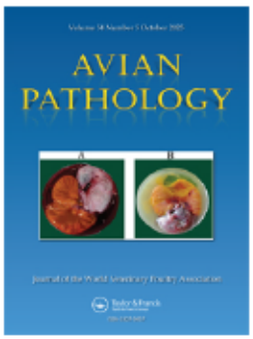

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ORIGINAL ARTICLE

Long-term protection of turkeys with a live clonal monoxenic *Histomonas meleagridis* vaccine

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ABSTRACT

Histomonosis, caused by the protozoan parasite *Histomonas meleagridis*, is a major concern in turkey production due to the lack of licenced drugs and vaccines. Despite various studies on experimental vaccination, the duration of immunity of such a vaccine remains unclear. This study evaluated the long-term efficacy of an attenuated clonal monoxenic *H. meleagridis* culture in turkeys, focusing on its protective effects. Day-old turkeys were vaccinated orally using a frozen vaccine culture directly, without additional multiplication, and challenged 12 weeks later. The vaccine caused no adverse clinical signs, consistent with prior studies. Instead, vaccinated birds had an improved weight gain and higher body-weight at 42 days. Vaccine uptake was confirmed by the detection of histomonad DNA in faeces starting 14 days post-vaccination, coinciding with the first sampling time point, with 60–70% of birds testing positive by 49 days. Considering all sampling time-points before the challenge, every sampled vaccinated turkey secreted histomonads at least once. Following the challenge, analysed clinical scores showed a more than 20-fold reduction in disease severity in vaccinated birds compared to controls, and survival rates were remarkably higher in the vaccinated group (90%) than in non-vaccinated controls (16%). Overall, this study supports the long-term efficacy of the attenuated *H. meleagridis* vaccine, providing robust protection against histomonosis, reducing severity of clinical signs and a significant reduction of mortality, organ lesions as well as parasite burden. The vaccine's effectiveness, when administered at day-old, highlights its potential to prevent histomonosis, though challenges remain for widespread use in commercial turkey farming.

RESEARCH HIGHLIGHTS

- Long-term efficacy of attenuated clonal monoxenic *H. meleagridis* vaccine was evaluated in turkeys.
- Long-term protection: Vaccine-protected turkeys from histomonosis for up to 84 days.
- Survival: 90% of vaccinated turkeys survived vs. 16% of non-vaccinated birds.
- Body-weights: Vaccinated birds weighed more, at 42 and 91–105 days of life.
- Lesions: Fewer liver/caecal lesions in vaccinated birds.

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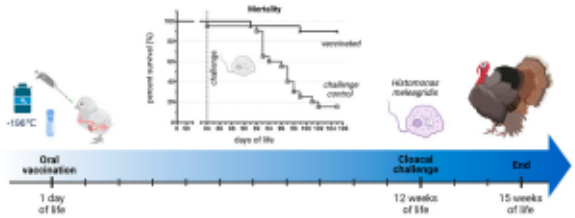
Duration of immunity; histomonosis; *Histomonas meleagridis*; vaccine; oral vaccination; long-term efficacy; turkey



Introduction

Histomonosis (also known as histomoniasis, infectious enterohepatitis, or blackhead disease) is a parasitic disease that primarily affects poultry, especially turkeys, and is caused by the protozoan *Histomonas meleagridis* (McDougald, 2005). Due to the severity of the disease in turkeys, entire flocks often have to be killed, which results in substantial compromise of bird welfare and high economic losses of this re-emerging disease (Hess *et al.*, 2015; Liebhart *et al.*, 2020). Histomonosis manifests initially with a reduction in feed intake (decreased appetite) followed by weight loss, inactivity, drooping wings (drowsiness), ruffled feathers, and diarrhoea with sulphur-coloured faeces (Barros *et al.*,

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2022). *Post-mortem* examination typically reveals necrotic lesions in the caeca and liver. While turkeys are most severely affected, other avian species can also become infected, with the severity of the disease varying across species (Hess *et al.*, 2015). In chickens, signs are generally less pronounced; however, *H. meleagridis* infection in laying hens and broiler breeders can lead to severe caecal inflammation clinically noticed by a decline in egg production (Esquenet *et al.*, 2003; Liebhart *et al.*, 2013). Furthermore, the correlation between histomonosis and colibacillosis is of high relevance (Paudel *et al.*, 2018).

For many years, histomonosis was well controlled by chemicals like arsenicals or nitro-heterocyclic compounds before the drugs were banned for use in poultry in several countries for reasons of consumer protection (for review, see Liebhart *et al.* (2017)). In the USA, arsenicals were used until being banned in 2016 resulting in the same situation as the EU with no licenced drugs left for prevention and treatment (Clark & Kimminau, 2017).

The first experiments on *in vitro* attenuation of *H. meleagridis* to be used for vaccination were published by Tyzzer (1936). However, the possibility of attenuating histomonads *in vitro* was later questioned, with an alternative hypothesis, suggesting that the coexistence of attenuated and virulent parasites in the same inoculum leads to the elimination of the virulent parasites (Lund *et al.*, 1966). Similarly, Beer *et al.* (2022) attributed lesions induced by *in vitro* attenuated histomonads, used as vaccine candidates that underwent 200 *in vitro* passages, to residual virulent histomonads within the vaccine isolates, albeit varying degrees of homologous and heterologous protection were recorded. In 2004, our laboratory established a clonal culture, the first of its kind, and implemented a completely new concept to create a live vaccine strain (Hess *et al.*, 2006). This clonal culture, generated through micromanipulation, was cultivated *in vitro* for approximately 3 years. Its efficacy and safety were confirmed through multiple studies, as previously summarized (Liebhart *et al.*, 2017). Amongst those, the high level of cross-protection was recently confirmed in a vaccination study, using the monoxenic vaccine candidate based on a genotype 1 histomonad strain with the *E. coli* strain 4CEF, to protect turkeys against a genotype 2 challenge (Hatfaludi *et al.*, 2022).

The duration of immunity (DOI), also termed duration of protection, is a critical aspect of vaccine efficacy. The principal parameters of protective efficacy include its nature, magnitude, onset, and duration. For example, in one of the previous studies, our laboratory demonstrated partial and complete protection when birds were challenged at 2 or 4 weeks post-vaccination (Liebhart *et al.*, 2010). Such study design is well in line with other previous experiments applying immunization at either 1 or 14 days of age, with challenge conducted at 4–6 weeks of age (Hess *et al.*, 2008; Sulejmanovic *et al.*, 2016; Lagler *et al.*, 2021; Beer *et al.*, 2022). Consequently, none of the studies to date has addressed DOI, a highly relevant subject given that field outbreaks, though less frequent, have been reported beyond the periods experimentally tested so far (Sulejmanovic *et al.*, 2017; Hauck *et al.*, 2018; Lüning *et al.*, 2023).

Therefore, the purpose of this study was to determine the DOI of an experimental vaccine, obtained by attenuation of a virulent *H. meleagridis* strain through repeated passaging for over 350x in culture medium, against a heterologous field isolate. For this, turkeys were vaccinated with the attenuated prototype histomonad vaccine at 1-day post-hatch, challenged at 84 days of life, and monitored for a total trial duration of 105 days of life.

Material and methods

Preparation of cultures for inoculation

Vaccine strain

The *in vitro* attenuated monoxenic clonal culture, *H. meleagridis* genotype 1/Turkey/Austria/2922-C6/04 co-cultivated with the bacterial strain *E. coli* 4CEF was cryopreserved and used for vaccination (totalling 351 passages: 290 times xenic/five times *E. coli* DH5a/ 56 times *E. coli* 4CEF) (Hatfaludi *et al.*, 2022). The frozen culture was thawed before vaccination by placing it in a water bath for 1 min at 37°C and thereafter resuspended in a vaccine carrier HuveGel® (Huvepharma NV, Antwerp, Belgium) (4% solution in Medium 199 supplemented with Earle's salts, L-glutamine, 25 mM HEPES and L-amino acids (Gibco™, Invitrogen, Paisley, UK)). Cells were not multiplied after thawing and before vaccination. Viable *H. meleagridis* cell count was determined using trypan blue and a Neubauer haemocytometer (Sigma-Aldrich, St. Louis, MO, USA) to adjust the vaccine dose to 10^4 cells/dose in 300 µl.

Challenge strain

The xenic clonal culture, *H. meleagridis*/Turkey/Germany/4114-C16/05 passaged seven times (Sulejmanovic *et al.*, 2016) was used for challenge. For this, the frozen *H. meleagridis* challenge strain was thawed at 37°C and cells were grown in cultivation medium, Medium 199 supplemented with Earle's salts, l-glutamine, 25 mM HEPES, and l-amino acids (Gibco™, Invitrogen, Paisley, UK), 10% horse serum (Sigma-Aldrich, Darmstadt, Germany), and 0.25% [w/v] sterilized rice starch (Carl Roth, Karlsruhe, Germany) for two additional passages, each for 72h at 40°C.

Housing of birds

A total of 40 1-day-old turkey poults (Hybrid Converter, Claeys Hatchery bvba, Kruisem, Belgium) were marked using sub-cutaneously fixed tags for individual identification before placement. The birds were divided into two groups: a vaccinated/challenged group and a challenge control group with 10 female and 10 male turkeys each. Groups of 20 birds were housed in two pens measuring 2 m^2/group during the first week to facilitate the circulation of the vaccine among vaccinated birds; thereafter, the pen size was increased to 4 m^2/ group. On day 49, all birds, as they had grown, were reallocated to pens measuring 2 m^2 at the same bird site, with each pen housing four turkeys: two males and two females.

The floor was covered with wood shavings in a bedding thickness of about 5 cm. One commercial pan feeder with a feed reservoir together with four drinking nipples was provided per pen.

The trial site was equipped with dynamic ventilation with an inlet centrally on the front side and an extraction unit at the back. The heat was provided by controlled gas-powered heaters. Ventilation and heating were regulated automatically. The ambient temperature (maximum/minimum) of the bird accommodation was recorded daily. The light scheme was regulated by a 24-h clock. Light duration decreased from 24–19 h over the first 6 days, with 30–40 lux intensity. From day 7 till the end of the trial, light was set at 18 h/day with 10–20 lux intensity. Pens were placed in such a way that light and environmental conditions were similar for all pens. Water and unmedicated commercial feed were provided *ad libitum* except for 4 h of feed restriction immediately after vaccination and challenge, similar to a previous trial (Hess *et al.*, 2006).

Design of the bird trial

On the first day of life, the birds of the vaccinated/challenged group were orally vaccinated with 10^4 cells of attenuated *H. meleagridis* genotype 1 with *E. coli* 4CEF (passage 351) in 300 µl HuveGel® solution by gavage using a syringe with small flexible tube, while control birds were left untreated. At 84 days of life, all birds, allocated either to the vaccinated/challenged group or challenged-only group were infected twice via the cloaca, each time with 0.5×10^5 cells of *H. meleagridis*/Turkey/Germany/4114-C16/05 (passage number 9) in 300 µl per bird (600 µl in total) using a conventional 1 ml pipette (Eppendorf AG, Hamburg, Germany). Birds were weighed at days 7, 42, 84, and 105 of life or when they were culled, and blood samples were collected on days 42, 84, and 105 of life. Five additional turkeys were killed at day-old for blood sampling.

Clinical monitoring and post-mortem investigation

Birds from each group were examined daily for any adverse clinical signs, especially apathy as well as reduced feed and water intake or diarrhoea. Clinical scoring was applied ranging from 0–3 with 0 – bird active with no clinical signs; 1 – slightly weak, dropping wings, and depressed; 2 – weak with ruffled feathers, reluctant to move, apathy; 3 – bird unable to move or stand, eyes closed, and intensified breathing.

Birds that were culled for welfare reasons due to histomonosis or at the end of the trial (day 105) were euthanized by the intravenous administration of thiopental (NadiMed, Kontich, Belgium) and subsequent exsanguination. During necropsy, lesion scores (LS) of the caecum and liver were determined. For this, an already established scoring system of pathological changes in the caecum and liver was applied (Bleyen *et al.*, 2009): LS 0 represents no lesion whereas LS 1–4 indicate mild to severe pathological changes. In detail, caecum lesion scoring system: 0 = no pathological changes; 1 = few scattered small lesions, but little or no thickening of the mucosal wall; 2 = yellow and foamy contents, lesions prominent but discreet and some bleeding and thickening of the mucosa; 3 = enlarged caeca, empty or filled with blood and/or yellow caseous abnormal lumen contents, thickened walls and confluent lesions, entire caecum involved; 4 = death from histomonosis, distended caeca with fragile walls, and necrotic lesions. Liver lesion scoring system in detail: 0 = no pathological changes; 1 = few small foci (off-white and variable in appearance) visible on the surface of the liver; 2 = lesions covering one-half of the liver surface; 3 = necrotic lesions (often large) covering more than 50% of the liver surface; 4 = death from histomonosis, with coalescing, necrotic lesions all over the liver surface. The veterinarians, performing lesion scoring, were entirely blinded. To ensure blinding, the birds for necropsy were collected from pens by the bird caretakers and delivered to the veterinarian. Bird caretakers were not blinded.

Detection of H. meleagridis DNA in cloacal swabs and organ tissues

To monitor parasite excretion cloacal swabs were taken weekly from the same five male and five female birds per group until the day of challenge (except at 21 days of life when no swabs were taken). Immediately after sampling, each swab was frozen at −20°C until further processing. Swabs were individually detached using a scalpel blade into a 2 ml Eppendorf tube containing 250 µl 1×PBS (Gibco™, Invitrogen, Paisley, UK) and the DNA extracted using the IndiSpin Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany) based on the manufacturer's instructions.

Following *post-mortem* examination, tissue samples of the liver and caecum were collected from all birds for DNA extraction, using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) based on the manufacturer's instructions. Approximately 25 mg of each tissue sample was placed in 2 ml Eppendorf tubes, 180 µl ATL buffer, and 20 µl proteinase K were added and incubated overnight at 56°C and 450 rpm in a thermomixer. For each sample, 200 µl was used for DNA extraction by an automated isolation machine (Qiagen QIAcube, Hilden, Germany).

qPCR (based on the 18S rRNA gene) was applied for screening the cloacal swabs and tissue samples for the presence of *H. meleagridis* DNA, as described previously (Sulejmanovic *et al.*, 2019). Ct values below 40 were considered positive.

Histopathology

Detection of histomonads in the liver was performed by histopathology. Briefly, samples of the organs were removed during necropsy and preserved in 10% neutral buffered formalin. The tissues were dehydrated in graded ethanol solutions, cleared in Neo-Clear™ (Sigma-Aldrich), and embedded in paraffin. Using a microtome (Microm HM 360, Microm Laborgeräte GmbH, Walldorf, Germany), 5 µm sections were cut, transferred to glass slides (Superfrost plus, Menzel-Gläser, Braunschweig, Germany) and dried. Afterwards, the sections were deparaffinized in Neo-Clear™, rehydrated in graded ethanol solutions, and stained with haematoxylin and eosin following a routine protocol before microscopic examinations were performed on an Olympus BX53 microscope equipped with the Olympus DP72 camera (Olympus Corporation, Tokyo, Japan). Histopathology of liver samples was assessed as follows: n = normal; mf = multi-focal mononuclear cell infiltration; inh = inflammation/necrosis/histomonads.

Serology

Blood samples were collected on days 1, 42, 84, and 105 of life, kept overnight at 4°C followed by centrifugation at 3300× *g* for 12 min and stored at −20°C until further processing. *H. meleagridis*-specific antibodies were measured in sera by an indirect sandwich ELISA following a protocol previously established (Windisch & Hess, 2009). Based on optical densities (OD) measured at a wavelength of 450 nm, OD-values above 0.450 were considered positive.

Statistical analysis

To determine differences in clinical scores, mean values area under the curve (AUC) were calculated using the trapezoid rule and graphically displayed with the AUC calculated between the groups using the Wilcoxon rank-sum test (Prism 5, GraphPad Software, Inc., San Diego, CA, USA). The results ($P < 0.05$) indicate a statistically significant difference. The mean qPCR Ct values were analysed with a two-way ANOVA test (Prism 5, GraphPad Software, Inc.). Mean lesion score data were analysed for variance using the General Linear Model, package, SAS Institute Inc. (2002) and compared using Duncan's multiple range tests, and a $P \leq 0.05$ was considered to indicate a statistically significant difference To evaluate both the mean optical density (OD) values of antibody titres and the average body weight, linear regression models were applied using the "lm" function from the base R package (R software, version 3.2.5 or later). Differences were considered statistically significant at $P < 0.05$.

Results

Clinical signs

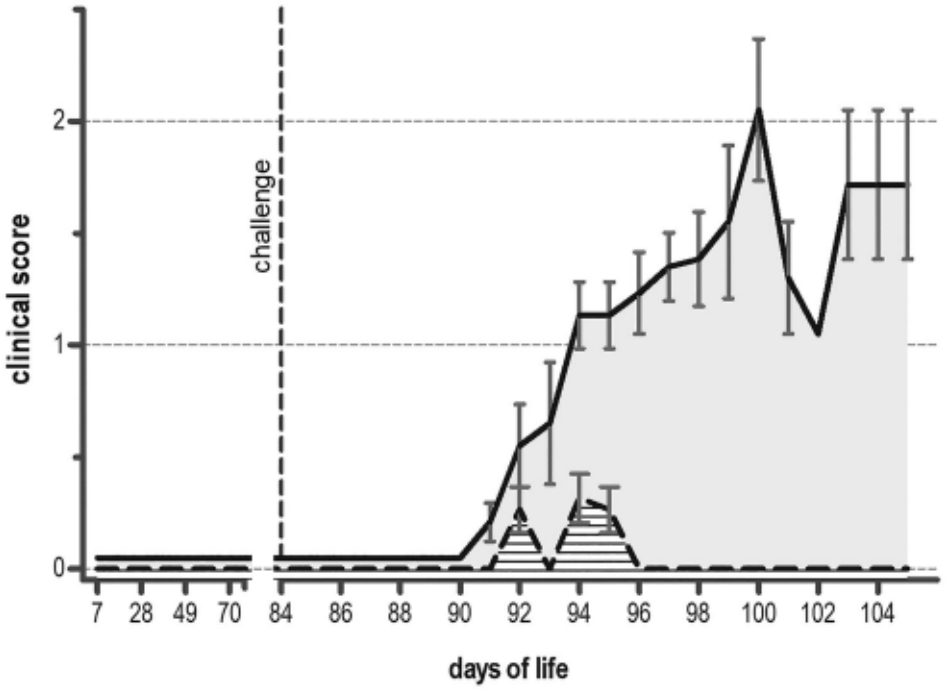
No adverse clinical signs or deaths were noticed before challenge at day 84 with the score remaining 0 for all birds in both groups. In Figure 1, the mean clinical scores and standard deviations for each treatment group are displayed starting from day 84. The AUC was calculated for each bird across all time points, and the group mean, standard deviation of the AUC, and sample size (*n*) for the corresponding period were determined. During the post-challenge period (days 84–105), the vaccinated/challenged group showed a significantly lower AUC for clinical scores compared to the challenge control group, with total AUC values of 0.84 versus 17.99 ($P < 0.05$).

Body-weight

The body-weight of each bird was recorded, and values were averaged at day old, day 42, the day of challenge (84) and at the end of the study (105) or at their death if it occurred earlier (91–105 days of life). Throughout the trial period up to the challenge, the body-weight of vaccinated turkeys remained higher than that of non-vaccinated birds. There were no statistically significant differences in the weight gain of the birds between the groups on the day of challenge (84 days of life) (Figure 2). At 42 and 91–105 days of life, the mean body-weight was significantly higher in the vaccinated/challenged group compared to birds from the challenge control group ($P < 0.05$).

Detection of H. meleagridis in cloacal swabs

In the vaccinated/challenged group excretion of histomonads started 14 days post-vaccination when



		total area	std. error	no. of birds
———	challenge control	17.99	3.29	18
– – –	vaccinated/ challenged	0.84	0.32	3 **

Figure 1. Mean clinical scores over time of turkeys following live vaccination at day-old with *H. meleagridis*/Turkey/Austria/2922-C6/04-4CEF (passage 351) and challenge with Hm/Turkey/Germany/4114-C16/05 (passage 9) on day 84, together with the challenge control group. Clinical signs were observed on the individual level and then averaged per group/day. Error bars indicate the standard deviation of the means. The shaded region represents the calculated area under the curve (AUC) for each group to evaluate the vaccine efficacy. ** statistically significant ($P < 0.05$) difference in the calculated AUC between the groups using the trapezoid rule and the Wilcoxon rank-sum test for the number of birds with clinical scores (data points).

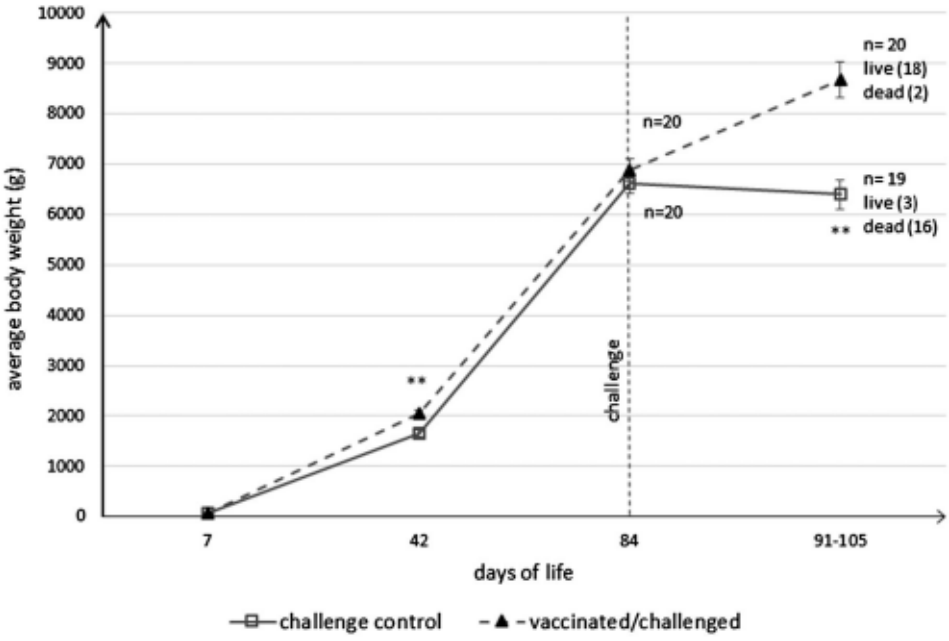


Figure 2. Average body weight of turkeys following live vaccination at day-old and challenge at day 84. Body-weight, at the end of the study, was calculated from 18 live and two dead birds in the vaccinated group, and from 3 live and 16 dead birds in the unvaccinated group. Error bars indicate the standard error of the means. ** statistically significant ($P < 0.05$) mean body-weight difference between the groups.

Table 1. Detection of parasitic *H. meleagridis* DNA by qPCR in cloacal swabs and organs, lesion scores, and histopathology of liver samples following live vaccination at day-old and challenge on day 84, together with the challenge control group.

Group	Sex	No.	qPCR of weekly cloacal swabs (days post-vaccination)[a]										Caecum		Liver		
			14	28	35	42	49	56	63	70	77	84[b]	Lesion score[c]	qPCR	Lesion score[d]	qPCR	Histology[e]
Challenge control	♂	1	–	–	–	–	–	–	–	–	–	–	3	+	4	+	inh
	♀	2	–	–	–	–	–	–	–	–	–	–	Dead				n.a.
	♂	3	–	–	–	–	–	–	–	–	–	–	3	+	4	+	inh
	♀	4	–	–	–	–	–	–	–	–	–	–	3	+	4	+	inh
	♂	5	–	–	–	–	–	–	–	–	–	–	3	+	4	+	inh
	♀	6	–	–	–	–	–	–	–	–	–	–	3	+	4	+	inh
	♂	7	–	–	–	–	–	–	–	–	–	–	3	+	4	+	inh
	♀	8	–	–	–	–	–	–	–	–	–	–	4	+	4	+	inh
	♂	9	–	–	–	–	–	–	–	–	–	–	3	+	4	+	inh
	♀	10	–	–	–	–	–	–	–	–	–	–	3	+	4	+	inh
	♂	11											1	+	4	+	inh
	♀	12											0	–	0	–	mf
	♂	13											3	+	4	+	inh
	♀	14											3	+	4	+	inh
	♂	15											1	–	0	–	n
	♀	16											3	+	4	+	inh
	♂	17											3	+	4	+	inh
	♀	18											1	–	0	–	mf
	♂	19											3	+	3	+	inh
	♀	20											3	+	4	+	inh
Vaccinated/challenged	♂	21	–	–	–	–	–	–	–	+	+	+	4	+	4	+	inh
	♀	22	–	–	–	–	–	–	–	–	+	–	1	+	0	–	mf
	♂	23	–	–	–	–	+	+	+	–	–	+	1	+	1	–	mf
	♀	24	–	–	–	–	–	+	+	–	+	–	1	+	0	–	mf
	♂	25	+	–	+	–	+	+	+	+	+	–	1	+	0	–	mf
	♀	26	+	–	–	–	+	–	+	+	–	+	3	+	1	–	mf
	♂	27	+	+	–	+	+	+	+	+	–	+	1	–	0	+	mf
	♀	28	+	–	–	+	+	+	+	+	+	+	1	–	0	–	n
	♂	29	–	+	–	+	–	+	+	+	+	+	0	+	0	–	mf
	♀	30	–	–	–	–	+	–	–	+	+	+	1	+	0	–	n
	♂	31											1	–	0	–	n
	♀	32											0	+	0	–	n
	♂	33											3	+	4	+	inh
	♀	34											1	–	1	–	mf
	♂	35											1	+	0	–	n
	♀	36											0	–	0	–	n
	♂	37											1	+	0	–	mf
	♀	38											1	–	0	–	mf
	♂	39											1	+	2	–	n
	♀	40											1	+	0	–	mf

[a]No swabs were taken on day 21, dead-euthanized due to limping before the challenge.
[b]Challenge at 84 days of age.
[c]Caecum lesion scoring system from 0 to 4 was applied; 0 represents no lesions, whereas 1–4 indicate mild to severe pathological changes.
[d]Liver lesion scoring system from 0 to 4 was applied; 0 represents no lesions, whereas 1–4 indicate mild to severe pathological changes.
[e]Histopathology of liver samples: n = normal (without lesions); mf = multi-focal mononuclear cell infiltration; inh = inflammation/necrosis/histomonads.
n.a.: not applicable.

sampling commenced. Considering all time-points before the challenge, every sampled bird (10 out of 20) of this group secreted histomonads at least once (Table 1). By far, most histomonads were detected from vaccinated birds 49 days post-vaccination with 60–70% of the sampled turkeys testing positive (Figure 3).

Mortality

No histomonosis-related mortality occurred before challenge. One bird in the challenge control group was culled on day 84 (before the challenge) due to severe limping. Mortality due to histomonosis started 8 days post challenge (dpc) in unvaccinated birds. At 7 and 15 dpc, two birds died in the vaccinated group. Overall, 90% of the vaccinated and challenged birds survived, compared to 15.8% survival of challenge control turkeys (Figure 4).

Lesion scores in the liver and caeca

All birds that died were necropsied and lesions were scored for histomonosis to assess the gross pathology and physical damage due to the parasitic infection. Figure 5 shows the lesion scores recorded for the liver and caeca for all birds grouped according to their scores. Significantly lower mean liver lesion scores were observed in the vaccinated/challenged group (0.65 vs 3.32, $P < 0.05$) compared to the challenge control group. Particularly, 14 out of 20 birds had no liver lesions (score 0) (Table 1). Furthermore, the mean lesion scores in the caeca were significantly lower in the vaccinated/challenged group (1.2 vs

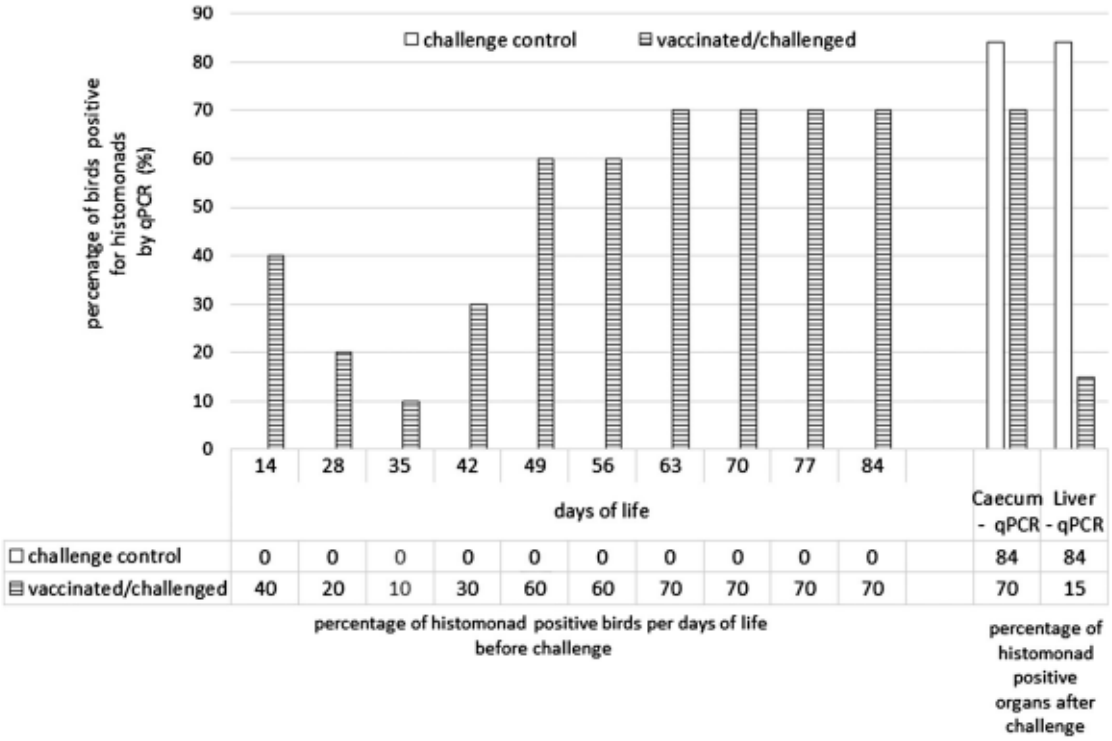


Figure 3. Detection of parasitic *H. meleagridis* DNA (%) by qPCR from cloacal swabs taken from vaccinated birds from 14 to 84 days of life, until the time of challenge. No swabs were taken on 21 days of life. The percentage of histomonad-positive qPCRs from organ samples collected during *post-mortem* investigations is shown on the right.

2.58, $P < 0.05$) compared to the challenge control group.

Histopathology

H. meleagridis-induced lesions in the liver, shown by histopathology are illustrated in Figure 6 and the findings in samples of each bird are listed in Table 1. Overall, the staining revealed typical lesions of histomonosis (inflammation/necrosis/histomonads) in 16 birds of the challenge control group and only in two birds in the vaccinated/challenged group (Table 1). No distinct signs of inflammation or necrosis (normal) were observed in the livers of seven vaccinated and challenged birds, compared to only one non-vaccinated bird. In addition, in the livers from 11 vaccinated/challenged birds, mononuclear cell infiltration was noted, likely due to increased inflammation, though histomonads were not observed.

Detection of H. meleagridis in organ samples

qPCR detection in necropsied organs revealed histomonads in 84% of livers from unvaccinated birds, compared to 15% in vaccinated and challenged birds. Similarly, a higher proportion of positive caecum samples was observed in unvaccinated birds (84%) compared to vaccinated birds (70%). Individual findings are given in Table 1.

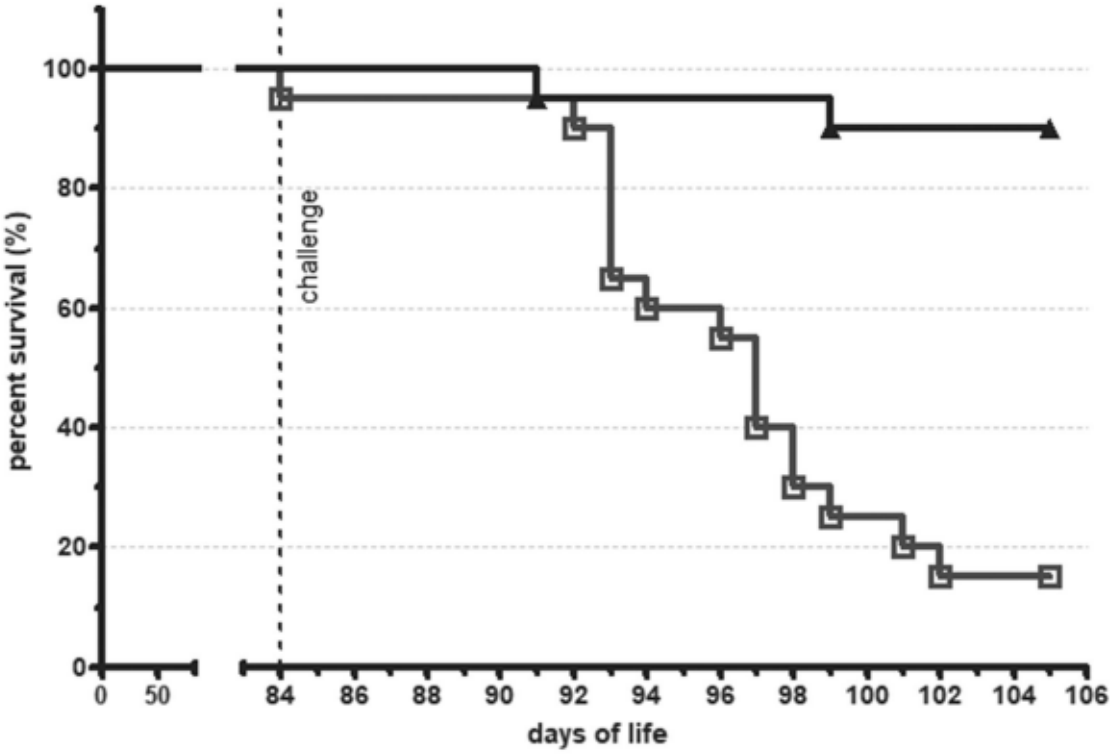
Serology

The mean *H. meleagridis* antibody titres of vaccinated birds on the day of challenge (84 days of life) and on day 105 were higher than those of the challenge control group (Figure 7). The difference between the titres of vaccinated and unvaccinated birds was statistically significant on day 84, before the challenge (0.865 vs 0.504, $P < 0.05$). Following the challenge, the titres in the sera of vaccinated birds increased more compared to those in non-vaccinated birds.

Discussion

Despite numerous experimental vaccination studies to prevent histomonosis, none have specifically addressed the duration of immunity in turkeys, the primary focus of the present study. First, it could be demonstrated that the developed prototype vaccine caused no adverse clinical signs for up to 12 weeks (84 days) of life prior to challenge and had no impact on the birds' weight gain. This finding aligns with an earlier study in which turkeys were vaccinated at 1 day of age and performance was monitored up to 16 weeks of age (Liebhart *et al.*, 2010). However, unlike the current work, the earlier study did not include a challenge, and the parasite was grown in a xenic background, making the two approaches somewhat different.

The results of this study confirm the effectiveness of the attenuated *H. meleagridis* vaccine in providing



		no. of birds	survival %	
▲	vaccinated/ challenged	18	90,0	**
□	challenge control	3	15,8	

Figure 4. Kaplan–Meier graph showing per cent survival per day after challenge and in total (%) of vaccinated and non-vaccinated birds following live vaccination on the first day of life and challenge on day 84. No histomonosis-related mortality occurred before the challenge. One bird in the challenge control group was culled on day 84 (before the challenge) due to severe limping. ** statistically significant ($P < 0.05$) higher survival in the vaccinated group.

significant protection against histomonosis in turkeys. This protection is evidenced by reduced clinical signs, improved survival rates, and minimized pathological damage. These findings are consistent with prior research demonstrating that vaccination can effectively mitigate the clinical impact of *H. meleagridis* and enhance poultry health and productivity. Vaccination also offers different levels of cross-protection against heterologous *H. meleagridis* strains, albeit depending on the vaccine strain (Ganas *et al.*, 2012; Sulejmanovic *et al.*, 2016; Beer *et al.*, 2022; Hatfaludi *et al.*, 2022; Chen *et al.*, 2024).

The observed clinical scores were analysed using the area under the curve (AUC) methodology, a quantitative approach widely applied in pharmaceutical and clinical studies to assess cumulative response over time (Derendorf *et al.*, 2000). AUC integrates both the magnitude and duration of clinical signs, providing a comprehensive measure of disease severity. The analysis revealed a significantly lower AUC (over 20-fold) for clinical scores of vaccinated/challenged turkeys compared to non-vaccinated birds following the challenge. This suggests that vaccination markedly reduces disease severity after challenge. Additionally, vaccinated birds showed higher body-weights throughout the trial, particularly at 42 and 91–105 days of life, supporting the notion that the vaccine helps maintain growth and productivity even after challenge.

The vaccine uptake was confirmed by the detection of histomonad DNA in the faeces starting 14 days post-vaccination, with 60–70% of the birds testing positive by 49 days of life. Intermittent excretion of live histomonads was demonstrated by re-isolation in earlier studies (Sulejmanovic *et al.*, 2013; Hatfaludi *et al.*, 2022), where cells were regrown in culture medium and examined microscopically. In contrast, qPCR is much less laborious, and the current data indicate a higher sensitivity albeit no validation was yet performed.

The most striking result was the substantial difference in survival rates, with 90% of vaccinated birds surviving the challenge compared to just 16% of unvaccinated challenge controls. This difference supports the long-term efficacy of the vaccine in preventing histomonosis-related mortality. The histopathological examination further confirmed these results, showing significantly fewer lesions in the liver and caeca of vaccinated birds. Additionally, fewer liver and caeca samples of vaccinated birds

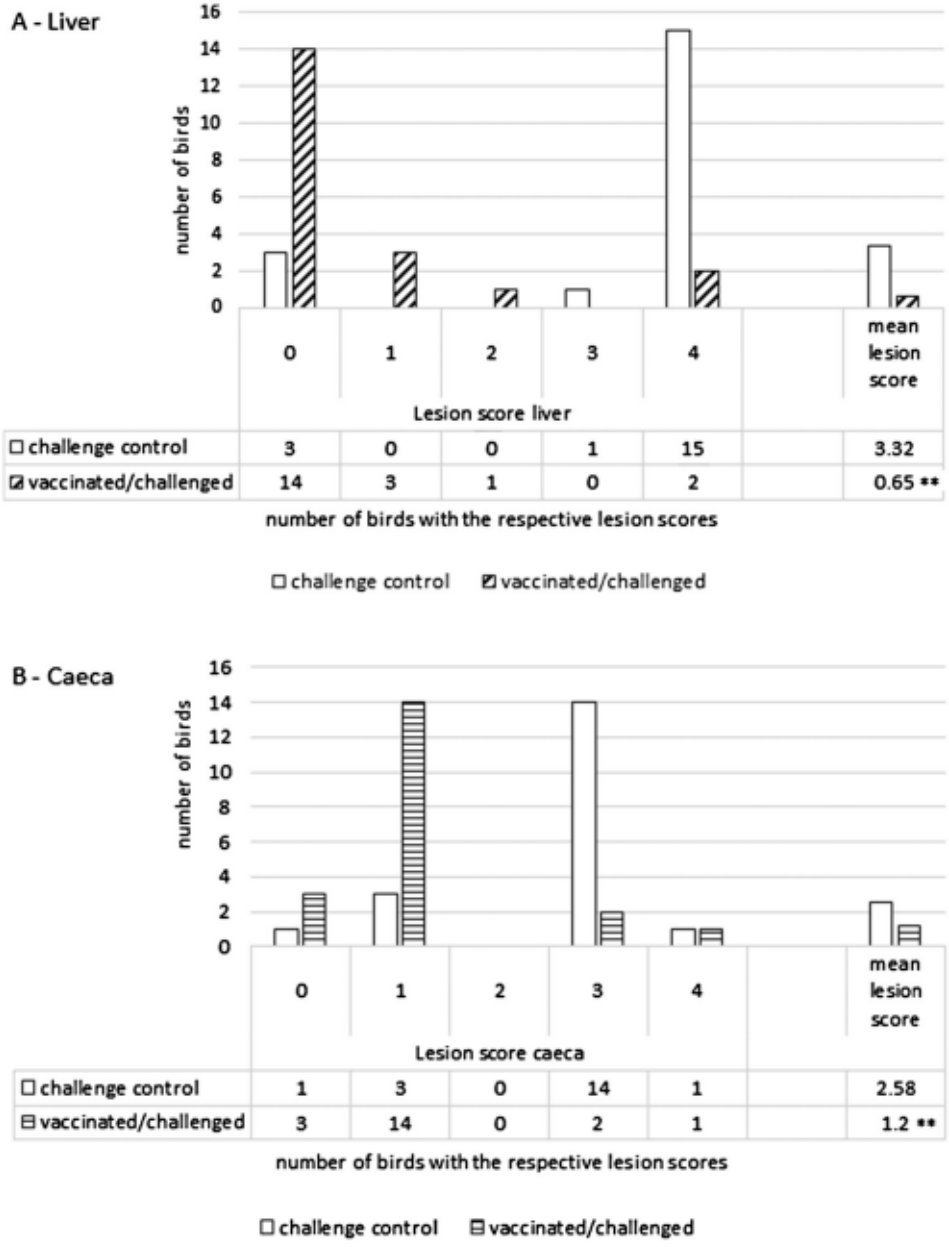


Figure 5. Lesion scores (0–4) in the liver (A) and caeca (B) recorded from vaccinated and non-vaccinated turkeys following live vaccination on the first day of life and challenge on day 84. Columns show the number of birds with each respective lesion score. ** statistically significant ($P < 0.05$) mean lesion score differences between the groups.

tested positive by qPCR, and histology detected fewer visible histomonads. These results confirmed that the monoxenic vaccine effectively limits parasite replication and minimizes tissue damage (Ganas *et al.*, 2012). This suggests that while the vaccine does not eliminate *H. meleagridis*, it significantly reduces parasite burden and prevents substantial tissue damage, allowing the vaccine to confer robust protection against the disease (Hess *et al.*, 2008; Ganas *et al.*, 2012).

Similar to this report, different previous immunization trials have demonstrated that the new live clonal monoxenic *H. meleagridis* vaccine candidate provides robust protection against a range of histomonad strains (Lagler *et al.*, 2021; Hatfaludi *et al.*, 2022). This study further shows that a high level of cross-protection is also available at 12 weeks (84 days) of life following day-old vaccination, indicating long-term resistance and ability to minimize local lymphocyte disturbances induced by infection (Mitra *et al.*, 2017). The occurrence of multifocal mononuclear cell areas in liver samples of vaccinated turkeys might be caused by the challenge 12 weeks later, consequently minimizing the infiltration of the parasite. Lymphoid cell aggregates had previously been detected after vaccination but to a lesser extent in challenged turkeys 4 weeks post-vaccination (Kidane *et al.*, 2018). However, those cells were positive for interferon-gamma and interleukin 10 mRNA, reflecting their potential for immune defence. Similar cellular effects were noticed in the caeca, although the cells were more disseminated in the tissue. While the possibility of a continuous local

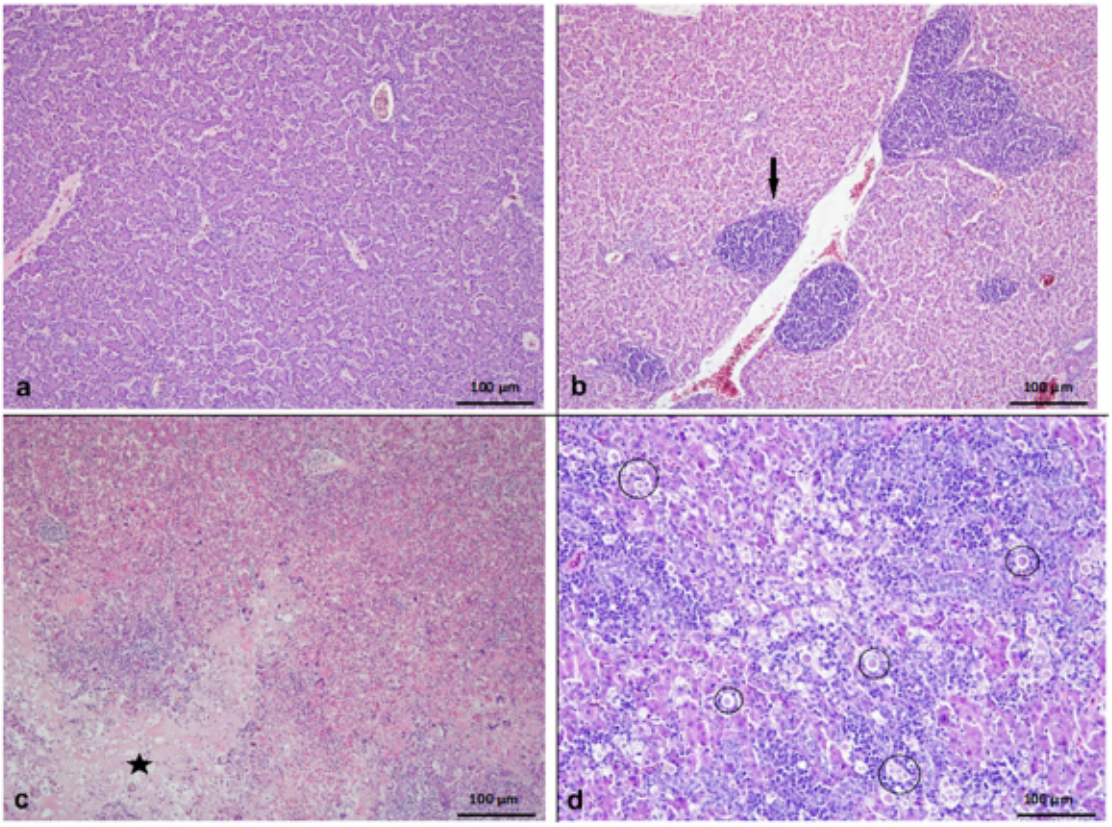


Figure 6. Histopathology for the detection of *H. meleagridis* and assessment of organ changes in liver samples of turkeys. Challenge control (a) liver without lesion (normal); (b) liver with multifocal infiltrations of mononuclear cells (arrow); (c) liver with necrosis and inflammation (asterisk); (d) multiple histomonads (circles) in the lesion.

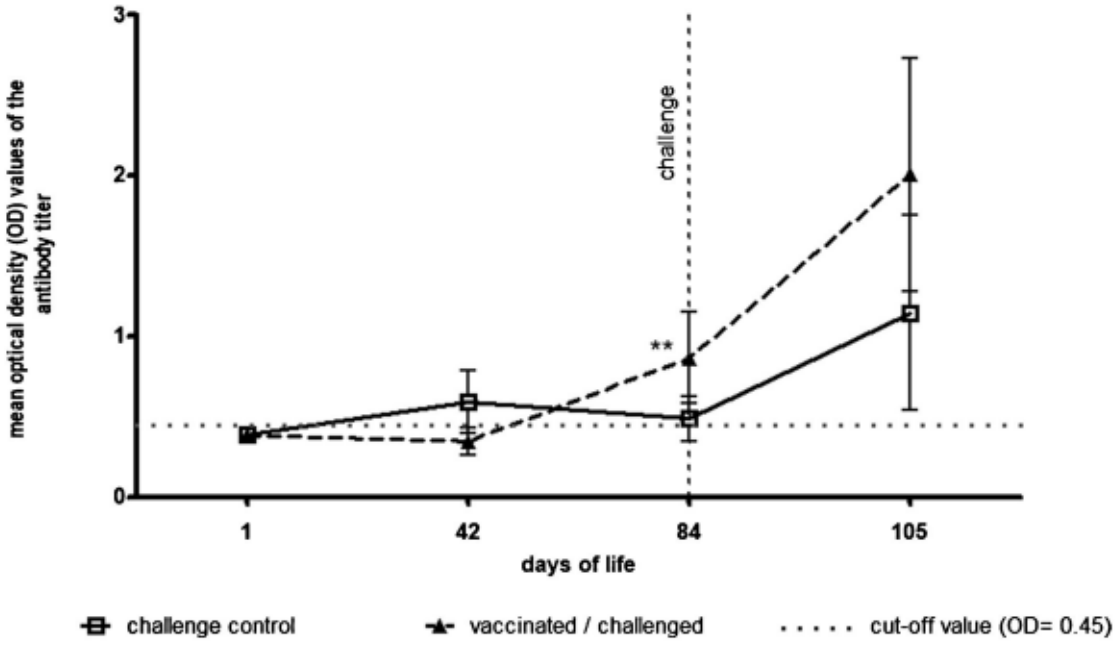


Figure 7. Mean antibody titres (optical density: OD values) per group and per sampling day of turkeys following live vaccination on the first day of life and challenge on day 84, together with the challenge control group. Error bars indicate the standard deviation of the means. ** statistically significant ($P < 0.05$) mean OD value difference.

immune response in the caeca due to parasite circulation and reinfection cannot be ruled out, a systemic immune response may contribute to protection. In this context, Lagler *et al.* (2021) found indications for a systemic cellular response in vaccinated turkeys and suggested a Th1-dominated immune response after infection with the parasite. In the latter paper, turkeys were challenged 4 weeks after vaccination, but the immune traits identified also appeared to be important for continued protection against later challenges. In

agreement with this, in the current study vaccinated birds developed a steeper incline and higher levels of serum antibodies after challenge compared to naïve birds, supporting a priming effect of vaccination.

This study demonstrates that the attenuated *H. meleagridis* vaccine provides significant protection against histomonosis in turkeys, as evidenced by improved clinical scores, higher survival rates, reduced lesions, and lower parasite burden in tissues, even at 12 weeks (84 days) of life after day-old vaccination, with no boosters required. Importantly, this vaccination was administered on the first day of life orally, which further emphasizes the vaccine's practicality for commercial turkey farming and the impact on histomonosis-associated losses. However, it needs to be mentioned that application remains a certain challenge to establish this vaccine as a valuable tool in the future for managing histomonosis in commercial turkey production.

Ethical statement

The bird trial and all the included procedures on experimental birds were approved by the Ethical Committee of Poulpharm BVBA, Izegem, Belgium (study code: P21263-FP) and took place at the Poulpharm animal site in Heestert, Belgium.

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