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PERFORMANCE AND INFECTION DYNAMICS WITH *EIMERIA* SPP. IN BROILERS MEDICATED WITH *ARTEMISIA ANNUA* IN COMPARISON WITH LASALOCID AND KEPT IN FIELD CONDITIONS

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Summary

During this study we followed performance and dynamic of infection with *Eimeria* spp. in chicken-broilers reared in field condition and medicated with *Artemisia annua* as natural coccidiostatic in comparison with lasalocid. For this purpose in a flock from a farm were designed three groups of 100 randomly selected broilers of mixed sex each. The chicks were treated for all their life-span with *A. annua* dried leaves in-feed (1.5%), essential oil of *A. annua* via drinking water (0.15 ml/l water) and with Tween 70 (0.75 ml/10 l of water). The remaining chicks from the flock were in-feed medicated with lasalocid (125g/tonne of feed). Number of oocysts per gram faeces was monitored every 2 days from 16 to 38 days of age. When chickens were 25 days species of *Eimeria* were determined by PCR. Furthemore, clinical apperence, mortality, lesion score, body weight gain and feed conversion were registred. Three species of *Eimeria* were identified in the flock and groups: *E. acervulina, E. tenella* and *E. maxima.* Chikens in-feed medicated with lasalocid or *A. annua* sheded significantly less oocysts ($p<0.001$) than untreated chickens (Tween group), but chickens in-feed medicated with lasalocid sheded significantly less oocysts ($p<0.03$) than chickens treated with *A. annua.* Between *A. annua* groups there wasn't difference statistic significantly (dried leaves = 8,300; essential oil = 9,742). Clinical signs and bloody diarrhoea were registered only in untreated chickens (Tween group). The lesion score at 15 days age of treated chickens was below 0.5, while in Tween group was 1.26; the highest lesion score was noticed in caecum. The mortality percentage was between 2 and 4. Body weight gain and feed conversion ratio were higher in chickens medicated with lasalocid, followed by chicks medicated with essential oil of *A. annua.*

Key words: *Artemisia*, artemisinin, *Eimeria*, poultry, field

Coccidiosis remains one of the most costly diseases of the poultry industry. It is caused by apicomplexan parasites belonging to the genus *Eimeria* which develop in the epithelial cells of the gut. In poultry, there are seven recognized species: *E. acervulina, E. tenella, E. maxima, E. necatrix, E. mitis, E. praecox* and *E. brunette* (41). In broiler chickens frequently are found *E. acervulina, E. tenella* and *E. maxima* (18, 21, 32, 37).

Prevention of diseases is based on in-feed prophylaxis with anticoccidial drugs during lifespan of broilers with a withdrawal period before slaughtering, and more rarely with live vaccines (28, 33, 38). Approximately 84% of broilers were prevented with coccidiostats and 12% by vaccination in Europe in 2006 (12). In Romania, prevention of coccidiosis in broiler farms is based exclusively on use of coccidiostats, vaccination being used partially only in layers and breeders (personal observation). Large-scale and long-term use of anticoccidial drugs over 70 years has led to the development of drug-resistance *Eimeria* spp. strains (5, 33), and consecutively to important economic losses caused mainly by poor weight gain and high feed consumption (19, 23). In the world, the economic losses due to subclinical coccidiosis are estimated at more than 3 billion US$ annually (6).

Taking in account these considerations there is a strongly need for inexpensive alternatives for coccidiosis control. Moreover, in organic farming medication with classical drugs is prohibited or restricted (11). For instance, in case of coccidiosis in some countries coccidiostats are allowed (UK) and in others not (Austria) (26, 43). Live vaccines proved to be efficient, but they are expensive and have adverse effects on early chick growth (40). Nevertheless, vaccines are recommended to restore drug sensitivity of resistant *Eimeria* strains (40).

In the last 15th years, studies on the inhibitory effects of natural products on *Eimeria* and/or on the protective immunity of the host were made (30). Dried leaves of *Artemisia annua* have been used in traditional Chinese medicine for over 2 millennia (25), and sesquiterpene lactone from *A. annua* exhibits high efficacy against several stages of *Plasmodium* (15). Also, there are studies showing that artemisinin limits the replication of *Eimeria* spp. in chickens (2, 4, 31, 42).

In this study, we investigated the anticoccidial effects of *A. annua* (leaf powder and essential oil) in broilers kept in field condition and compared with a classical coccidiostatic, lasalocid.

Materials and methods

Animals and experimental design

The experiment took place in house four of broiler farm S.C. Oprea Avicom S.R.L. located in Crăieşti, Mureş county. In the house was introduced a flock of 19,500 chickens Ross 308 obtained from the hatchery of the same commercial society (Venchi-Sighişoara, Mureş county). Chickens were vaccinated against Newcastle disease three times (aged 9, 10 and 24 days) and against Gumboro disease two times (aged 12 and 18 days). Feed and water were offered *ad libitum*.

The lighting programme consisted in alternative periods of light and dark as follows: 1st and 2nd days, 23 hours light and one hour dark; 3th to 5th day, 22 hours light and two hours dark; 6th to 21st days, 19 hours light and five hours dark; 22nd day, 20 hours light and four hours dark; and from 23th day till the slaughtering 21 hours light and three hours dark. The light intensity was of 60-70 lux for all period of rearing.

The flock was divided in four groups: (i) group Aa-p of 100 chickens, treated with *A. annua* GERM07 as powder in feed (15 kg powder per ton of feed; 1.5%); (ii) group Aa-eo of 100 chickens, treated with *A. annua* ROMN08 as essential oil in water (0.15 ml per liter of water); (iii) group Tw80 of 100 chickens, treated with Tween 80 in water (0.75 ml per 10 liters of water); and (iv) group *Las* of remaining chickens, in-feed medicated with lasalocid (Avatec®150 G; Alpharma, Belgium; 125 g per ton of feed). Groups Aa-eo and Tw80 were received feed free of coccidiostatics. All treatments were applied from day-old to 36 days-old.

Artemisia* products and HPLC-UV analysis of *Artemisia annua

A. annua L. was used as (i) *A. annua* leaf powder obtained from Anamed A3 cultivar (GERM07) and as (ii) essential oil obtained from a Romanian variety of *A. annua* (ROMN 08). Leaf powder of GERM07 was obtained by grounding dried leaves with artisanal machinery. The essential oil of ROMN 08 was obtained through a hydro distillation process of plants vegetative parts, blooms and flowers (except long stems and branches) using artisanal machinery.

GERM 07 and ROMN 08 *A. annua* strains were analysed by HPLC-UV analysis to quantify the concentration of artemisinin, deoxyartemisinin, dihydroartemisinic acid, and artemisinic acid, following the published protocol of Ferreira and Gonzalez (13).

Observations and analytical procedures

Efficacy of *Artemisia annua* as a natural coccidiostatic was evaluated by (i) clinical signs; (ii) mortality rate; (iii) oocysts shed per gram of faeces (McMaster counting technique); (iv) lesion score; (v) body weight; and (vi) feed conversion ratio. Also, *Eimeria* species were determined when chickens were 26-days-old.

For oocysts counting, pooled fecal samples were collected every two days from 16 to 38 days-old. At each sampling time, 40 fresh droppings were collected per group and mixed, resulting in approxiamtely 200 g faeces per group. Sodium chloride (sp.gr. 1.20) solution was used as flotation solution.

Oocysts from samples collected on day 26th of age were isolated, purified and concentrated from faeces with saturated salt solution (36) and sporulated in 2.5% potassium dichromate solution. At the end, polymerase chain reaction was done to identify the species of *Eimeria.*

Lesion score was evaluated at 15 and 35 days-old for 10 chicks per group each time using a score of 0–4 (24).

Deoxyribonucleic Acid (DNA) extraction

DNA extraction from sporulated oocysts of each group was performed with the commercial kit Isolate Fecal DNA Kit (Bioline; cat. no. BIO-52038) following the manufacturer's instructions using 200µl of sporulated oocysts suspension. The DNA was stored at −20°C till analysis.

Polimerase Chain Reaction (PCR)

Eimeria species were identified by single PCR assay using species-specific primers targeting the internal transcribed spacer-1 (ITS-1) as previously described by Schnitzler et al. (34, 35) and Haug et al. (20). Each reaction mixture of 25 µl contained: 2µl DNA sample; 25 pmol of species-specific reverse and forward primers; 12.5 µl MyTaq™ Mix (Bioline, cat. no. BIO-25041); 9µl ultra-pure water (PCR Water, cat. no. BIO-37080, Bioline); and 0.5µl 1% bovine serum albumin. We used Houghton strains of all seven Eimeria species that infect chickens obtained from VLA (Veterinary Laboratory Agency Weybridge, UK) as positive controls and distilled water as negative control.

The amplification was performed in *MyGenie*™ 96 Gradient Thermal Block (Bioneer). The cycling parameters for the amplification consisted of an initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation (95°C, 15 s), annealing (58 or 65°C, 15 s) and extension (72°C, 10s), with a final extension at 72°C for 3 min.

The PCR products (8 µl), mixed with loading buffer (2 µl), were separated on a 1.5% agarose gel by electrophoresis, stained with SYBR® Green I Nucleic Acid Gel Stain (Invitrogen). Specific fragments were identified by size using a 100 bp ladder under UV light (BioDoc-It® Imaging Systems, UVP®, VWR International LLC).

Statistical analysis

Normal probability plots were generated based on oocysts per gram of faeces, and lesional score, to verify that data followed an approximate normal distribution (10). Groups were compared using Paired-Samples T Test. Analyses were performed using SPSS software version 19 (7).

Results and discussions

HPLC-UV analysis of *A. annua*

GERM07 *A. annua* leaf powder contained 0.75% artemisinin (Art), 0.18% dihydroartemisinic acid (DHAA), and 0.03% artemisinic acid (AA) (Fig. 1).

ROMN 08 *A. annua* plants vegetative parts, blooms and flowers contained 0.2% artemisinin (Art), and 0.3% artemisinic acid (AA).

Although deoxyartemisinin (DOArt) was also present in both samples, the compound lacks the peroxide bridge found in artemisinin, having a single oxygen atom in the bridge, and believed to have no biological activity (29).

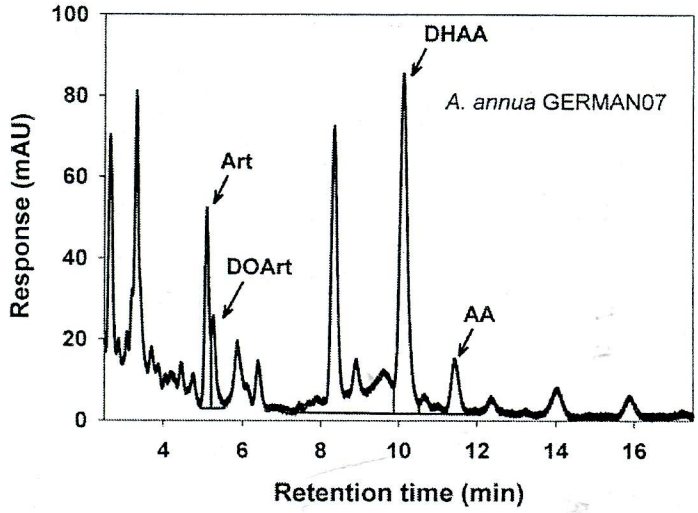


Fig. 1. HPLC-UV (192 nm) chromatogram of a 10-µL injection of GERM 07 (leaf powder). Quantification is in g/100g dry weight.

***Eimeria* species**

Chickens from all groups sheded oocysts of *E. acervulina, E. tenella* and *E. maxima* (Fig. 2).

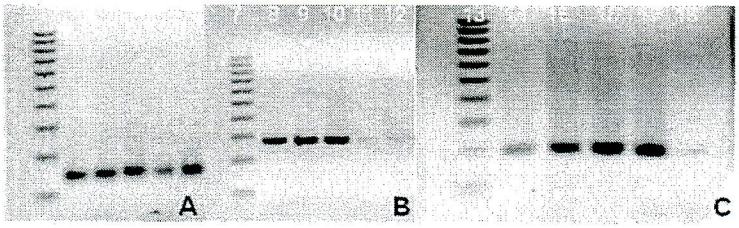


Fig. 2. *E. acervulina* 145 pb (A); *E. tenella* 278 pb (B); *E. maxima* 205 pb (C). Lines (L)1,7, and 13 represent the standard molecular weight of 100 bp, and lines 2, 8 and 14 positive controls (Houghton strains). Group Las: L3, L9, L15; Group Tw80: L4, L10, L16; Group Aa-p: L5, L11, L17; Group Aa-eo: L6, L12, L18.

Worldwide, in broilers are commonly found *E. acervulina*, *E. tenella, E. maxima* and *E. praecox* (21, 27, 32). During an epidemiological study by PCR in Romanian broiler farms were identified *E. acervulina, E. tenella, E. maxima* and *E. praecox* (16, 17).

Clinical signs and mortality

Clinical signs as weakness, inappetence, polidipsia, and diarrhoea appeared to 12 chickens from Tw80 group at 19 days of age. Three days later, half of chicks presented bloody diarrhoea, and four of them died after another 3 days (Table 1). The mortality rate to the other groups was lower, as follows: 3.3% in Las group; and 2% in *A. annua* groups (Table 1).

Table 1

Clinical aspects including weakness, inappetence, polidipsia, bloody diarrhoea, and mortality in the experimental groups

Group	Clinical signs	Bloody diarrhoea	Mortality
Las	0	0	3.3%
Aa-p	0	0	2/100
Aa-ue	0	0	2/100
Tw	12/100	6/100	4/100

Legend: Las – group treated with lasalocid 125 g per ton of feed; Aa-p – group treated with A. annua as powder, 15 kg per ton of feed; Aa-ue – group treated with A. annua as essential oil, 0,15 ml/l of water; Tw80 – group treated with Tween 80, 0,75 ml/10 l of water.

In a previous study after experimental infection with 10,000 oocysts of *E. tenella* and in-feed treatment with 1.5% *A. annua* as leaf powder, 30% of broilers presented clinical signs of coccidiosis, but no mortality (9).

OPG and lesion score

The number of oocysts excreted by the chickens from experimental groups during rearing period is presented in Fig. 3. The OPG was significantly higher (p=0,002) in group Tw80 (mean 25,383). Besides, chickens treated with *A. annua* either leaf powder (GERM07 strain) or essential oil (ROMN08 strain) excreted with 67.3% (mean 8,300), and 61.6% (mean 9,742) respectively less oocysts than those treated with Tween 80. Chickens treated with lasalocid sheded the fewest oocysts (mean 750).

We observed the same trend for oocysts production regardless of group; the peak was earlier for Tw80 group (22 days) and later for Las group (26 days); as for *Artemisia* groups an intermediate peak (24 days) between Tw80 and Las groups was noticed (Fig. 3).

The lesion scores by groups and segments of the intestine (duodenum, jejunum, and caecum) are presented in Table 2, at 15 and 35 days of age. The highest lesion score was registered in Tw80 group (3.8; 2.6), followed by Aa-p (1.6; 1.4) and Aa-ue (1.2; 0.8) groups, and the lowest in Las group (0.6; 1.0). When chickens were 15 days, the percentage of lesion score reduction in *A. annua* groups was about 57.9-68.3, being higher in chickens treated with essential oil. In comparison to chicken treated with lasalocid the reduction was 84.1%. However, at 35 days, the lesion score was below 1, in all experimental groups, less in Tw80 group for caecum (Table 2).

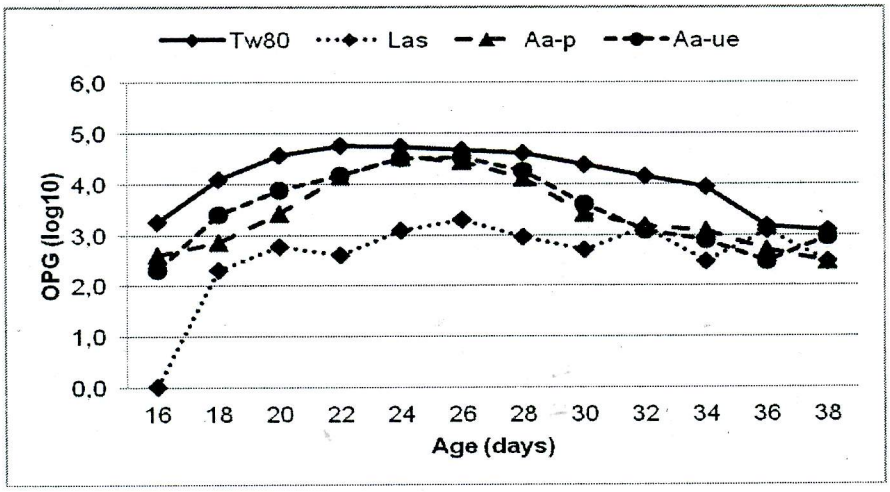


Fig. 3. Effect of *A. annua* as leaf powder (GERM07) (*Aa-p*), and essential oil (ROMN08) (Aa-eo) on the fecal oocyst output in field condition in comparison with lasalocid (Las) and tween 80 (Tw80)

Table 2

Lesion score in the experimental groups at 15 (a), and 35 (b) days age (average of 10 chicks)

Group	Duodenum		Jejunum		Caecum		Total	
	a	b	a	b	a	b	a	b
Tw80	1.0	0.8	0.8	0.8	2.0	1.0	3.8	2.6
Las	0.3	0.4	0.2	0.4	0.1	0.2	0.6	1.0
Aa-p	0.4	0.8	0.4	0.2	0.8	0.4	1.6	1.4
Aa-ue	0.4	0.6	0.2	0.2	0.6	0.0	1.2	0.8

Legend: a – 15 days age; b – 35 days age; Tw80 – group treated with Tween 80, 0,75 ml/10 l of water; Las – group treated with lasalocid 125 g per ton of feed; Aa-p – group treated with A. annua as powder, 15 kg per ton of feed; Aa-ue – group treated with A. annua as essential oil, 0,15 ml/l of water.

Previously when we used 1,500 and 10,000 oocyst of *E. tenella* (Houghton strain) for experimental infection and 1.5% *A. annua* leaf powder in diet, the oocysts output reduction was higher, between 87.9 and 90.8% and the reduction of lesion score similar, between 55.5 and 56.3% (8, 9).

Body weight gains (BWG) and feed conversion (FCR)

Overall, chickens in-feed treated with lasalocid 125 ppm had the best performances: BWG of 62.0 g/day, and FCR of 1.86. The Tw80 group presented the lowest BWG till 41 days, when Aa-p group presented the lowest BWG (Table 3). It is interesting as Aa-p group had among the best BWG till before slaughtering,

and then changed. Even before slaughtering chickens treated with Tw80 had a good BWG, they consumed more feed (2.12) for one kilogram of body weight than the other groups. Chickens treated with *A. annua* used between 1.96-1.99 kilograms of feed for one kilogram body weight.

Table 3

Body weight gain and feed conversion in experimental groups following *Artemisia annua* diets

Group	FCR	BWG					
		7 days	14 days	21 days	28 days	35 days	41 days
Tw80	2.12	17.7	32.3	49.6	49.0	67.9	62.0
Las	1.86	18.0	33.4	50.0	59.3	65.7	65.0
Aa-p	1.99	18.6	38.4	44.4	60.1	71.8	52.2
Aa-ue	1.96	18.0	35.7	57.3	54.0	66.0	63.6

Legend: FCR – feed conversion ratio; BWG – body weight gain; Tw80 – group treated with Tween 80, 0,75 ml/10 l of water; Las – group treated with lasalocid 125 g per ton of feed; Aa-p – group treated with A. annua as powder, 15 kg per ton of feed; Aa-ue – group treated with A. annua as essential oil, 0,15 ml/l of water.

Almeida et al. (3) in medium and low-growing chickens found no significant differences in feed consumption between groups, treated or not with *A. annua*, even before *Eimeria* infection chicks treated with *A. annua* had significantly lower average body weight gains. The authors suggested that the bitter taste of plant had interfered negatively with the broilers' performance before the build-up of coccidia infections.

The studies made on effect of *A. annua* against coccidial infection in chickens varied greatly in results. On the plant side, this variation was probably a consequence of artemisinin concentration caused by either different chemotypes or seasonal variation (39), although different methods of drying can also change artemisinin concentration in leaves (14). On the animal side, it was probably a consequence of routes of administration, experimental design (*Eimeria* species, oocysts dose, etc.), and different susceptibilities of *Eimeria* species and strains used in the studies. In general, most of them provided a good control against *E. tenella* infection, but not for *E. acervulina* and *E. maxima* (1, 4).

Conclusions

The results obtained by us in chickens kept in field condition suggested that *A. annua* as leaf powder or essential oil prevents successfully clinical coccidiosis. Moreover, *A. annua* can be a good candidate for preventing coccidiosis in organic farming.

Acknowledgements

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PERFORMANCE AND INFECTION DYNAMICS WITH *EIMERIA* SPP. IN BROILERS MEDICATED WITH *ARTEMISIA ANNUA* IN COMPARISON WITH LASALOCID AND KEPT IN FIELD CONDITIONS

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INTRODUCTION

Coccidiosis remains one of the most costly diseases of the poultry industry. It is caused by apicomplexan parasites belonging to the genus *Eimeria* which develop in the epithelial cells of the gut. In poultry, there are seven recognized species: *E. acervulina, E. tenella, E. maxima, E. necatrix, E. mitis, E. praecox* and *E. brunetti* (19). In broiler chickens frequently are found *E. acervulina, E. tenella* and *E. maxima* (6,8, 14, 16).

Prevention of disease is based on in-feed prophylaxis with anticoccidial drugs, and more rarely with live vaccines (11, 15, 17). Approximately 84% of broilers were prevented with coccidiostats and 12% by vaccination in Europe in 2006 (4). In Romania, prevention of coccidiosis in broiler farms is based exclusively on use of coccidiostats, vaccination being used partially only in layers and breeders (personal observation). Large-scale and long-term use of anticoccidial drugs over 70 years has led to the development of drug-resistance *Eimeria* spp. strains (3, 15), and consecutively to important economic losses caused mainly by poor weight gain and high feed consumption (7, 9)

Taking in account these considerations there is a strongly need for inexpensive alternatives for coccidiosis control. Live vaccines proved to be efficient, but they are expensive and have adverse effects on early chick growth (18). Nevertheless, vaccines are recommended to restore drug sensitivity of resistant *Eimeria* strains (18).

In the last 15th years, studies on the inhibitory effects of natural products on *Eimeria* and/or on the protective immunity of the host were made (12). Dried leaves of *Artemisia annua* have been used in traditional Chinese medicine for over 2 millennia (10), and sesquiterpene lactone from *A. annua* exhibits high efficacy against several stages of *Plasmodium* (5). Also, there are studies showing that artemisinin limits the replication of *Eimeria* spp. in chickens (1,2, 13, 20).

In this study, we investigated the anticoccidial effects of *A. annua* (leaf powder and essential oil) in broilers kept in field condition and compared with a classical coccidiostatic, lasalocid.

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MATERIAL AND METHODS

Animals and experimental design

- Four groups of 100 chickens (Table 1)
- Feed free of anticoccidials (except group Las)
- Treatment from day-old to 36 days-old

Table 1

Group	Treatment
Las	Lasalocid 125 g/ton of feed
Aa-p	*A. annua* GERM07, powder 15 kg per ton of feed
Aa-eo	*A. annua* ROMN08, essential oil 0.15 ml per liter of water
Tw80	Tween 80 0.75 ml per 10 liters of water

Artemisia products

a) A. annua leaf powder from Anamed A3 cultivar (GERM07)
b) Essential oil from a Romanian variety of *A. annua* (ROMN 08)

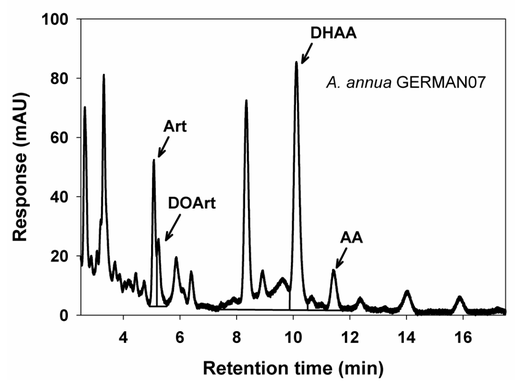
Observations and analytical procedures

1. Analysis of *Artemisia annua* (GERM 07 and ROMN 08) by HPLC-UV.
2. *Eimeria* species determined by PCR from faeces collected on day 26 of age.
3. Efficacy of *Artemisia annua* as a natural coccidiostatic evaluated by:
- clinical signs;
- mortality rate;
- oocysts shed per gram of faeces (McMaster counting technique);
- lesion score;
- body weight and feed conversion ratio.

RESULTS

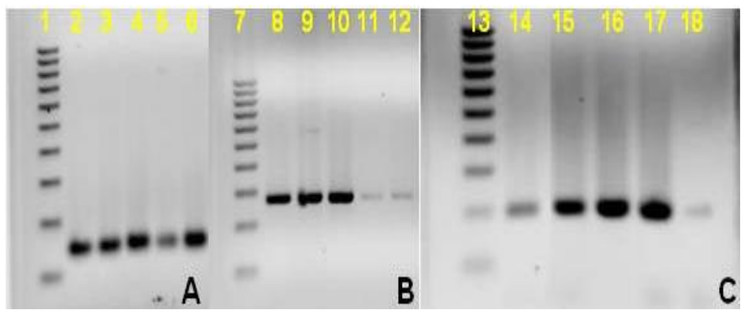
HPLC-UV analysis of *A. annua*

	GERM07	ROMN 08
Artemisinin	0.75	0.2
Deoxyartemisinin	-	-
Dihydroartemisinic acid	0.18	-
Artemisinic acid	0.03	0.3



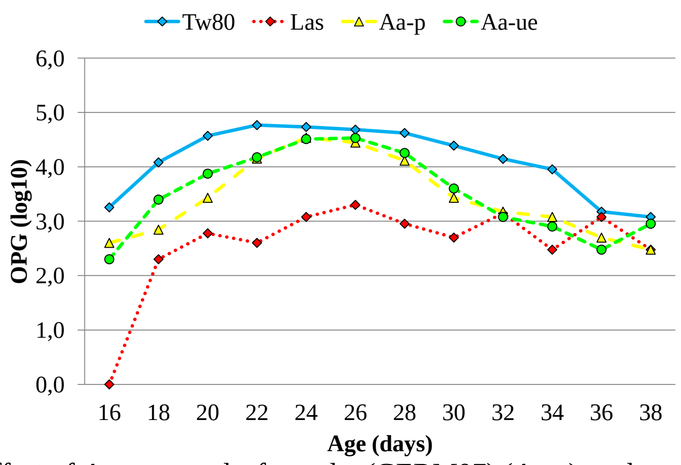
Eimeria species

Chickens from all groups sheded oocysts of *E. acervulina*, *E. tenella* and *E. maxima* (Fig. 1)

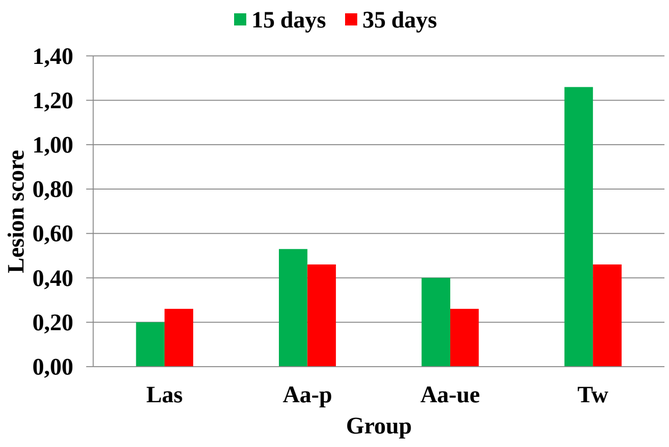


E. acervulina 145 pb (A); *E. tenella* 278 pb (B); *E. maxima* 205 pb (C).
Lines (L)1,7, and 13 represent the standard molecular weight of 100 bp.
Lines 2, 8 and 14 positive controls (Houghton strains).
Group Las: L3, L9, L15; Group Tw80: L4, L10, L16;
Group Aa-p: L5, L11, L17; Group Aa-eo: L6, L12, L18.

OPG and lesion score

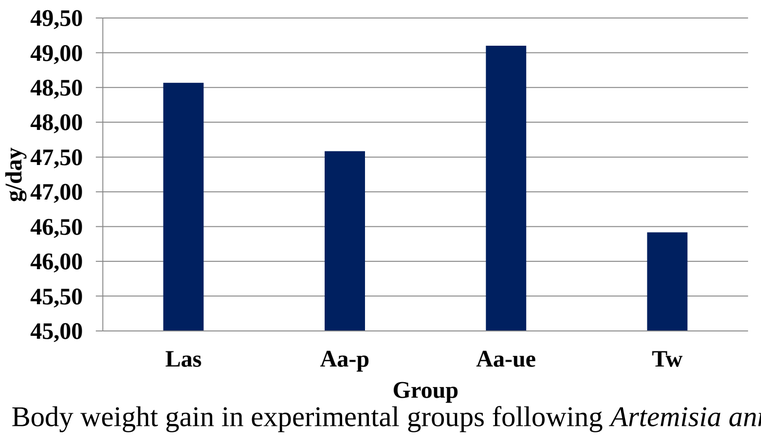


Effect of *A. annua* as leaf powder (GERM07) (*Aa-p*), and essential oil (ROMN08) (Aa-eo) on the fecal oocyst output in field condition in comparison with lasalocid (Las) and tween 80 (Tw80)

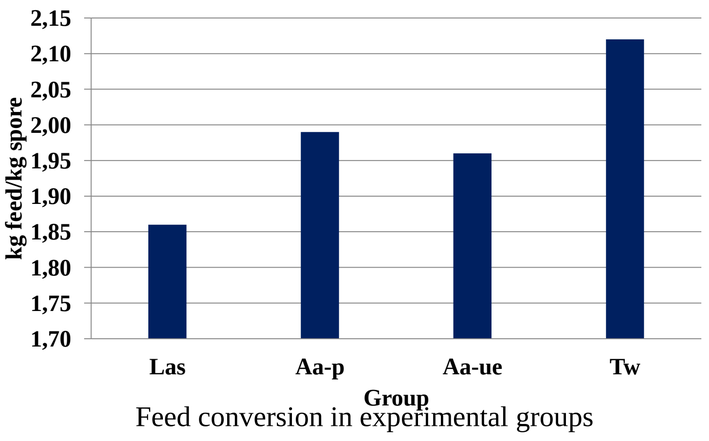


Lesion score in the experimental groups at 15 and 35 days age (average of 10 chicks)

Body weight gains (BWG) and feed conversion (FCR)



Body weight gain in experimental groups following *Artemisia annua* diets



Feed conversion in experimental groups

CONCLUSIONS

The results obtained by us in chickens kept in field condition suggested that *A. annua* as leaf powder or essential oil prevents successfully clinical coccidiosis. Moreover, *A. annua* can be a good candidate for preventing coccidiosis in organic farming.

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