping glass dispensers. Flasks were properly identified and stored in the fridge until needed.

One week before the trial, flasks with the Ethanolic herbal extracts of *A. annua* and *C. longa* were transported to the experimental station at Korin Agropecuária Ltd. and kept in the fridge before its use. One day before allocating the chicks to the pens, the ethanolic concentrated solutions were mixed with 155 liters tap water using the same volume depending on the dosage: (1.5 mL of each ethanolic extract in T6, 2.5 mL for T7 and 3.6 mL for T8). The content of active ingredients was estimated by HPCL according to Ferreira and Gonzales (2009). 1.15mg artemisinin/mL was found for the *A. annua* ethanolic extract (unpublished data) while from proximal calculations, the ethanolic extract of *C. longa* had an estimated concentration of 4.6 mg curcumin/mL. In accordance to relevant literature on curcumin and artemisinin contents in raw materials and in herbal ethanolic extracts (Souza and Gloria, 1992; Ferreira et al., 2011 respectively) we estimated that the three dosages supplemented represented a 1:4 ratio of artemisinin:curcumin (Table 1).

**Transmission by contact and monitoring strategy**

At day 8 three randomly chosen broilers per pen (n= 18 per treatment) were artificially inoculated by gavage with a 1 mL distillated water suspension containing 5x10⁴ *E. maxima* + 2x10⁵ *E. acervulina* sporulated oocysts. These broilers were nominated "seeders" and were raised with contemporary broilers with the aim of transmitting the disease by contact (Velkers et al., 2010). “Seeders” were removed from the pens nine days post inoculation and harvested (cervical dislocation) for scoring of lesions (n= 5 per treatment) following Johnson and Reid...
(1970) and supported by illustrations provided by Anon (1990). Results of scoring of lesions are presented by the mean (± SD) for lesions caused by *E. acervulina* (duodenum), *E. maxima* (mid part of small intestine) and averaged for the total lesions observed.

To assess infection dynamics spread by the “seeders”, five birds were randomly selected in each pen and nominated as “tracers”. Twice weekly starting three days post inoculation of “seeders”, the “tracers” were placed in communitarian cages (one cage per pen) during 15 minutes for pooled samplings of fecal samples. In total, eight pooled fecal samples per pen were examined during the trial. In the same day of faeces collection, pooled samples (n= 48) were analysed by a modified McMaster technique using saturated NaCl solution with 50% glucose monohydrate as flotation fluid with a sensitivity of 20 oocysts per g of faeces (OPG) (Almeida et al., 2012). Results are presented as the mean ± SEM of oocyst excretion (OE) for each treatment.

**Performance attributes**

Consumption of feed per pen was monitored and recorded every week. Individual body weight of the same ten contemporary broilers in each pen (not “seeders” or “tracers”) (n=60/treatment) was taken every week. We estimated performance attributes by considering the mean body weight at 24 hours post hatch, 22 days-old, and when broilers were 43 days of age before slaughter. With this strategy, we could estimate body weight gain, daily gain, feed intake and feed conversion rate for two main periods - before and after expected infection and for the total experiment (1-22d; 23-43d and 1-43 days of age). Results of performance attributes are presented as the mean ± SEM and averaged for each treatment combination.

**Statistical Analysis**

Broilers in our study were allocated to pens in a completely randomized design. Categorical data on lesion score was firstly analysed by linear regression to identify any possible effects attributed to different dosages and followed by multiple range tests. Data on mortality were analysed by the Chi-square test. The infection dynamics was analysed by a mixed
procedure (PROC MIX) in SAS (SAS, 2000) where the statistical unit was the logarithmical transformed value for each OPG observation according to the following model:

\[ Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + A_{k(ij)} + \varepsilon_{ijk} \]

where \( Y_{ijk} \) was the natural logarithm of the number of oocysts per gram of faeces (OPG) for each pen in each sampling date; \( \mu \) = the mean value; \( \alpha_i \) = the treatment, \( i = 1,2,3,4,5,6,7,8 \) (Uninfected negative control; Vaccinated, infected; Coccidiostat, infected; Untreated, Infected; Regano®, Infected; Dose 1 (LOW) of herbal extracts, Infected; Dose 2 (MEDIUM) of herbal extracts, Infected; Dose 3 (HIGH) of herbal extracts, Infected); \( \beta_j \) = the OPG sampling date; \( j = 1 \) to 8; \( (\alpha\beta)_{ij} \) = the interaction treatment \( \times \) OPG sampling date; \( A_k \) = the random effect of pen number (1 to 48) and \( \varepsilon_{ijk} \) = the residual; Sampling date was defined as repeated measurement and \( \varepsilon_{ijk} \) was assumed to have a multivariate distribution where observations from different treatments were uncorrelated while observations from different weeks were assumed to have a toeplitz (Type = TOEP) correlation structure. Differences in performance attributes were accessed by analysis of variances with one-way ANOVA tests and the dependent variables were the mean values for each attribute observed for each pen (body weight gain, feed intake and feed conversion rate) and the independent variables were the treatments. P-values less than or equal to 0.05 were considered statically significant.

**RESULTS**

Figure 1A illustrates the mean scores (±SD) of lesions observed in the duodenum of the “seeders”. As expected, no lesions were observed for the broilers in the negative control group (T1) and for broilers supplemented with the chemical coccidiostat (T3). However, vaccinated broilers (T2) presented the highest mean of scores with no differences from broilers in the positive controlled group (T4).

In addition, broilers supplemented with Regano® (T5) did not differ from vaccinated broilers (T2) in terms of lesions caused by *E. acervulina*. Broilers supplemented with LOW dose of the herbal mixture (T6) presented an intermediary mean score of lesions, not different from
broilers supplemented with Regano® (T5), and not different to the other two doses of the herbal mixtures (T7 and T8). The two higher doses of the mixture *A. annua* and *C. longa* presented less lesions compared to the vaccinated broilers (T2), to broilers supplemented with Regano® (T5) and to broilers in the positive control group (T4). However, higher scores were observed for the mixed treatments (T6, T7 and T8) when compared with broilers supplemented with the chemical coccidiostat (T3) and with broilers in the negative control group (T1).

The scoring of lesions in the mid-intestinal mucosal surface (Fig 1B) indicated that broilers receiving the two highest dosages of the herbal combination (T7 and T8) did not differ from broilers supplemented with the chemical coccidiostats (T3). In addition, vaccinated broilers (T2) presented the smallest score of lesions in the mid part of the intestine while broilers supplemented with Regano® (T5) presented the highest score of lesions, similar to the infected non treated broilers (T4) and to broilers supplemented with the LOW dose of the herbal combination (T6).

When the sum of lesions (duodenum + mid intestine) was considered, a regression (p=0.04) was observed showing a dose dependent factor when supplementing the mixture *A. annua* and *C. longa* in the ratio 1:4 artemisinin:curcumin (Figure 1C). In summary, for each increase in dose supplementation (T4–T6–T7–T8), the mean score of lesions was reduced by a factor of 0.3.

Mortality was low in the first part of the experiment (1-22d) and no records were observed for broilers supplemented with the chemical coccidiostat. However, in the second growing period (22-43d), a trend in reduced mortality (p=0.08) was observed for the broilers treated with the highest dosage of the herbal combination T8 (0.0%) compared to mortality of birds treated with the chemical coccidiostat T3 (5.6%). Based on the oocyst excreted per g of faeces (OPG), broilers receiving the smallest dose (LOW) of the herbal combination (T6) excreted numerically more oocysts, similar to the infected, untreated (positive control) broilers (T4) and with broilers supplemented with Regano® (T5) (Table 2).
Broilers in the two groups T6 and T4 presented the highest infection peaks at 15 days post inoculation (PI) and a second peak at 22 days PI compared with broilers supplemented with the chemical coccidiostat (T3), in the uninfected group (T1) and with broilers receiving the MEDIUM dose of the combination *A. annua* and *C. longa* extracts (T7) (Figure 2). Considering the total amount of oocysts excreted, broilers supplemented with MEDIUM (T7) and HIGH (T8) levels of herbal ethanolic extracts presented intermediary OPG not different from the one recorded for vaccinated broilers (T2) and for broilers in the uninfected group (T1), and not statistically different from the broilers supplemented with the chemical coccidiostats (Table 2).

Broilers supplemented with the LOW dose of the botanical mixture (T6) and broilers vaccinated (T2) presented the smaller feed intake for the initial period (1-22d) compared to broilers in the uninfected group (T1) (Table 2). In the final period investigated (22-43d), broilers supplemented with Regano® (T5) and broilers vaccinated (T2) presented the highest feed intake compared with broilers in the negative control group (T1). When all experimental period is considered, feed intake was higher for the broilers consuming Regano® (T5) and smaller in the group of broilers consuming the HIGH dose of the botanical mixture (T8).

In the initial period, body weight gain (BWG) was higher for the broilers supplemented with the chemical coccidiostats (T3) but not different compared to broilers in the negative control group (T1). In addition, BWG in this period (1-22d) was not different when compared to broilers supplemented with the LOW dose of herbal mixture (T6) (Table 2). Broilers vaccinated (T2), supplemented with Regano® (T5) and broilers supplemented with the HIGH dose of the herbal mixture (T8) presented the smallest BWG.

In the second period (22-43d), after the dissemination of oocysts by the “seeders”, supplementation of chemical coccidiostats in the diet remained as the most efficient treatment in terms of BWG (Table 2). However, it was not different if compared with the vaccinated broilers (T2), broilers in the control group (T1) and the ones supplemented with the Regano® (T5). No differences in FCR were observed in all periods investigated. For the total period (1-43d), BWG was higher only for the broilers supplemented with the chemical coccidiostats. In addition, feed intake was impaired for the broilers supplemented with the Regano® (T5) and vaccinated (T2)
when compared to broilers supplemented with the HIGH dose of the botanical mixture (T8) for the total period investigated (Table 2).

DISCUSSION

To our knowledge this is the first study investigating to combined use *A. annua* and *C. longa* (ethanolic extracts) against coccidiosis in chickens. The study was performed based on reports on synergistic effects of the combination artemisinin:curcumin against malaria (Padmanaban et al., 2012; Nandakumar et al., 2006) and that synergistic effects caused by adding curcumin were not obtained in a ration 1:2 due to a high concentration of artemisinin (Isacchi et al., 2012). Our results suggest a dose-dependent effect (in a 1:4 ratio) against dual infections caused by *Eimeria acervulina* and *E. maxima* based on reduced lesions score, excreted oocysts, performance attributes, and mortality.

In general, broilers in the uninfected group (T1) showed no lesions in the mucosal surface attributed to infections caused by *E. acervulina* and, apparently, a very low score (mean of 0.4) attributed to infections caused by *E. maxima* (Figures 1A and 1B). However, when OPG counting was considered (Table 2 and Figure 2), “tracers” located in two replicated pens in this group (T1) started oocyst excretion at 18 to 23 days of age.

From one to 22 days of age, broilers in the negative control group (T1) presented no differences in BWG compared to broilers receiving the chemical coccidiostat (T3) but higher BWG if compared with broilers supplemented with the SMALL dose of the herbal mixture (T6). Feed intake and FCR were not impaired when compared with broilers consuming the chemical coccidiostat (Table 2). However, when broilers were 25 to 30 days old, oocysts were found in two replicates of this group (T1). Even with the strict hygiene measures applied during the study, broilers in all six replicates of the uninfected group (T1) were contaminated and excreted oocysts in the final part of their growing period (22-43d). Even with BWG for the second period not different compared to broilers consuming chemical coccidiostats (T3), when the total period is taking in to account (1-43d) this has led to a smaller BWG when comparing T1 with broilers supplemented with chemical coccidiostat (T3) (Table 2). However, Rosen (1995), in a review
with more than 1200 studies, concluded that supplementation with ionophores improves performance attributes of fast growing broilers, suggesting that the low infection experienced in “tracer” broilers for group T1 did not influence performance attributes and would be compatible with uninfected broilers. Nevertheless, flies, ants, other insects, and also the technician boots and hands can serve as vectors and highlight the opportunism and fast dissemination of the *Eimeria* parasites (Henken, 1994).

In contrast, broilers in the positive group (T4) presented the highest score of lesions (Figure 1) and higher excreted amount oocysts, without increasing mortality, compared to other groups (Table 2). Broiler “seeders” were artificially challenged with dual coccidial infections at 8 days of age and euthanized for scoring of lesions 9 days post inoculation, after sufficient time to contaminate the litter in each pen in accordance with the pre-patent period of *E. acervulina* and *E. maxima* parasites (Eckert et al., 2005). From this strategy, it was possible to assess the effects of treatments on intestinal lesions and at the same time allow “seeders” to infect contemporary broilers by contact (Velkers et al., 2010) imitating the natural transmission pathway occurring at commercial farms.

Broilers supplemented with chemical coccidiostats (T3) presented no lesions attributed to *E. acervulina* (Figure 1A) while few lesions attributed to *E. maxima* were observed (Figure 1B). In group T3, the parasites were not completely suppressed. According to Butaye et al. (2000) and with Martin et al., (1997), and more recently, Abbas et al. (2011), this points to a build-up of parasite resistance. By observing the infection dynamics from the consecutive OPG counting (Figure 2), a successful suppression in parasites reproduction was observed until broilers were 22 days of age. From this point ahead, apparently the drug of choice (salinomycin 12%) was not 100% efficient in suppressing the infection (Figure 2). However, even without complete suppression of oocyst excretion, performance attributes were superior for the group treated with coccidiostats (T3).

Vaccinated broilers presented the highest mean score of lesions (2.4) in the duodenum mucosal surface (Figure 1A). Perhaps the time of the *E. acervulina* challenge, provoked by the artificial inoculation (2 x 10^5 oocysts), was too close to vaccination time, suggesting that the time
interval between vaccination and challenge was not sufficient to allow a proper immunization, in agreement with Oviedo-Rondón et al. (2006). This is also in accordance with the theory that antibodies released after an initial challenge take time to induce protection by blocking the development and replication of parasites (Crane et al., 1988; Hafeez et al., 2007; Anwar et al., 2008).

Vaccinated broilers (T2) presented the lowest mean score of lesions (0.4) in the mid part of the small intestine, the site of *E. maxima* infections, comparable to uninfected controls (T1). On the one hand, vaccination could have been efficient in providing immunization against *E. maxima*, quite differently from what was observed with the absence of protection against *E. acervulina*. On the other hand, heavier challenges caused by *E. acervulina* may lead to inhibition of *E. maxima* to develop (Mathis, 2005). The former hypothesis sounds more plausible as the lesions caused by *E. acervulina* were higher for broilers in T2 (Figure 1A).

Khatafalla et al. (2011) reported a dose-dependent effect of curcumin against *E. tenella* sporozoites *in vitro*. However, there is no literature referring to the use of a mixture of *A. annua* and *C. longa* ethanolic extracts against coccidiosis in chickens. We observed a dose-dependent response when supplementing ethanolic extracts of *A. annua* and *C. longa* equivalent of a 1:4 ratio of artemisinin:curcumin. However, when accessing the scoring of lesions in the mid-intestinal mucosal surface caused by *E. maxima* infections (Fig 1B), broilers in T7 and T8 did not differ from broilers supplemented with the chemical coccidiostats (T3).

In terms of total oocyst excreted by “tracers” among the groups, broilers supplemented with MEDIUM and HIGH doses were not different compared to broilers supplemented with the chemical coccidiostats (Table 2). On the one hand, the two higher dosages of the mixtures *A. annua* and *C. longa* (T7 and T8) provided some protection compared to the untreated group (T4), although not statistically different for the total OPG (Table 2). Also, (as supported by Figure 1) total lesion score was significantly lower for groups T7 and T8 than for group T4 (infected, untreated). For the total period investigated (1 – 43d), groups T7 and T8 had numerical lower mortality rates (4.1 and 1.3% respectively) than the group (T3) treated with the chemical coccidiostat (5.6%). Even though they were not different for total oocyst excreted (Table 2), the
infection dynamics (Figure 2) illustrates that the broilers supplemented with the MEDIUM dose had a smaller but not statistically different infection peak than broilers supplemented with the HIGH dose, comparable to broilers supplemented with the chemical coccidiostat.

Although one could argue that the interval between samplings for OPG did not capture the highest period for occyst extraction, in our opinion, the combination of measured parameters (OPG, lesion score, and performance attributes) were sufficient to capture the differences observed among treatments.

For performance attributes, a numerical reduction in BWG was observed in the initial period (1-22d) suggesting that dose influenced weight gain. It could also be an indication of the presence of anti palatable components in ethanolic extracts, thus leading broilers to restrict feed intake as reported when supplementing dried leaves of *A. annua* to broilers (Almeida et al., 2012). Even though differences in feed intake were observed between groups, FCR was not impaired during this period (1-22d) refuting this hypothesis.

Strategies to control coccidiosis in conventional production systems are dependent on chemical drugs while in organic systems live vaccines or commercial preparations of plant-based products are used (Abbas et al., 2012). Some studies associate vaccination with botanical products. For example, Waldenstedt (2003) suggested vaccination in combination with oregano containing products for increased intestinal health of chickens, and thus reduce the effects caused by coccidiosis.

Before (1-22d) and after the coccidial challenge (22-43d), broilers supplemented with the chemical coccidiostat showed higher performance attributes in our study. However, broilers supplemented with the HIGH dose of the botanical mixtures (T8) presented smaller feed intake (13 to 22 g/day) compared with vaccinated broilers (T2) and broilers supplemented with Regano® (T5) without any differences in BWG and FCR (Table 2).

From the conditions experienced in this study, we concluded that broilers supplemented with chemical coccidiostats presented superior performance attributes during the trial due to a
better induction of protection against dual infections caused by *E. acervulina* and *E. maxima* parasites. However, despite lower BWG for the two highest doses of the extracts mixture, the protection against lesions, and trend in reducing mortality and OPG counts provided by the HIGH dose of the combined *A. annua* and *C. longa* ethanolic extracts, compared to the positive control, suggests that the supplementation of herbal extracts in drinking water can be a feasible alternative method for coccidia control in organic production systems. It is affordable, practical to be implemented at farm level, and is residue free. Other studies to investigate dosages and modes of action of this combination are warranted. In addition, supplementation of herbal extracts in combination with other management practices may help farmers engaged in the production of broilers to reduce the use of synthetic drugs.

ACKNOWLEDGEMENTS

The authors wish to thank Evandro Possamai, Dayana Pereira and Pedro Lorga from Korin agropecuaria Ltd. for the logistical and technical support during the study.

FINANCIAL SUPPORT

The Danish Ministry of Science, Technology and Innovation is thanked for financing collaboration among Brazilian and Danish scientists. Aarhus University, SOAR - Research School for Organic Agriculture and Food Systems and Korin Agropecuária Ltd. - are thanked for supporting our study.
REFERENCES


Table 1 – Description of treatments, acronyms, supplementation strategy and age of the broilers when treatments were supplemented

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acronym</th>
<th>Delivering strategy *</th>
<th>Age of the broilers (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control (untreated, uninfected)</td>
<td>T1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vaccinated¹, infected²</td>
<td>T2</td>
<td>Drinking water</td>
<td>1</td>
</tr>
<tr>
<td>Coccidiostat³, infected</td>
<td>T3</td>
<td>Dietary inclusion</td>
<td>1-39</td>
</tr>
<tr>
<td>Positive control (untreated, infected)</td>
<td>T4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Regano®⁴, Infected</td>
<td>T5</td>
<td>Drinking water</td>
<td>1-39</td>
</tr>
<tr>
<td>Dose 1 of Ethanolic herb extracts of <em>A. annua</em> + <em>C. longa</em> (11 ppb Artemisinin + 45 ppb Curcumin), Infected</td>
<td>T6</td>
<td>Drinking water</td>
<td>1-42</td>
</tr>
<tr>
<td>Dose 2 of Ethanolic herb extracts of <em>A. annua</em> + <em>C. longa</em> (19 ppb Artemisinin + 74 ppb Curcumin), Infected</td>
<td>T7</td>
<td>Drinking water</td>
<td>1-42</td>
</tr>
<tr>
<td>Dose 3 of Ethanolic herb extracts of <em>A. annua</em> + <em>C. longa</em> (27 ppb Artemisinin + 107 ppb Curcumin), Infected</td>
<td>T8</td>
<td>Drinking water</td>
<td>1-42</td>
</tr>
</tbody>
</table>

¹ Livacox vaccine (Merial Ltd. Brazil) consisted of attenuated oocysts of *E. acervulina*, *E. maxima* and *E. tenella*.

² Three (3) “seeders” per pen (10%/pen) aged 8-days-old where orally inoculated with 1ml suspension containing $5 \times 10^4$ *E. maxima* and $2 \times 10^5$ *E. acervulina* sporulated oocysts. “Seeders” were removed from the pens at 17 days-old, 9 days post-inoculation.

³ 50 ppm Nicarbazin + Narazin (Maxiban®) supplemented from 1 to 22 days of age and 66 ppm Salinomycin 12% (Coxistac®) supplemented from 22 to 39 days of age.

⁴ 42 g/Kg Carvacrol + 1.25g/Kg Timol (Regano®).

* Preparation of mixtures were performed every second day. 155 liters of tap water were mixed with dosages of herbal ingredients in T5, T6, T7 and T8.
Table 2 – Performance attributes for each treatment* for two distinct periods under investigation and for the entire period investigated.

Periods consisted of 1 to 22 days of age, 22 to 43 days of age and 1 to 43 days of age. Degree of infection is presented by the mean OPG (oocyst per gram of faeces) for the total period. All results presented are in LS means ± SEM.

<table>
<thead>
<tr>
<th>Performance attributes</th>
<th>p values</th>
<th>NEGATIVE CONTROL (T1)</th>
<th>VACCINE (T2)</th>
<th>CHEMICAL COCCID. (T3)</th>
<th>POSITIVE CONTROL (T4)</th>
<th>REGANO (T5)</th>
<th>LOW DOSE (T6)</th>
<th>MEDIUM DOSE (T7)</th>
<th>HIGH DOSE (T8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Period BWG (g)</td>
<td>0.01</td>
<td>838 ± 15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>789 ± 13&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>873 ± 09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>818 ± 14&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>779 ± 15&lt;sup&gt;de&lt;/sup&gt;</td>
<td>836 ± 12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>821 ± 13&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>768 ± 16&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Period FI (g/d)</td>
<td>0.01</td>
<td>68.9 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.6 ± 0.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>66.2 ± 0.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>65.1 ± 1.1&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>65.6 ± 1.2&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>63.7 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>64.5 ± 1.4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>61.6 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1-22 d FCR&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.38</td>
<td>1.71 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.67 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.59 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.67 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.75 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.59 ± 0.03&lt;sup&gt;de&lt;/sup&gt;</td>
<td>1.65 ± 0.06&lt;sup&gt;de&lt;/sup&gt;</td>
<td>1.70 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mort. (%)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.56</td>
<td>3.3 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7 ± 1.2&lt;sup&gt;de&lt;/sup&gt;</td>
<td>1.7 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 0.8&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Second Period BWG (g)</td>
<td>0.05</td>
<td>2010 ± 28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2002 ± 35&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2103 ± 29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1993 ± 44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2013 ± 27&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1916 ± 35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1946 ± 37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1964 ± 39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Period FI (g/d)</td>
<td>0.02</td>
<td>177.3 ± 2.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>187.7 ± 5.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>180.9 ± 3.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>180.1 ± 2.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>194.1 ± 5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>177.4 ± 2.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>183.0 ± 2.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>175.1 ± 3.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>22-43 d FCR&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.08</td>
<td>1.80 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.88 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.71 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.85 ± 0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.91 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.87 ± 0.05&lt;sup&gt;de&lt;/sup&gt;</td>
<td>1.93 ± 0.06&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.87 ± 0.04&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mort. (%)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.20</td>
<td>2.5 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.1 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total period BWG (g)</td>
<td>0.01</td>
<td>2848 ± 36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2791 ± 41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2976 ± 34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2811 ± 46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2792 ± 34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2787 ± 39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2767 ± 42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2732 ± 47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>period FI (g/d)</td>
<td>0.02</td>
<td>124.4 ± 1.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>127.2 ± 4.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>125.5 ± 1.7&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>123.6 ± 1.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>132.0 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>121.4 ± 1.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>125.1 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>118.4 ± 1.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1-43 d FCR&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.10</td>
<td>1.79 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.85 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.71 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.81 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.90 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.80 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.86 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.82 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mort. (%)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.59</td>
<td>5.9 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.7 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>k OPG&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.05</td>
<td>7.4 ± 0.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.3 ± 0.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.3 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.1 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.5 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.6 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8 ± 1.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.7 ± 1.5&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Number in a row marked with different letters are significantly different (p<0.05). *Treatments consisted of: T<sub>1</sub>= Uninfected negative control (untreated, uninfected); T<sub>2</sub>= Vaccinated, infected; T<sub>3</sub>= Coccidiostat, infected; T<sub>4</sub>= Untreated, Infected (positive control); T<sub>5</sub>= Regano®, Infected; T<sub>6</sub>= Dose 1 (LOW) of herbal extracts, Infected; T<sub>7</sub>= Dose 2 (MEDIUM) of herbal extracts, Infected; T<sub>8</sub>= Dose 3 (HIGH) of herbal extracts, Infected. 1BWG = Body weight gain for the period specified (g). 2FI = Feed intake for the period specified (g/broiler/day). 3FCR = Kg feed consumed / body weight gain in Kg for the period specified. 4Mort. (%) = Percentage mortality observed for the period specified. 5k OPG = 100 x OPG.
Figure 1 – Mean scores of gross lesions ± SD for duodenal (A), mid intestine (B) and total (C) lesions.

Bars in the same figure marked with different letters are significantly different (p<0.05).

* Treatments with different letters are significantly different at P < 0.05. Treatments consisted of:

- T<sub>1</sub> = Negative control (untreated, uninfected);
- T<sub>2</sub> = Vaccinated, infected;
- T<sub>3</sub> = Coccidiostat, infected;
- T<sub>4</sub> = Untreated, Infected (positive control);
- T<sub>5</sub> = Regano®, Infected;
- T<sub>6</sub> = Dose 1 (LOW) of herbal extracts, Infected;
- T<sub>7</sub> = Dose 2 (MEDIUM) of herbal extracts, Infected;
- T<sub>8</sub> = Dose 3 (HIGH) of herbal extracts, Infected.
Figure 2 - Effect of treatments* on coccidia oocyst excretion after 8 consecutive samplings from “tracer broilers”. Curves are presented representing the variation in the mean OPG (oocyst per gram of faeces) for the 6 replicates pens for each of the date of faeces collection.

* Treatments consisted of: T<sub>1</sub> = Negative control (untreated, uninfected); T<sub>2</sub> = Vaccinated, infected; T<sub>3</sub> = Coccidiostat, infected; T<sub>4</sub> = Untreated, Infected (Positive control); T<sub>5</sub> = Regano®, Infected; T<sub>6</sub> = Dose 1 (LOW) of herbal extracts, Infected; T<sub>7</sub> = Dose 2 (MEDIUM) of herbal extracts, Infected; T<sub>8</sub> = Dose 3 (HIGH) of herbal extracts, Infected.