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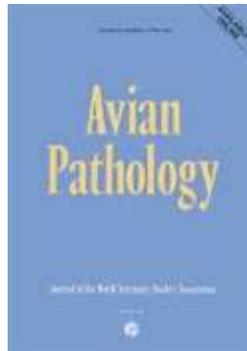
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***In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys**

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In vivo effect of herbal products against *Histomonas meleagridis* in turkeys

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In vivo effect of herbal products against *Histomonas meleagridis* in turkeys**Harold M.J.F. van der Heijden^{*}, Wil J.M. Landman****Summary**

Histomoniasis is a serious disease in poultry. All chemotherapeutics with known efficacy against its causative agent, *Histomonas meleagridis*, have been banned from use as prophylactic or therapeutic use in production animals. In a search for possible alternatives, the *in vivo* effects of the herbal products Enteroguard™ and Protophyt™ were examined. Two-week-old turkeys allocated in 13 groups of 18 birds were either sham inoculated (negative control group) or with 100 or 3162 or 200000 histomonads per bird. Control groups (no feed additives, dimetridazole, or Histostat-50™) were included in the study. No morbidity or mortality was observed in the negative control group or in the groups inoculated with 100 histomonads per bird. Mortality was 100% in the groups inoculated with 200,000 histomonads per bird and either untreated (positive control group), or receiving Protophyt SP™, Protophyt™ SP and Protophyt™ B, Enteroguard™, or Histostat-50™. Mortality was 17% in the dimetridazole treated group. In the groups inoculated with 3162 histomonads per bird, mortality was 100% for the positive control group and the group receiving Enteroguard™, and 94% in the group receiving Protophyt™ SP. In this study Enteroguard™ or Protophyt™ were not found to be effective against histomoniasis.

Introduction

Histomoniasis (infectious enterohepatitis, blackhead disease) is a disease of galliforms and other species of birds, mainly affecting the liver and caecae. The severity of the disease varies over the different species. In turkey flocks, for example, mortality can be very high, whereas in chicken symptoms are generally less severe (McDougald, 1997). The causative agent of histomoniasis, *Histomonas meleagridis*, is transmitted between flocks through the vector *Heterakis gallinarum* (Graybill & Smith, 1920). Within a flock direct lateral transmission of *H. meleagridis* between birds occurs (Hu & McDougald, 2003).

Effective chemotherapeutics like nifursol and nitroimidazoles are not allowed anymore in both the US and the EU (McDougald, 2005). In addition, the EU also banned arsenical compounds (Byrne, 2001). Therefore, there is an urgent need for alternative antihistomonal products (Hu & McDougald, 2004). In the field, herbal products are used to prevent outbreaks of histomoniasis (Hafez & Hauck, 2006). However there are only a few scientific *in vivo* studies backing up the efficacy of such products against *H. meleagridis*. Duffy *et al* (2005) observed a positive effect of Natustat™, a yeast-derived mannonoligosaccharide combined with organic mineral nutrients and plant extracts on caecal and liver lesions scores but not on mortality. In a recent study (Hafez & Hauck, 2006) Protophyt™ SP (feed additive) and Protophyt™ B (drinking water additive) were found to reduce mortality following experimental inoculation with *H. meleagridis* from 50% (untreated group) to 20% (additives), with no mortality in the control group.

In the present study the antihistomonal effects of Enteroguard™ and Protophyt™ are examined on turkey poults that were experimentally inoculated with three different inoculation doses of histomonads. Enteroguard™ is a product based on garlic and cinnamon, with allicin and cinnamaldehyde as possibly active compounds. Protophyt™ is based on volatile oils extracted from garlic, cinnamon, rosemary and lemon.

Material and Methods

Medicated feed and drinking water. Non-medicated turkey starter feed (Arkervaart-Twente, no. 94040, Nijkerk, the Netherlands) and non-medicated turkey feed (Arkevaart-Twente, no. 94041, Nijkerk, the Netherlands) were mixed with the different antihistomonal products: either 200 ppm dimetridazole (1,2 dimethyl-5-nitroimidazole, Sigma, D4025, Zwijndrecht, the Netherlands) or 375 ppm Histostat-50™ (no. 560101, Alpharma Inc., Fort Lee, USA), or 3000 ppm Protophyt SP™ (Phytosynthèse, Riom, France) or 500 ppm Enteroguard™ (Orffa, Giessen, The Netherlands). To ensure an adequate distribution of the product in the feed the following feed mixing procedure was used. A maximum quantity of 25 kg of feed was mixed per run. After accurate weighing of the product it was transferred to a plastic bag. Approximately 500 g of feed was added and thoroughly mixed. Subsequently, the contents of the bag were transferred to a larger plastic bag which contained approximately 2.5 kg of feed, followed by thorough mixing. During the next mixing step another 2.5 kg of feed was added and mixed again. Thereafter, the contents of the bag were mixed with the remainder of the 25 kg feed for approximately 15 minutes using a blender (Naturamix, no. 19091, Haarlem, the Netherlands). The medicated feed was collected in a labeled paper bag. All of the medicated feed needed for the whole experimental period, except for the dimetridazole feed, was produced within two days and stored at room temperature until used. The dimetridazole feed was freshly mixed every week and stored at 4°C in the dark, as recommended by the producer. The blender was thoroughly cleaned between products using a brush, followed by operation of the blender with 5 kg of non-medicated feed that was subsequently discarded. Drinking water with a herbal additive (Protophyt™ B) was freshly prepared every day by adding 2 ml Protophyt B™ per litre of water.

Assessment of feed mixing procedure. Samples were taken from the feeds with dimetridazole and Histostat-50™ and analyzed for dimetridazole and arsenic content for assessment of the mixing procedure.

Dimetridazole was quantified by HPLC after extraction with acetonitril/methanol. Briefly, 5 g of feed was moistened with 15.0 ml water. After 5 min, 35.0 ml of a mixture (1:1) of acetonitril (Fisher Scientific, Loughborough, United Kingdom) and methanol (Biosolve, Valkenswaard, the Netherlands) was added. The suspension was shaken for 30 min at room temperature. The extract was filtered through a paper filter (Schleicher, Dassel, Germany) and eluted over 4 g neutral aluminiumoxide (Fisher Scientific) packed in a glass column (25 ml). The first 2 ml eluted filtrate was not used. The extract was diluted 10 times with mobile phase. The mobile phase consisted of 170 ml sodium-acetate buffer (0.01 mol/l pH 6,0) with 30 ml acetonitril. The HPLC column was a 3.0 mm C18 Chromspher packed with 40 µm reversed phase material (Varian, Middelburg, the Netherlands). Dimetridazole was quantified against standard material (Rhône-Poulenc, Amstelveen, the Netherlands) at 320 nm. All handling was done under yellow light to protect degradation of dimetridazole. The detection limit was 0.8 mg/kg. The recovery of the method is between 90 and 95 % in the range of 2 to 500 mg/kg.

Arsene was determined by ICP. Briefly, 1 g of feed to which 6 ml 70% HNO₃ (Baker Chemicals, Deventer, the Netherlands) was added, was destructed in a magnetron digestion unit (Milestone Inc., Shelton, USA) until a clear destruate remained. After complete solubilisation, 50 ml of water was added. Arsene was measured with an ICP (Optima 3300 DV, Perkin Elmer, Waltham, USA).

Experimental setup. In two separate rooms (6.85 x 4.48 m) A and B, 13 stainless steel wire pens (1.3 x 1 m) were constructed. On the left side of both rooms four pens (group no. 1 to 4,

and 8 to 11, respectively) were positioned at a distance of 40 cm, while on the right side of the rooms three (group no. 5 to 7), or two (group no. 12 and 13) pens, were placed, also at a distance of 40 cm. The side walls of all pens were covered with plastic to minimize the risk of cross-infections between pens. Approximately 8 cm of wood shavings was used as bedding litter. The experimental rooms were only accessible for qualified personnel, and involved changing of footwear and overall clothing.

One-day-old BUT Big 6 poult (120 female and 120 male) were kept separately in two pens for one week, with non-medicated drinking water and feed (turkey starter) *ad libitum*. The lighting program was: 2 hrs of darkness and 22 hrs of light.

After one week all birds were tagged (Swifttack), individually weighed and divided into 6 weight classes per sex. All birds of each weight class were then randomly distributed over the 13 pens. Each group consisted of 9 female and 9 male poult. Until the termination of the experiment at the age of 6 weeks, light was provided for 16 h per day.

The experimental groups no. 1 to 7 (Table 1) in room A were given a “standard” inoculation dose of 200000 histomonads per bird, whereas the birds in room B received either a low inoculation dose of 3162 histomonads per bird (groups no. 8, 10, and 12) or a very low inoculation dose of 100 histomonads per bird (groups no. 9, 11, 13). The inoculations were done during two consecutive days in room A, and room B, respectively. The negative control group (no. 2), i.e. no treatment and no inoculation, was directly positioned next to the positive control group (no. 1), i.e. inoculated but not treated. During handling of the animals throughout the experiment (feed supply, removal of dead birds from the pens, etc.) the negative control group was always handled last in order to detect possible cross-infection. At the age of 7 days, i.e. at transfer of the animals to the experimental groups, the starter feed with additives was supplied *ad libitum*. However, in group 7 non-medicated feed and drinking

water was continued until two days before inoculation, when starter feed with Protophyt SP™ and drinking water with Protophyt B™ was supplied to the birds.

At the time of inoculation at two weeks of age, all groups changed from turkey starter feed to turkey feed with the appropriate feed additive for the remainder of the experimental period (until 6 weeks of age).

After inoculation, the groups were inspected three times a day and dead birds removed from the pens. When birds were very sick and did not eat for two consecutive inspections, these were euthanized by injection with T61.

H. meleagridis strain. Strain /Deventer/NL/AL327-type I/03 (Van der Heijden *et al*, 2006), a Dutch field strain that was successfully propagated in culture (Van der Heijden *et al*, 2005) was used for inoculation. On two consecutive days, the isolate was resuscitated, cultured in Dwyer's medium (Dwyer, 1970) and subcultured once in modified Dwyer's medium (Van der Heijden *et al*, 2007a), consisting of Medium 199 with Hanks salts (Gibco-BRL, Grand Island, USA) with 10% heat-inactivated horse serum (Ginco-BRL) and 0.096% w/v of white rice powder (Arrowhead Mills Inc., Hereford, USA). After pooling of the contents of four tissue culture flasks; a 1:5 pre-dilution was made in pre-warmed Medium 199 with Hanks salts and counted using a Bürker-Türk haemocytometer and phase contrast microscopy at 200x magnification. Subsequently the suspensions were further diluted in M199 medium to obtain a concentration of 200000 histomonads/ml and counted before and after the inoculation on both inoculation days. On the second inoculation day the 200000 histomonads/ml suspension was further diluted in order to obtain the desired inoculation doses (Table 1). The inoculation doses were aliquoted for each experimental group by pipetting 25 ml into pre-warmed 50 ml tubes which were kept warm until used.

Inoculation. The turkeys were essentially inoculated by the method of Chappel (1975). The birds were inoculated intraclonally with 1 ml of histomonad-suspension using a 10 ml syringe (Pharma-Plast) with a blunt tip. After inoculation a finger was placed over the cloaca for 30 seconds to prevent voiding of the inoculum. Subsequently the birds were suspended in inverted position for a maximum of 5 min but at least for the time that the cloaca was observed pulsating (usually for approximately one minute after stimulation). In only three out of 234 cases voiding of the inoculum was seen (birds in group no. 3, 7 and 12), and after which these turkeys received a new inoculum. No birds were seen discharging after return to their pens. The negative control group was inoculated with 1 ml of M199 medium instead of histomonads.

Postmortem examination. Thorough postmortem examination was performed on all experimental birds either during the experiment (in case of mortality or severe disease) or at the end of the study at the age of 6 weeks. Birds that died during the experiment were kept at 4°C before being subjected to postmortem examination within one or two days.

Six weeks after inoculation the remaining turkeys were transported in boxes to the postmortem room and euthanized using CO₂. At necropsy, liver and caecal lesions were scored on a scale ranging from 0 (no lesions) to 4 (McDougald & Hu, 2001).

Statistical analysis. The time of survival in the groups with different treatments was analyzed using one-way analysis of variance (AOV). Group means were adjusted using Bonferoni's method. The residuals were assessed for being normal distributed. The liver and caecum lesion scores between groups were analyzed using the nonparametrical Kruskal-Wallis comparison test. Statistical analysis was performed using Statistix 8 (Analytical Software, Tallahassee, USA).

Results

Assessment of the feed mixing procedure. The average dimetridazole level of the feed mixed with dimetridazole was 182 ppm (desired dose 200 ppm) while the average arsenic level of the feed mixed with Histostat-50™ was 68 ppm (desired dose 63 ppm), which corresponds with 405 ppm Histostat-50™ (desired dose 375 ppm).

***H. meleagridis* inoculation.** Not a single turkey died in the negative control group (no treatment, sham inoculated) while at postmortem the livers and caecae of all birds did not show abnormalities (all were scored 0). In the experimental groups inoculated with the standard dose of 200,000 histomonads per animal (Figure 1), mortality was high in most of the groups. The first turkeys died from histomoniasis around day 10 p.i. In the positive control group (no feed additive), and the groups treated with either Protophyt SP™ or Enteroguard™ at day 13 p.i. approximately 50% of the animals had died, while 50% mortality was (slightly) delayed by one or two days in the experimental group with combined therapy with Protophyt SP™ and Protophyt B™ and in the group treated with Histostat-50™. At day 16 to 18 p.i. almost all animals had died in the positive control group and in the groups treated with either Protophyt™ or Enteroguard™, while 100% mortality was delayed for 3 days in the group medicated with Histostat-50™. In the experimental group that received dimetridazole the first two turkeys died at 17 days p.i. Nevertheless, mortality in this group was limited to 17% at 30 days p.i. when the experiment was terminated. Surprisingly, 13 of the 15 surviving animals in this group presented typical lesions in caecae and liver at necropsy.

The different treatments of the groups in which the turkeys were inoculated with 200,000 histomonads per bird had a significant effect (one-way AOV, $P < 0.05$) on the time of survival, also when the dimetridazole group was left out of the analyses (Table 2). Only in the

groups receiving dimetridazole or Histostat-50™ did birds die significantly later than in the positive control group (Bonferroni's pair-wise comparisons test, $P < 0.05$). Among these groups the effect of treatment was significant for the liver lesion scores (Kruskal-Wallis, $P < 0.05$) but not for caecal lesion scores ($P > 0.05$). Pair-wise comparisons revealed no differences between the groups in liver lesion scores.

In the three groups inoculated with the low dose of 3162 histomonads per animal (Figure 2) the results were very similar. The first turkeys died in the positive control group (no feed additive) at 7 days p.i, even prior to the positive control group inoculated with 200000 histomonads per animal. In the other two groups treated with Protophyt SP™ or Enteroguard™ mortality was first seen at 9 or 12 days p.i., respectively. In all three groups 50% mortality occurred at 15 days p.i. which was two days later than the groups inoculated with 200000 histomonads per animal. Finally, maximum mortality occurred at 19 days p.i., also slightly delayed compared to the groups inoculated with 200000 histomonads per bird. The single turkey in the Protophyt SP™ group that survived throughout the experiment had no lesions in liver or caecae upon necropsy.

No significant effect of treatment in the groups inoculated with 3162 histomonads per bird was found, neither for number of days of survival (one-way AOV, $P > 0.05$), nor for caecal or liver lesion scores (Kruskal-Wallis, $P > 0.05$).

In the three experimental groups inoculated with 100 histomonads per animal and either non-treated (positive control; no feed additive), or treated with Enteroguard™ or Protophyt SP™ no mortality was seen and the livers and caecae were normal (score 0) at postmortem.

No differences were seen in mortality of female or male turkeys after experimental inoculation with *H. meleagridis* (Figure 3). Additionally, there was no significant relation (regression analysis; $P > 0.05$) between the weight of the poults at the start of the experiment

and the number of days the bird survived inoculation, even if female and males results were analyzed separately. In the groups inoculated with 100 histomonads per bird (i.e. no mortality), the overall consumption of feed with either no additive, Protophyt™ SP or Enteroguard was 32.5, 33.1, or 32.3 kg/group, respectively.

Discussion

The ban of effective chemotherapeutics against *H. meleagridis* in commercial poultry (Byrne, 2001) propelled the search for alternative antihistomonal products. In the present study two herbal products were tested for a possible anti-histomonal effect. However, both Protophyt™ and Enteroguard™ had no effect on mortality following inoculation of turkey poults with 200000 histomonads per bird. In the positive control group, the groups receiving either Enteroguard™ or Protophyt™ SP as a feed additive, and in the group of turkeys that received Protophyt™ SP as feed additive and Protophyt™ B in drinking water, mortality was 100%. The latter finding is in agreement with the results of a field study in turkeys (Chossat, 2002), but in contrast with a recent *in vivo* experiment (Hafez & Hauck, 2006). In the latter study turkeys provided with Protophyt™ SP in the feed six days before inoculation and Protophyt™ B in the drinking water three days before inoculation showed 20% mortality instead of 50% mortality in the positive control group after experimental inoculation with 147500 histomonads per bird. This difference was only just significant (Chi-Square; $P = 0.047$). Moreover, their birds were housed in cages, which makes re-infections through direct lateral transfer as under practical conditions less likely. The only positive effect of the Protophyt™ SP and B treatment in the present study was a slight delay in mortality of one or two days in comparison with the other groups. Here, both Protophyt™ SP and Protophyt™ B were

supplied only two days before inoculation. Possibly more time is needed for the products to partly protect birds against histomoniasis following an inoculation with *H. meleagridis*.

Surprisingly, the mortality in the group medicated with Histostat-50™ was 100%. Nitarsone (4-nitrophenyl-arsonic acid) is the active compound of this product, and has been reported to be highly effective for preventive use in feed although it is probably histomonastatic since a relapse has been described after withdrawal of medication (McGuire & Morehouse, 1952). In addition, some mortality was seen in the experimental group medicated with dimetridazole, especially toward the end of the experiment at 4 weeks after inoculation. Moreover, a large proportion of the surviving turkeys showed severe lesions at necropsy and it is likely that more turkeys would have died had the experiment been continued. Since dimetridazole is considered to be highly effective (McDougald, 2005) this result was unexpected. It is unlikely that this was caused by a slightly lower dose of dimetridazole, 182 ppm instead of 200 ppm, in the feed, since the 50% protective dose of dimetridazole was found to be 38 ppm (Lucas, 1961) and with 100 ppm (McGuire *et al*, 1963) or 125 ppm (Lucas & Goose, 1965) close to 100% protection against histomoniasis was obtained. Also, the recovery of the dimetridazole test was determined during the validation of the assay and found to be 87% in the range of 5 to 100 ppm. It is expected that for higher concentrations up to 200 ppm the recovery is approximately the same. Therefore the actual dimetridazole concentration in the feed was probably close to the desired 200 ppm.

A dose-response relation between the size of the infective dose and mortality was reported by Lund (1955). After inoculation with 10^5 , 10^4 , 10^3 , 10^2 , or 10 histomonads per turkey mortality was 100%, 82%, 25%, 5%, and 5%, respectively. But the infective dose in itself probably also does not explain the results of the present experiment. Most inoculation studies used infective doses between 10^5 and 10^6 histomonads per animal (McDougald, 2005). In addition, in the present experiment a lower (3,162) and a much lower (100) inoculation

dose were examined, although only in positive control groups and groups of birds having either Protophyt SP™ or Enteroguard™ as feed additive. In the groups inoculated with 3162 histomonads per bird again all birds of the positive control group and the group treated with Enteroguard™ died. In the group of turkeys that was treated with Protophyt SP™ only a single bird survived the experiment. Thus, also at lower inoculation doses, the herbal products had no or hardly any effect on mortality. In all three groups inoculated with 100 histomonads per bird, however, not a single bird died during the experiment or showed lesions at necropsy.

The relative high virulence of the strain used might explain the fact that mortality was high, despite a relative low inoculation dose. This might also be the cause of the considerable morbidity in the dimetridazole treated group towards the end of the experiment. This is however speculative since there have been no reports on the effect of chemotherapeutics on strains of different virulence, and it needs further investigation.

The inoculation procedure (Chappel, 1975) was probably very effective as none of the experimental poult (except for a few birds that were re-inoculated) showed discharge of the inoculum after returning to their pens. This may also have contributed to the higher mortality found compared to other *in vivo* studies using a different intracloacal inoculation method. On the other hand, infected birds are known to shed *H. meleagridis* as early as two days after inoculation (McDougald, 2005; Hess *et al*, 2006). Since the experimental turkeys were housed on litter direct lateral transmission of the parasite (Hu & McDougald, 2003) causing re-infections could have contributed to the higher mortality.

A possible explanation for the high mortality in the Histostat-50™ medicated group of birds may be that the administration of this feed additive started just one week before inoculation. This may have been too short for prophylactic treatment. Nitarsone, the active compound in Histostat-50™ is a prophylactic rather than a curative drug (Hu & McDougald, 2004).

Cross contamination between groups, potentially masking antihistomonal effect of a certain product, was highly unlikely. The negative control group (no medication, no inoculation) that was placed directly next to the positive control group with an inoculation dose of 200,000 histomonads per turkey remained free from clinical disease throughout the experiment and the birds showed no lesions at necropsy. The same was true in the experimental groups inoculated with 100 histomonads per bird as these were placed next to the groups inoculated with 3162 histomonads per bird and also remained free of disease, while almost every bird in the neighbouring pen died of histomoniasis.

No difference was found between female and male birds regarding the susceptibility to an experimental inoculation with *H. meleagridis*.

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Figure 1. *Survival of turkeys in experimental groups following inoculation with 200000 histomonads per turkey.*

Figure 2. *Survival of turkeys in experimental groups following inoculation with 3162 histomonads per turkey.*

Figure 3. *Comparison of survival of male and female turkeys among all groups after experimental inoculation with H. meleagridis.*

Table 1. *Experimental groups and inoculation dose*

Exp. room	Groups	Feed additive	Dose (histomonads/bird)
A	1	no (pc ^a)	200,000
	2	no (nc ^b)	0
	3	dimetridazole	200,000
	4	Histostat-50	200,000
	5	Protophyt SP	200,000
	6	Enteroguard	200,000
	7	Protophyt SP/B	200,000
B	8	no (pc)	3,162
	9	no (pc)	100
	10	Protophyt SP	3,162
	11	Protophyt SP	100
	12	Enteroguard	3,162
	13	Enteroguard	100

^aPositive control.^bNegative control.

Table 2. Comparison of mortality day, and caeca and liver lesions scores of turkeys that died following inoculation with *H. meleagridis*

Dose (Hm/bird)	Feed additive	n ^a	Mortality day		Caeca lesions		Liver lesions	
			Mean	SD ^a	Mean	SD	Mean	SD
0	no additive	0	-	-	-	-	-	-
100	no additive	0	-	-	-	-	-	-
	Protophyt SP	0	-	-	-	-	-	-
	Enteroguard	0	-	-	-	-	-	-
3162	no additive	18	15.1	2.68	3.9	0.24	3.1	0.90
	Protophyt SP	17	15.1	2.30	3.9	0.24	3.0	0.79
	Enteroguard	18	15.5	1.69	4.0	0.00	3.3	0.49
200000	dimetridazole	3	25.7 ^A	7.25	3.3	0.58	3.3	0.58
	Histostat-50	18	16.2 ^B	3.25	3.9	0.32	3.0	0.59
	Protophyt SP/B	18	14.5 ^{BC}	1.74	3.9	0.32	3.4	0.51
	no additive	18	13.7 ^C	1.15	3.9	0.24	3.3	0.46
	Protophyt SP	18	13.3 ^C	1.45	3.9	0.24	3.6	0.50
	Enteroguard	18	13.5 ^C	1.82	4.0	0.00	3.4	0.51

^aNumber of birds that died during the experiment.

^bStandard deviation.

Means with different superscripts are significantly ($P < 0.05$) different among the groups with the same infective dose.

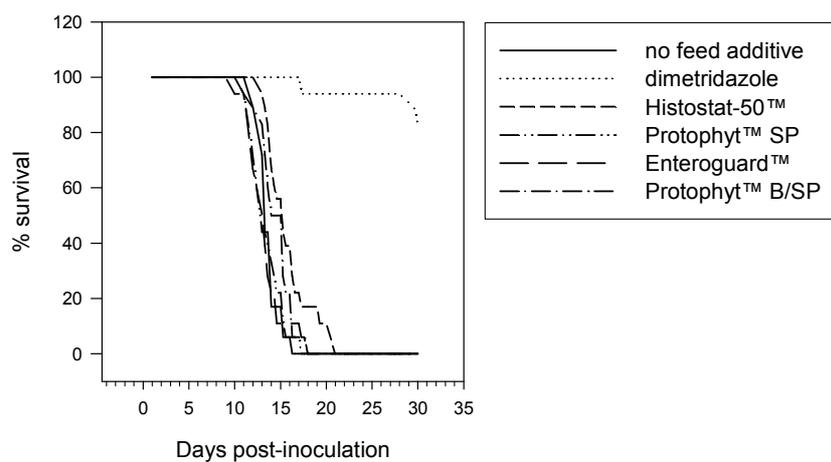


Figure 1. Survival in experimental groups following inoculation with 200,000 histomonads per turkey.

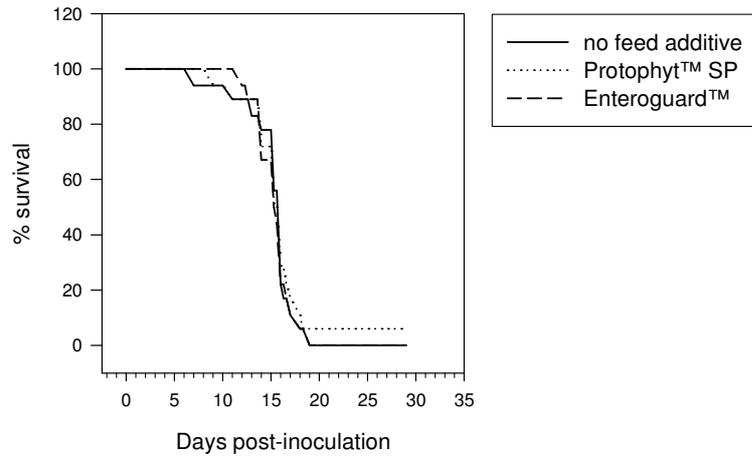


Figure 2. Survival in experimental groups following inoculation with 3,162 histomonads per turkey.

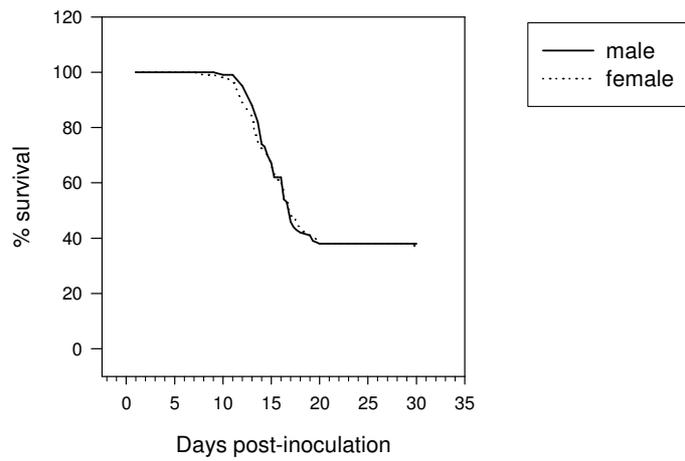


Figure 3. Comparison of survival of male and female turkeys among all groups after experimental inoculation with *H. meleagridis*.