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Application of an experimental prototype vaccine against histomonosis (Blackhead disease) in turkeys

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Declaration of authorship

I hereby certify that the submitted work has been composed by me and is based on my own work. No other person's work has been used without due acknowledgment in this work. All references and verbatim extracts have been quoted. This work has not been submitted or published elsewhere.

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ABSTRACT

Histomonosis is a severe disease in turkeys. The ban of all effective chemical drugs used earlier for the control of this disease obliged researchers and laboratories to search for new alternatives. Among these alternatives, an experimental vaccine based on a clonal culture of *Histomonas meleagridis* attenuated *in vitro* was shown to be effective in turkeys against a virulent challenge. However, the features dealing with its application in turkeys to improve the efficacy of vaccination have not been studied in detail. This study investigated the effect of an edible gel (HuveGel®) used as a carrier for the oral administration of oral vaccines as well as two direct-fed-microbials (DFMs) (Broilact® and B-Act®) on the vaccine uptake.

In two distinct experiments, day-old turkeys were vaccinated with a single dose of a vaccine consisting of 10^4 *in vitro* attenuated *Histomonas meleagridis* with different applications. In experiment I, 4 groups of 8 birds each were used. A control group vaccinated via the cloacal route with the prototype vaccine alone was compared to three other groups vaccinated via the oral route with the prototype vaccine co-administered with HuveGel®. Of these three groups, one group received Broilact® in the drinking water and another group received B-Act® in the feed. In experiment II, the effect of HuveGel® when combined with the vaccine was investigated. A sham inoculated negative control group of 10 birds was compared to two other groups of 12 birds each vaccinated via the oral route with or without co-administration of HuveGel®.

No clinical signs, mortalities, or negative effect on the body weight of the birds were observed, independent of the vaccine preparation, the DFM, and the route of vaccination. Vaccine uptake was significantly higher in the group vaccinated via the cloacal route, the group whose vaccine was co-administered with HuveGel®, and the group supplemented with Broilact®. No liver lesions were observed, however, some minor cecal lesions with a low mean lesion score were observed in all vaccinated groups. Among them, the group supplemented with Broilact® had the highest mean lesion score (1.25) and the highest number of birds with cecal lesions (87.5% of birds). In experiment I, PCR and IHC confirmed the presence of histomonads in >94% of the cecal samples in the group vaccinated via the cloacal route (PV/c) and the group supplemented with Broilact® (PV/o+HuveGel+Broilact). In the other groups, histomonads were hardly detected. In experiment II, PCR and IHC confirmed the presence of histomonads in 100% of the cecal tissues in the group whose vaccine was co-administered with HuveGel® (PV/o+HuveGel) while in the group vaccinated via the oral route with the prototype vaccine alone (PV/o) no histomonads were detected.

Overall, the use of HuveGel® for oral application of the *Histomonas* vaccine improved vaccine uptake and may even be superior to the cloacal route. Adding Broilact® to day-old turkeys together with the *Histomonas* vaccine enhanced the vaccine uptake but triggered slight cecal lesion.

ZUSAMMENFASSUNG

Histomonose ist eine schwere Krankheit bei Puten. Das Verbot aller wirksamen chemischen Medikamente, die früher zur Bekämpfung dieser Krankheit eingesetzt wurden, zwang Forscher und Labore, nach neuen Alternativen zu suchen. Unter diesen Alternativen erwies sich ein experimenteller Impfstoff mit *in vitro* attenuiertem *Histomonas meleagridis* auf der Grundlage einer Klonkultur als wirksam bei Puten gegen eine virulente Herausforderung. Die Merkmale, die sich mit seiner Anwendung bei Puten zur Verbesserung der Wirksamkeit der Impfung befassen, wurden jedoch nicht im Detail untersucht. Diese Studie untersuchte die Wirkung eines essbaren Gels (HuveGel®), das als Träger für die orale Verabreichung von oralen Impfstoffen verwendet wird, sowie von zwei direkt gefütterten Mikroben (DFMs) (Broilact® und B-Act®) auf die Impfstoffaufnahme.

In zwei unterschiedlichen Experimenten wurden eintägige Truthähne mit einer Einzeldosis eines Impfstoffs geimpft, der aus 10^4 *in vitro* attenuierten *Histomonas meleagridis* mit unterschiedlichen Anwendungen bestand. In Experiment I wurden 4 Gruppen von jeweils 8 Vögeln verwendet. Eine Kontrollgruppe, die über die Kloake mit dem Prototypimpfstoff allein geimpft wurde, wurde mit drei anderen Gruppen verglichen, die über den oralen Weg mit dem Prototypimpfstoff zusammen mit HuveGel® geimpft wurden. Von diesen drei Gruppen erhielt eine Gruppe Broilact® im Trinkwasser und eine andere Gruppe B-Act® im Futter. In Experiment II wurde die Wirkung von HuveGel® in Kombination mit dem Impfstoff untersucht. Eine negative Kontrollgruppe von 10 Vögeln wurde mit zwei anderen Gruppen von 12 Vögeln verglichen, die jeweils oral mit oder ohne gleichzeitige Verabreichung von HuveGel® geimpft wurden.

Es wurden keine klinischen Anzeichen, Todesfälle oder negativen Auswirkungen auf das Körpergewicht der Vögel beobachtet, unabhängig von der Impfstoffzubereitung, dem DFM und dem Impfweg. Die Impfstoffaufnahme war signifikant höher in der Gruppe, die über die Kloake geimpft wurde, der Gruppe, deren Impfstoff zusammen mit HuveGel® verabreicht wurde, und der Gruppe, die mit Broilact® ergänzt wurde. Es wurden keine Leberläsionen beobachtet, jedoch wurden in allen geimpften Gruppen einige geringfügige Zökumläsionen mit einem niedrigen mittleren Läsionswert beobachtet. Unter ihnen hatte die mit Broilact® ergänzte Gruppe den höchsten mittleren Läsionswert (1.25) und die höchste Anzahl an Vögeln mit Zökumläsionen (87.5 % der Vögel). In Experiment I bestätigten PCR und IHC das Vorhandensein von Histomonaden in > 94 % der Zökumproben in der Gruppe, die über die Kloakenroute (PV/c) geimpft wurde, und der Gruppe, die mit Broilact® (PV/o+HuveGel+Broilact) ergänzt wurde. In den anderen Gruppen wurden Histomonaden

kaum nachgewiesen. In Experiment II bestätigten PCR und IHC das Vorhandensein von Histomonaden in 100 % der Zökumgewebe in der Gruppe, deren Impfstoff zusammen mit HuveGel® (PV/o+HuveGel) verabreicht wurde, während in der Gruppe, die nur mit dem Prototyp-Impfstoff (PV /o) über den oralen Weg wurden keine Histomonaden nachgewiesen.

Insgesamt verbesserte die Verwendung von HuveGel® zur oralen Verabreichung des *Histomonas*-Impfstoffs die Impfstoffaufnahme und könnte sogar der Kloakenroute überlegen sein. Die Zugabe von Broilact® zu eintägigen Truthähnen zusammen mit dem *Histomonas*-Impfstoff verbesserte die Impfstoffaufnahme, löste jedoch leichte Zökumläsionen aus.

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LIST OF ABBREVIATIONS

dpv Day Post Vaccination

DFM Direct-Fed-Microbial

ELISA Enzyme-Linked Immunosorbent Assay

GE Gross Examination

IHC Immunohistochemistry

LS Lesion Score

ME Microscopic Examination

PCR Polymerase Chain Reaction

PME Postmortem Examination

NC Negative Control Group

1. INTRODUCTION

Histomonosis, also known as blackhead disease, is an infectious disease of gallinaceous birds (Hess and McDougald, 2020).

For decades, arsenicals, nitrofurans, and nitro-imidazoles were used and the disease was well controlled. However, the withdrawal of these drugs in the EU and the USA due to drug residue concerns has led to the re-emergence of the disease (Hess et al., 2015; Liebhart et al., 2017). Today, no drug or vaccine against histomonosis is commercially available. The sole measure to control the disease is limiting its transmission once it enters flocks through farm management techniques (Liebhart et al., 2017).

Previous attempts to immunize birds with attenuated strains of *H. meleagridis* showed some degree of success but the overall results were inconclusive (Tyzzer, 1934; Tyzzer, 1936; Lund, 1959; Lund et al., 1966). In all these studies the attenuated histomonads were administrated via the cloacal route to birds over 2 weeks of age. In addition, a booster was often required to maintain a certain level of immunization. However, vaccination of 14-day-old turkeys with an *in vitro* attenuated cloned strain of *H. meleagridis* via the cloacal route resulted in successful immunization of the birds (Hess et al., 2008). Other studies showed that the vaccine based on an *in vitro* attenuated cloned strain of *H. meleagridis* was effective and safe in day-old turkey via the oral route (Liebhart et al., 2010; Liebhart et al., 2011). The vaccine did not revert to virulence (Sulejmanovic et al., 2013) and was effective against heterologous strains (Sulejmanovic et al., 2016). All these advances make the vaccine based on an *in vitro* attenuated cloned strain of *H. meleagridis* an ideal candidate for the development of a commercial *Histomonas* vaccine but application under field conditions has to be investigated. For practical reasons, administration in hatcheries on the day of hatch via the oral route without a booster would be ideal.

The involvement of certain cecal bacteria is essential in the initiation of histomonosis in the host (Bilic and Hess, 2020). Studies on gnotobiotic turkeys and chickens showed that according to the bacterial strains supplied with the protozoan culture, the disease can be severe (lesions in both ceca and liver), moderate (lesions in the ceca only) or absent (no lesions) (Springer et al., 1970). Chicks hatch with an immature adaptive immune system and almost non-existent gut microbiota with the major colonization of the chicks' gut occurring after hatching (Smith et al., 2022; Kogut, 2022). The development and maturation of the gut (adaptive) immune system after hatch are linked to microbial colonization of the gut (Schokker et al., 2017; Rodrigues et al., 2020; Zenner et al., 2021). Thus, the faster the establishment of

the gut microbiota, the earlier the maturation of the gut immune system (Rubio, 2019). Numerous studies showed that the modulation of the gut microbiota around the time of hatch through inoculation of microbiota derived from adult birds or certain direct-fed-microbials (DFMs) resulted in accelerated maturation of chicks' gut microbiota and, therefore, the acceleration of their immunocompetence (Lee et al., 2010; Gao et al., 2017; Meijerink et al., 2020).

DFMs can consist of mono-strains like *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Enterococcus*, *Saccharomyces,* etc., or multi-strains of bacteria (Patterson and Burkholder, 2003). Certain strains of DFMs (also called immunobiotics) can enhance the mucosal immune responses. The mechanisms of action of immunobiotics are multifactorial and not well-characterized. Proposed mechanisms include cytokine activation (Brisbin et al., 2010), expression of Toll-like receptors (Sato et al., 2009), or enhancement of antibody production (Yang et al., 2005; Haghighi et al., 2005; Haghighi et al., 2006). Based on these data, DFMs could be used as immunoadjuvants to increase the vaccine response. However, few studies have investigated the effect of DFMs around the time of vaccination of birds. Co-administration of DFMs with a coccidiosis vaccine (Ritzi et al., 2016) or Newcastle disease and infectious bursal disease vaccine (Talebi et al., 2008) in day-old chicks enhanced resistance following a virulent challenge. Moreover, the administration of DFMs to day-old chicks minimized the immunological stress against a challenge with lipopolysaccharides (Li et al., 2015).

Administration of live oral vaccines is challenging because of the harsh gastrointestinal environment. A method of delivering live oral vaccines in an edible gel is used in hatcheries in recent years. This method showed an increase in intestinal vaccine uptake and therefore vaccine effectiveness (Jenkins et al., 2012).

Based on these data, the objective of the current study was to test new methods for applying an experimental prototype vaccine against histomonosis to day-old turkeys.

Two hypotheses have been advanced:

(1) A diluent gel (HuveGel®) could improve the uptake of the *Histomonas* vaccine in day-old turkeys and therefore enhance its effectiveness.

(2) Provision of two direct-fed-microbials (Broilact® and B-Act®) to day-old turkeys could accelerate the establishment of their initial microbiota and thus improve the uptake of the *Histomonas* vaccine.

1. LITERATURE OVERVIEW

2.1. HISTOMONOSIS: A GENERAL OVERVIEW

Histomonosis, also known as blackhead disease (Cushman, 1893), infectious enterohepatitis (Smith, 1895), or histomoniasis (Tyzzer, 1920), is an infectious disease of gallinaceous birds (Hess and McDougald, 2020). Histomonosis is of great significance in commercial turkeys and chickens because of its impact on the health and welfare of infected flocks as well as the substantial economic losses and shortfalls (Hess et al., 2015). These consist of high rates of morbidities and mortalities observed in turkeys, reaching in some cases 100% of the flock (McDougald, 2005), and a decrease in the zootechnical performances of chickens (Liebhart et al., 2013). The causative agent is a single-cell anaerobic protozoan called *Histomonas meleagridis* (Hess and McDougald, 2020). Two stages of *H. meleagridis* are known; (i) flagellated cecal lumen-dwelling form; occurring in a non-amoebiform (3–16 µm in size) and owning one single anterior flagellum which is lost during the invasion of the host's tissues, and (ii) non-flagellated tissue form; occurring in an amoebiform (6–20 µm in size) (Honigberg and Bennett, 1971).

Histomonosis has been reported in a wide range of galliform birds. However, the clinical manifestation of the disease and the lesions differ according to the host species infected. In turkeys, clinical signs are impaired general condition with sulfur yellow diarrhea and high mortalities. The lesions consist of ulcerative or hemorrhagic inflammation in the cecum (typhlitis) and multifocal areas of necrosis in the liver (hepatitis) (Hess and McDougald, 2020). The infection is less severe in chickens. Clinical signs are mild, mortalities are slightly increased, and the lesions are often restricted to the ceca (Hess et al., 2015; Hess, 2017). The routine diagnosis of histomonosis is based on clinical signs and the appearance of gross lesions in the ceca and liver. Direct identification of histomonads using microscopic or histopathological examination, or indirect identification using a PCR or immunohistological examination allows for confirming or refuting the clinical diagnosis (Hess and McDougald, 2020).

The literature review shows three stages in the evolution of research on histomonosis; (i) Emergence of the disease: From the first outbreak of histomonosis in 1894 in Rohde Island (Cushman, 1893) until the development of modern anti-histomonal drugs in the middle of last century, where management changes brought the disease under control, although it remained the first cause of death in commercial turkey flocks (McDougald, 2005). During this period, scientific research was focused on the disease and its causative agent, but also on the

development of effective drugs (McDougald, 2005). (ii) Disease control: From the introduction of effective antihistomonal drugs against histomonosis until their withdrawal in the late 1990s, where effective chemical drugs (dimetridazole and nifursol) have been used to control the disease. Thus, due to the regular use of these drugs, research on histomonosis has been neglected and little work was published. (iii) Re-emergence of the disease: From the withdrawal of effective chemical drugs used against histomonosis in Europe and the USA until today. Thus, after several decades of silence, histomonosis is resurfacing and reemerging on a massive scale in numerous areas mainly Europe and the USA, with devastating effects on commercial turkey farming (Liebhart und Hess, 2020).

Indirect oral transmission with a vector following ingestion of eggs of the intermediate host *Heterakis gallinarum* has long been considered the most significant route of transmission under field conditions (Springer et al., 1969). It is well known that unprotected histomonads do not survive in acidic environments (Bishop, 1938; Delappe, 1953). Therefore, due to the acidic environment of the digestive tract of birds, oral natural infection without a vector has long been considered insignificant. However, recent studies showed that histomonads can survive acid conditions inside the upper intestinal tract, thus rendering direct oral transmission without a vector feasible (Liebhart and Hess, 2009; Liebhart et al., 2010). However, direct oral transmission under field conditions remains controversial.

Pathogenesis of histomonosis is linked to (i) the affected host species (Lund and Chute, 1972) and (ii) the kind of cecal bacteria as demonstrated in trials with germ-free turkeys and chickens (Springer et al., 1970). After entering the digestive tract of the host via the cloacal or oral route, histomonads reach the cecum and invade the cecal mucosa causing typhlitis. Histomonads can cross the cecal intestinal barrier and spread to the liver via blood circulation leading to hepatitis. Sometimes other organs like the bursa of Fabricius and kidneys are also invaded (Singh et al., 2008; Hess and McDougald, 2020).

Histomonads have an obligate relationship with certain live cecal bacteria, although the role of the latter remains unclear. Indeed, the involvement of certain bacteria is vital for the histomonads, whether to grow and maintain in a culture medium (*in vitro*) or to cause disease in its host (*in vivo*). The different hypotheses concerning this obligate relationship histomonads-bacteria can be found at Bilic and Hess (2020).

In earlier studies xenic cultures (fresh cecal microbiota) were used for the *in vitro* cultivation of histomonads and all attempts to cultivate histomonads in an axenic culture medium were unsuccessful (Hauck et al., 2010). In monoxenic cultures, the cultivation of histomonads was successful in at least 8 species of Enterobacteriaceae. Among these 8 species, histomonads

had the best growth with *E. coli*. In other monoxenic cultures with Lactobacillaceae, Micrococcaceae, Brucelaceae, Corynebacteriaceae, Bacillaceae, and Pseudomonadaceae, the histomonads died after a few transfers (Stepkowski and Klimont, 1980). In a recent study, the best cultivation of histomonads was observed in monoxenic cultures with *E. coli*, then to a limited extent with *S. typhimurium* and *P. aeruginosa*. In monoxenic cultures with *C. perfringens* and *S. enteritidis,* the histomonads died after a limited number of transfers whereas monoxenic cultures with *E. faecalis* or *S. aureus* did not support the growth of histomonads (Ganas et al., 2012). The same study reported that the degree of attenuation of histomonads was not linked with the bacteria in the culture medium but rather to the number of passages *in vitro*. Moreover, the virulence of histomonads from monoxenic cultures with *E. coli* was nearly similar to that observed with xenic cultures when inoculated into turkeys, although the appearance of clinical signs took (about 1 week) longer (Ganas et al., 2012).

The inoculation of gnotobiotic turkeys or chickens with histomonads alone resulted in no lesions and did not induce disease, whilst when the cecal microbiota from conventional chickens or turkeys were introduced, histomonads led to lesions and induced a disease (Springer et al., 1970; Kemp, 1974). The use of gnotobiotic turkeys and chickens demonstrated that certain bacteria species introduced with histomonads resulted in no lesions and induced no histomonosis, other bacteria species induced mild to moderate histomonosis with cecal but not liver involvement, while other bacteria species induced severe histomonosis with cecal and liver lesions (Franker and Doll, 1964; Bradley et al., 1964; Bradley and Reid, 1966). Also, when histomonads and two species of bacteria i.e., *E. coli* and *C. perfringens,* were introduced into gnotobiotic turkeys, more lesions and more severe histomonosis were observed than when the histomonads were introduced with a single species of bacteria i.e., *E. coli* or *C. perfringens* (Springer et al., 1970). Moreover, when histomonads with *E. coli* and *C. perfringens* were used for inoculation, typical cecal and liver lesions were observed in all gnotobiotic turkeys, whilst mild atypical cecal lesions were observed in a limited number of gnotobiotic chickens (Springer et al., 1970).

The severity of histomonosis results from an interaction between (i) factors related to the host, i.e., breed of birds (Lotfi and Hafez, 2009), age of birds (Lund, 1969), and infection route (Farmer and Stephenson, 1949), (ii) those related to the parasite, i.e., the virulence of the strain (Lund, 1969) and the infective dose (Lund, 1955). Although the latter does not have an impact on disease outcome when a strain is highly virulent (Liebhart et al., 2008). And (iii) the cecal bacteria as mentioned above (Springer et al., 1970).

For a long time, arsenicals e.g., nitarsone (Jaquette and Marsden, 1947), nitrofurans, e.g., furazolidone, nifursol (Sullivan et al., 1972; Vatne et al., 1969) and nitro-imidazoles, e.g., dimetridazole (Flowers et al., 1965; Morehouse et al., 1968), have been used for the control of histomonosis. A recent review summarizes in detail earlier and current strategies used against histomonosis (Liebhart et al., 2017).

Today, after the withdrawal of all efficacious chemotherapeutics, no drugs or treatments have been approved to prevent or treat histomonosis in commercial flocks, neither in the EU nor in the USA (Liebhart et al., 2017; Clark and Kimminau, 2017). Moreover, it also seems unlikely that a new active ingredient will be able to obtain marketing authorization (MA) as long as the laboratories do not undertake research in this direction (Regmi et al., 2016). The sole realistic measure left for veterinarians and farmers to control histomonosis right now is to limit its entrance into a flock through farm management techniques, e.g. changing litter between flocks, separating flocks of different ages and species, and other biosecurity and hygiene measures (Liebhart et al., 2017). However, these remain insufficient to prevent the occurrence of an outbreak and the disease remains an increasing concern, especially in case of valuable turkey breeder flocks (Clark and Kimminau, 2017).

Passive immunization of birds with antisera from immune birds (Clarkson, 1963; Bleyen et al., 2009) and active immunization with live strains of *Histomonas wenrichi* (Lund, 1963) or inactivated strains of *Histomonas meleagridis* (Hess et al., 2008; Bleyen et al., 2009) failed to confer immunization against challenge with virulent strains of histomonads.

In earlier studies, some degree of success was achieved to immunize chickens and turkeys with *in vitro* attenuated histomonads but the overall results were inconclusive (Tyzzer, 1936; Lund, 1959; Lund et al., 1966). However, vaccination of 14-day-old turkeys with a clonal *in vitro* attenuated strain of *H. meleagridis* via the cloacal route resulted in full protection against a homologous virulent challenge (Hess et al., 2008). Subsequent studies showed that such a vaccine was also effective and had no negative effects on the growth of turkeys being vaccinated at day-old via the oral route (Liebhart et al., 2010). The vaccine was also found safe in both day-old turkeys and chickens (Liebhart et al., 2011). Other studies showed that the attenuation was stable with no reversion to virulence after serial passages *in vitro* (Sulejmanovic et al., 2013) or *in vivo* (Pham et al., 2013). Moreover, the vaccine was found effective against heterologous virulent strains (Sulejmanovic et al., 2016; Hatfaludi et al., 2022).

It is well-known that the gut microbiota composition of turkeys and chickens differs according to the age of the birds. In addition, other factors like breed, sex, feed, etc. also influence the composition of the gut microbiota (Maki et al., 2019). This suggests that the severity of histomonosis attributed to the age and breed of birds is the consequence of the different compositions of the caecal microbiota.

2.2. MICROBIOTA OF DAY-OLD TURKEYS

Limited studies have been conducted so far on the microbiota of turkeys. Most studies on the gut microbiota of birds concerned commercial chickens, often broilers older than 1 week of age.

Cecal microbiota varies according to the age of the birds. The bacterial microbiota of the ceca of adult commercial turkeys contains 4 major phyla; Firmicutes (66.3%), Proteobacteria (7.4%), Actinobacteria (3.2%), Bacteroidetes and 4 minor phyla; Verrucomicrobia, Synergistetes, from Elusimicrobia and Lentisphaerae with an abundance of two bacterial genera; *Olsenella* and *Rikenella* (Wei et al., 2016).

The review of the literature highlights three steps in the establishment of the gut microbiota in commercial birds (chickens or turkeys) hatched in commercial hatcheries; (i) initial colonization, (ii) succession over time, and (iii) maturation and stabilization (Rychlik, 2020).

Microbial colonization starts at hatch when microorganisms from the environment colonize the gastrointestinal tract and establish the first microbial inoculum of hatched birds called the initial gut microbiota (Stanley et al., 2013). It was long assumed that the eggs form in a sterile environment and that the birds hatch with a sterile intestine. However, recent research on broiler chicks reported that a few microbial colonizers can be transmitted from the hen to the embryo during oogenesis (Ding et al., 2017; Lee et al., 2019). However, this vertical transfer of microbiota remains marginal (Akinyemi et al., 2020). The studies investigating day-old commercial chick's microbiota describe an "almost haphazard" initial gut microbiota with significant variations (Polansky et al., 2016; Rychlik, 2020). These variations are observed between the different groups of day-old chicks and between the chicks within the same group (Scupham, 2009; Stanley et al., 2013). Some of these reported differences could be attributable to the true age of the day-old chicks at the time of the sampling as day-old chicks obtained from hatcheries can be less than 24 hours or more than 72 hours old at the time of distribution to farms (Richards et al., 2019). Studies on day-old commercial chicks showed that the bacterial colonization of the gut is rapid and significant with most colonization occurring in the first days after hatching (Lu et al., 2003; Ballou et al., 2016). Pedrosa et al. (2005) reported that upon arrival at the farm, the chicks already had complex communities of bacteria in their

intestinal tract (Pedroso et al., 2005). In another study, the cecum of chicks was estimated to contain 10^{10} bacteria/gram of cecal content one day after hatching. This number increased to 10^{11} on the third day and remains high for the following 30 days (Apajalahti et al., 2004). However, most of these differences are related to modern commercial practices in hatcheries. Studies have shown that chicks hatching in contact with an adult hen (like under natural conditions) receive most of their initial gut microbiota through vertical transfer from the mother with some input from the environment (Kubasova et al., 2019). However, in commercial hatcheries, the eggs are fumigated before their incubation and the chicks hatch in a clean environment with no contact with adult birds. Hence, hatched chicks get almost all of their initial gut microbiota from environmental sources (environment of hatcheries, human handlers, transport boxes, transport vehicles, etc.) instead of the natural maternal source (Stanley et al., 2013; Donaldson et al., 2017; Kubasova et al., 2019).

In a recent study, the investigation of the initial gut microbiota of commercial turkey poults at the time of hatching, before standard operations take place such as vaccination, sexing, etc. showed a variation in its composition between the different hatcheries and the time of sampling. Initial gut microbiota composition was also reported to be poor in diversity (Smith and Rehberger, 2018). The gut microbiota diversity increases as the birds encounter new microorganisms from environmental sources (litter, food, water, etc.) (Rychlik, 2020).

3. MATERIALS AND METHODS

3.1. Experimental materials

3.1.1. Prototype vaccine and culture medium

The *Histomonas* prototype vaccine (PV) used consisted of an attenuated mono-eukaryotic culture of histomonads labelled as *Histomonas meleagridis*/Turkey/Austria/2922-C6/04 (Hess et al., 2006) combined with a monoxenic culture of *E. coli* 4CEF (Ganas et al., 2012). In brief, *H. meleagridis*/Turkey/Austria/2922-C6/04 was cultivated in a culture medium containing a xenic culture of cecal bacteria and passaged for 290 *in vitro* before the establishment of monoxenic culture with *E. coli* DH5a (Ganas et al., 2012). After 5 further passages *in vitro* (totaling 295 passages) the *E. coli* DH5a bacterial strain was replaced with *E. coli* 4CEF using the same procedure. Additional 12 *in vitro* passages (totaling 307 passages) were carried out for experiment I *(290x xenic/ 5x DH5a/ 12x 4CEF)* and 44 *in vitro* passages (totaling 339 passages) for experiment II *(290x xenic/ 5x DH5a/ 44x 4CEF)* before the cryogenic freezing of the culture.

To prepare the vaccine inoculum, the frozen culture was thawed in a water bath for 1 min at 37°C. Viable histomonad cells count was determined using trypan blue a Neubauer hemocytometer (Sigma-Aldrich, St. Louis, MO, USA) to adjust the vaccine dose to 10^4 cells/dose in 300 µl of a culture medium (90% Medium 199 containing Earle's Salts, L-glutamine, 25 mM HEPES and L-amino acids (Gibco™), 10% heat-inactivated horse serum (Gibco™), and 0.25% rice starch (Sigma-Aldrich)) before being used as a vaccine. Histomonad cells were not multiplied after thawing and before vaccination.

3.1.2. Additives

HuveGel® (HuvePharma N.V., Antwerp, Belgium): HuveGel® is composed of maltodextrin, polyethylene glycerol, and powdered cellulose. In this study, the gel solution of HuveGel® was used as a carrier (or vehicle) for the oral administration of the prototype vaccine.

Broilact® (Orion Corporation, Espoo, Finland): Broilact® is a selected mixed culture derived from the cecal contents and scrapings of cecal walls of one single hen from 1987. Altogether 32 different types of bacteria have been isolated from Broilact®, including 22 strictly anaerobic rods and cocci, representing five genera, and 10 different facultatively anaerobic rods and cocci representing three genera. It is entirely free from all spore-forming organisms and contains only one Gram-negative facultative anaerobic rod, a well-characterized *E. coli* strain sensitive to all tested antibiotics (Schneitz and Hakkinen, 2016). The gut microbiota in Broilact® colonizes the intestinal epithelium and forms a complex ecosystem, which creates an

inhospitable environment for undesirable bacteria, inhibiting their ability to attach and multiply. This defence mechanism is known as competitive exclusion. Broilact® can be used for various species of fowl, including chickens, and turkeys, it is recommended for use in day-old chicks, as a single dose of 1 mg per chick, via the oral route.

B-Act® (HuvePharma N.V., Antwerp, Belgium): B-Act®, is a preparation containing viable spores of a strain of *Bacillus licheniformis* DSM 28710. B-Act® is a powder with a minimum declared content of 3.2 x 10^9 colony forming units (CFU) of *Bacillus licheniformis* DSM 287107 per gram of additive (spores concentrate, ~ 3% and calcium carbonate, ~ 97%). *Bacillus licheniformis* has been reported to improve gut health in day-old chickens. B-Act® is intended for use in feed for chickens and turkeys for fattening, chickens and turkeys reared for laying/breeding, and minor growing species for fattening or raised for laying/breeding at the proposed dose of 1.6 x 10^9 CFU/kg complete feeding stuff (Rychen et al., 2016).

3.2. Experimental animals and experimental design

The experimental trials were conducted with day-old commercial turkeys sourced from a commercial hatchery (Putenzucht Miko GmbH, Oberösterreich, Austria). Both experimental trials were discussed and approved by the institutional ethics committee and licensed by Austrian law (license numbers GZ 2020-0.028.651).

After receipt, each bird was tagged with an identification number using a tool designed for this purpose (Swiftack™, Heartland Animal Health Inv., Fair Play, MO, USA) and assigned into distinct groups containing the same male-female *ratio* as follows:

Experimental trial I: A total of 32 day-old turkeys were divided into 4 groups of 8 birds each kept until 6 weeks of age. At the beginning of the trial, 5 day-old turkeys were killed to collect blood for antibody detection by ELISA.

Experimental trial II: A total of 34 day-old turkeys were divided into 2 groups of 12 birds each and 1 control group of 10 birds and raised until 12 weeks of age. At the beginning of the trial, 5 day-old turkeys were killed to collect blood for antibody detection by ELISA.

In both experimental trials, each group of birds was raised in a floor pen of about 1 m^2 and housed on deep litter (about 10 cm depth) consisting of wood shavings. Each floor pen was equipped with one drinker, one feeder, and one infrared heating lamp. Non-medicated commercial feed (Vitakorn Futtermittel GmbH, Burgenland, Austria) and water were provided *ad libitum* throughout the experimental trials. The design of the experimental trials is shown in Table 1.

Biosecurity procedures were maintained throughout the experimental trials between the different vaccinated groups. New disposable overalls, overboots, bouffant caps, and gloves were used for each group to avoid cross-contamination.

3.3. Vaccination of birds

Two different vaccine preparations were prepared. The first vaccine preparation contained the prototype vaccine alone (PV) administered either via the cloacal (PV/c) or the oral route (PV/o). The second vaccine preparation contained the prototype vaccine + HuveGel® administered via the oral route (PV/o+HuveGel). Broilact® and B-Act® were prepared according to the manufacturer's instructions and either suspended in drinking water at a dose of 1.0 mg/bird and provided for 48 hours in the case of Broilact® (PV/o+HuveGel+Broilact) or mixed in feed at a dose of 1.6×10^9 CFU/kg of feed and provided for 72 hours in the case of B-Act® (PV/o+HuveGel+B-Act) (Table 1).

Oral vaccination of day-old turkeys was carried out using a syringe with a crop tube inserted about 2 cm into the oral cavity of birds, whereas cloacal vaccination was achieved using a standard pipette (Eppendorf, Austria) by inducing contractions of the cloaca due to the phenomenon of cloacal drinking (Sorvari et al., 1977). The negative control group in experimental trial II received 0.3 ml of culture medium (free from histomonads) via the oral route. After the administration of the vaccine preparation, the birds were deprived of feed and water for 5 hours.

Table 1: Experimental trial design and procedures

Groups	N. of birds	Additives			Route of vaccination	Vaccine Dose	Vaccine volume
		HuveGel®	B-Act®	Broilact®			
Experimental trial I							
PV/c	4♂ 4♀	–	–	–	cloacal route	10^4 cells/bird	0.3 ml
PV/o+HuveGel	4♂ 4♀	+	–	–	oral route	10^4 cells/bird	0.3 ml
PV/o+HuveGel+Broilact	4♂ 4♀	+	–	+			
PV/o+HuveGel+B-Act	4♂ 4♀	+	+	–			
Experimental trial II							
PV/o	6♂ 6♀	–	–	–	oral route	10^4 cells/bird	0.3 ml
PV/o+HuveGel	6♂ 6♀	+	–	–			
NC	5♂ 5♀	–	–	–		culture medium	0.3 ml

PV/c: Prototype vaccine alone via the cloacal route; **PV/o+HuveGel**: Prototype vaccine + HuveGel® via the oral route; **PV/o+HuveGel+Broilact**: Prototype vaccine + HuveGel®+ Broilact® via the oral route; **PV/o+HuveGel+B-Act**: Prototype vaccine + HuveGel®+ B-Act® via the oral route; **PV/o**: Prototype vaccine alone via the oral route; **NC**: Non-vaccinated negative control group. The NC received 0.3 ml of culture medium free from histomonads via the oral route. Minus sign (−) Yes; Plus sign (+) No.

3.4. Sampling scheme

Throughout both experiments, routine monitoring of the bird's health, cloacal swabbing twice a week together with weighing as well as blood sampling once a week were performed (Table 2).

In experiment I, all 32 birds were euthanized at 42 dpv, while in experiment II, 4 birds from the PV/o group and 4 birds from the PV/o+HuveGel group were euthanized at 42 dpv. The remaining 8 birds from each group in experiment II were euthanized at 84 dpv. In the negative control group, at 39 dpv, 1 bird died and 2 birds were euthanized to collect organs and blood. Later, at 42 dpv, 4 birds were euthanized, and at 46 dpv, the 3 remaining birds were euthanized.

Euthanasia was carried out using an intravenous injection of thiopental (Medicamentum Pharma GmbH, Allerheiligen am Mürztal, Austria) followed by bleeding the birds to death. All euthanized birds were necropsied and their organs were examined for gross lesions indicative of histomonosis. Afterwards, liver and cecal tissue samples were taken for further investigations.

Table 2: Summary of sampling

Type of Sample	Day post-vaccination (dpv)	Total samples	Examination type
Experimental trial I			
cloacal swabbing	0, 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39, 42	338	ME[a] + PCR
Weighing	0, 7, 14, 21, 28, 35, 42	-	-
blood collection	0, 28, 42	68	ELISA
organ collection	42	32	GE[b] + PCR + IHC
Experiment trial II			
cloacal swabbing	0, 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39, 42, 46, 49, 53, 56, 60, 63, 67, 70, 74, 77, 81	588	ME[a] + PCR
Weighing	0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 81	-	-
blood collection	0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 81	314	ELISA
organ collection	39, 42, 46, 84	34	GE[b] + PCR + IHC

[a] Microscopic examination, [b] Gross examination

3.5. Investigation of samples

3.5.1. Investigation of cloacal swabs samples

Throughout both experiments, a total of 926 cloacal swabs samples were collected from all birds to re-isolate live histomonads and detect *Histomonas* DNA by PCR. After sampling, each cloacal swab was inoculated into tubes containing 1.5 ml of culture medium as described above and incubated at +40 °C for 96h by sealing the culture tubes tightly. At the end of incubation, the cloacal swab cultures were examined using a light microscope before being

centrifuged at 15 000 rpm for 10 min (Hettich Rotanta 460, Hettich, Tuttlingen, Germany) and frozen at –20 °C for a subsequent PCR examination.

3.5.1.1. Re-isolation of live histomonads

Re-isolation of live histomonads was assessed using a light microscope (Olympus BX53 microscope, Olympus Austria GmbH, Vienna, Austria). Aliquots of 10 µl were taken from the incubated cloacal swab cultures and transferred to a glass slide for microscopic examination according to a previously performed protocol (Hess et al., 2006).

3.5.1.2. Detection of *Histomonas* DNA

DNA extraction: Pellet DNA was extracted from the frozen cloacal swab cultures using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) based on the manufacturer's instructions. After pelleting, 180 µl ATL buffer and 20 µl proteinase K were added and incubated overnight at 56°C and 450 rpm in a thermomixer. 200 µl of each sample were used for DNA extraction by an automated isolation machine (Qiagen QIAcube) according to the manufacturer's instructions.

qPCR: A qPCR protocol based on the 18S rRNA gene was applied for screening the samples for the presence of *Histomonas* DNA and ct values below 40 were considered positive (Sulejmanovic, 2019).

3.5.2. Investigation of serum samples

The humoral immune response was determined by detecting circulating IgG antibodies against *H. meleagridis*. For this, 382 blood samples of about 2 ml each were collected from the wing vein of all birds throughout both experiments. After sampling, blood was kept overnight at +4°C before being centrifuged at 3300 *x g* for 12 min (Hettich Rotanta 460, Hettich, Tuttlingen, Germany). The obtained sera were collected and stored in 1.5 ml tubes at –20°C until their examination using an indirect sandwich ELISA.

Indirect sandwich ELISA: Quantitative determination of anti-histomonas antibodies was performed using an indirect sandwich ELISA according to Windisch and Hess (2009). The cut-off for the ELISA was set based on OD measured at a wavelength of 450 nm (unpublished data). Levels of antibodies at or above the cut-off (OD-values ≥ 0.450) were considered to be positive

3.5.3. Postmortem investigation

3.5.3.1. Gross examination

Gross examination (GE) was carried out on the necropsied birds to inspect for gross lesions of cecal or liver involvement. Lesions in the ceca and liver were evaluated using a lesion score method ranging from 0 to 4 (Table 3). Birds having a cecal or liver lesion score ≥ 1 were considered positive for histomonosis (Windisch and Hess, 2009; Zahoor et al., 2011).

Table 3: Lesion scoring system (Windisch and Hess, 2009; Zahoor et al., 2011)

Lesion score	Ceca	Liver
LS 0	No pathological changes	No pathological changes
LS 1	Sporadic inflammation and/or mild thickening of the wall of one cecum	Few single punctiform necrosis up to 1 mm
LS 2	Sporadic inflammation and/or mild thickening of the wall of both ceca	Few single punctiform necrosis more than 1 mm or single punctiform necrosis disseminated throughout the liver up to 1 mm
LS 3	Inflammation and thickening of both ceca with liquid fibrin or sporadic fibrinous coagula in the lumen	Single punctiform necrosis disseminated throughout the liver more than 1 mm or some large necrosis areas
LS 4	Severe inflammation and necrosis of both ceca with compact fibrinous masses in the lumen of the ceca	Confluent necrosis throughout the liver

Mean lesion score: This was an average indicator of the intensity of gross lesions in the ceca of necropsied turkeys. The mean lesion score was calculated according to the following formula: Mean LS = $\frac{\sum LF}{\sum (LS \times LF)}$ LF: Frequency of lesions, LS: Score of lesions

After determining and assigning lesion scores, representative tissue samples were collected from both liver and ceca of each bird for a later PCR or immunohistochemical examination after fixation in 10% formalin.

3.5.3.2. Immunohistochemical examination of the organ samples

To investigate the immunohistochemical localization and spread of histomonads in host tissues, ICH was performed according to the protocol described by Singh et al. (2008). In short, representative tissue samples of ceca and liver were collected from each necropsied bird and fixed in 10% buffered formalin. After the samples were cut and placed into plastic cassettes, they were rinsed in water for 1h and thereafter dehydrated and embedded in paraffin. The paraffin-embedded samples of tissue were then cut in slices of 4 µm thickness using a microtome (Microm HM 360, Microm Laborgeräte GmbH, Walldorf, Germany) and mounted on glass slides (Superfrost plus, Menzel-Gläser, Braunschweig, Germany). The tissue samples were dewaxed and rehydrated, followed by retrieval of the antigen by heating the

slides in citrate buffer (pH 6.0) and blockage of endogenous peroxidase activity with 1.5% H_2O_2 in methanol. The sections were then briefly covered with a dilution of normal goat serum (Vector Laboratories, Burlingame, USA) before overnight incubation with the primary antibody (purified polyclonal anti-histomonad serum) at +4°C followed. After extensive washing with PBS, a secondary antibody (biotinylated anti-rabbit IgG antibody; Vector Laboratories) was applied to the sections. The Vectastain ABC Kit (Vector Laboratories) was used before *H. meleagridis* was visualized by the DAB Substrate Kit (Vector Laboratories). The applied method stains histomonads prominently brown in contrast to surrounding tissue that was counterstained with Mayer's haemalum (Merck, Darmstadt, Germany).

4. RESULTS

4.1. Clinical signs and mortalities

Throughout both experiments, no adverse reactions, clinical signs, or mortalities linked to the histomonosis prototype vaccine were recorded in the vaccinated birds.

4.2. Body weight of birds

The average starting live body weight of day-old turkey at the placement was about 65 g.

Experiment I: No significant difference in terms of body weight between the turkeys of the 4 groups at the end of the experimental trial at 42 days of age was observed neither in turkey toms nor turkey hens. The turkey hens in the PV/c group showed a slightly lower body weight than the other 3 groups of turkey hens, however, the difference was statistically not significant. From the 2nd week onwards in females and the 3rd week in males, slight differences in terms of body weight appeared between the 4 groups and the performance goals estimated for commercial Hybrid Converter turkeys at this age (Figure 1a).

Experiment II: The body weight of the birds from day 0 until about 3 weeks showed no difference between the PV/o and PV/o+HuveGel groups, regardless of their sex. Between 3 weeks and about 6 weeks of age, there was also no significant difference in body weight between the two groups and between the two sexes. From the 6th week and until the end of the experiment at the 12th week, it was observed that the average body weight of the birds of the PV/o group was lower than that of the PV/o+HuveGel group, both in males and in females, however, the difference was statistically not significant. The average body weight remained however lower than the performance goals estimated for commercial Hybrid Converter turkeys at this age, except in the females of the VC+HuveGel group (Figure 1b).

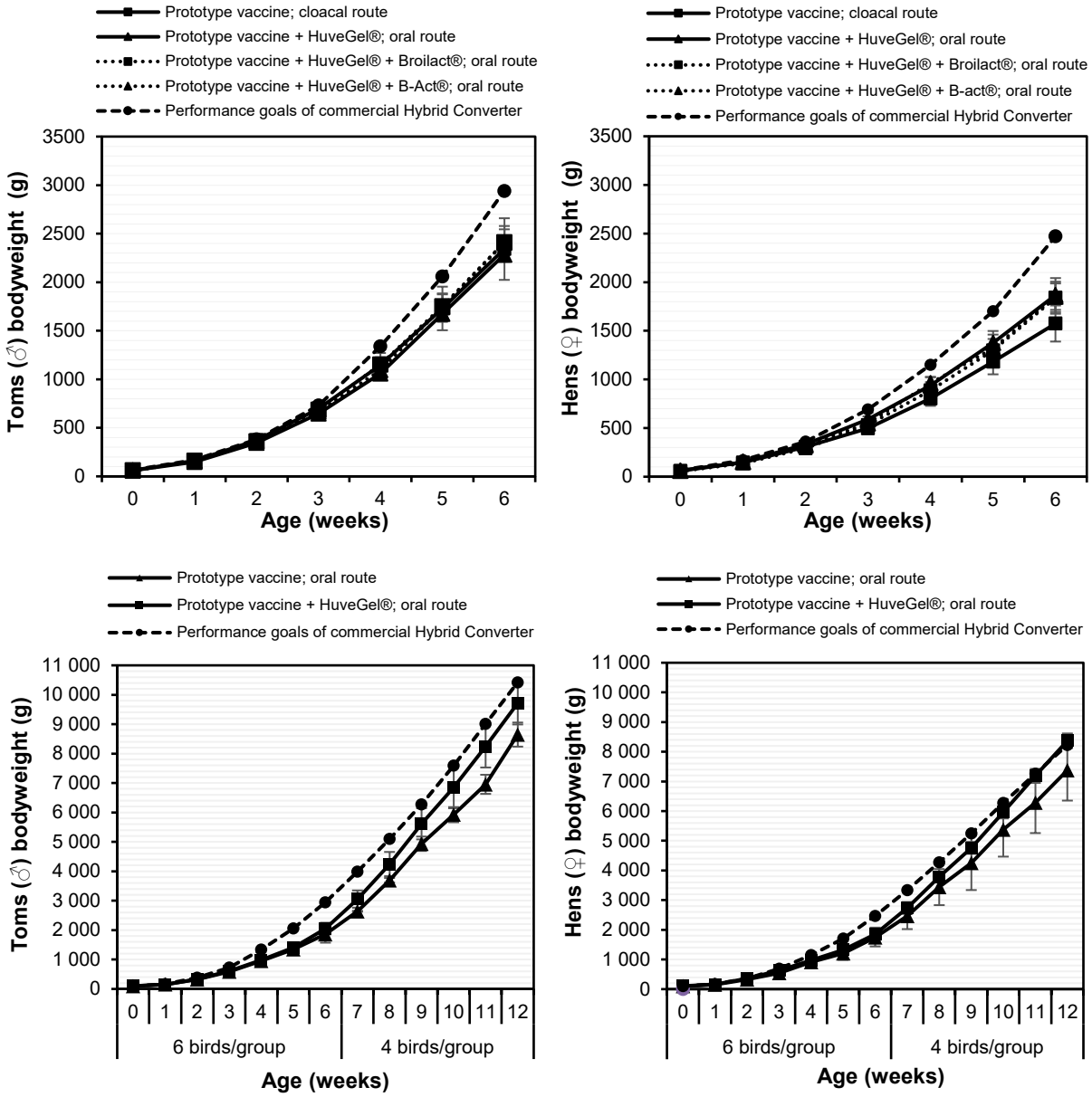


Figure 1: Average body weight of turkey toms (♂) and hens (♀) over time in both experiments.

4.3. Re-isolation of live histomonads and detection of *Histomonas* DNA from swab samples

Using microscopic examination on cloacal swab cultures collected from vaccinated birds at different stages of both experiments as well as on cultures of their cecal contents samples, live histomonads were re-isolated at least once from each vaccinated bird in both experiments. Shedding of live histomonads was first observed in samples from 7 out of 32 birds taken on 7 dpv in experiment I and from 1 out of 24 turkeys taken on 4 dpv in experiment II.

Using PCR on the cultured swab and cecal content samples, *Histomonas* DNA was detected from the 3rd dpv in 8 out of 8 (8/8) vaccinated birds in the PV/c group, 7 out of 8 (7/8) birds in the PV/o+HuveGel group, 8 out of 8 (8/8) birds in the PV/o+HuveGel+Broilact, and 6 out of 8 (6/8) birds in the PV/o+HuveGel+B-Act group in experiment I. In experiment II, *Histomonas* DNA was detected from the 4th dpv in 10 out of 12 (10/12) vaccinated birds in the PV/o group and 12 out of 12 (12/12) birds in the PV/o+HuveGel group.

In both experiments, the detection of live histomonads and *Histomonas* DNA from cloacal swab samples continued until the birds were euthanized. No live histomonads and *Histomonas* DNA were re-isolated or detected from cloacal swab and cecal content samples of non-vaccinated birds in the negative control group (Figures 2a and 2b).

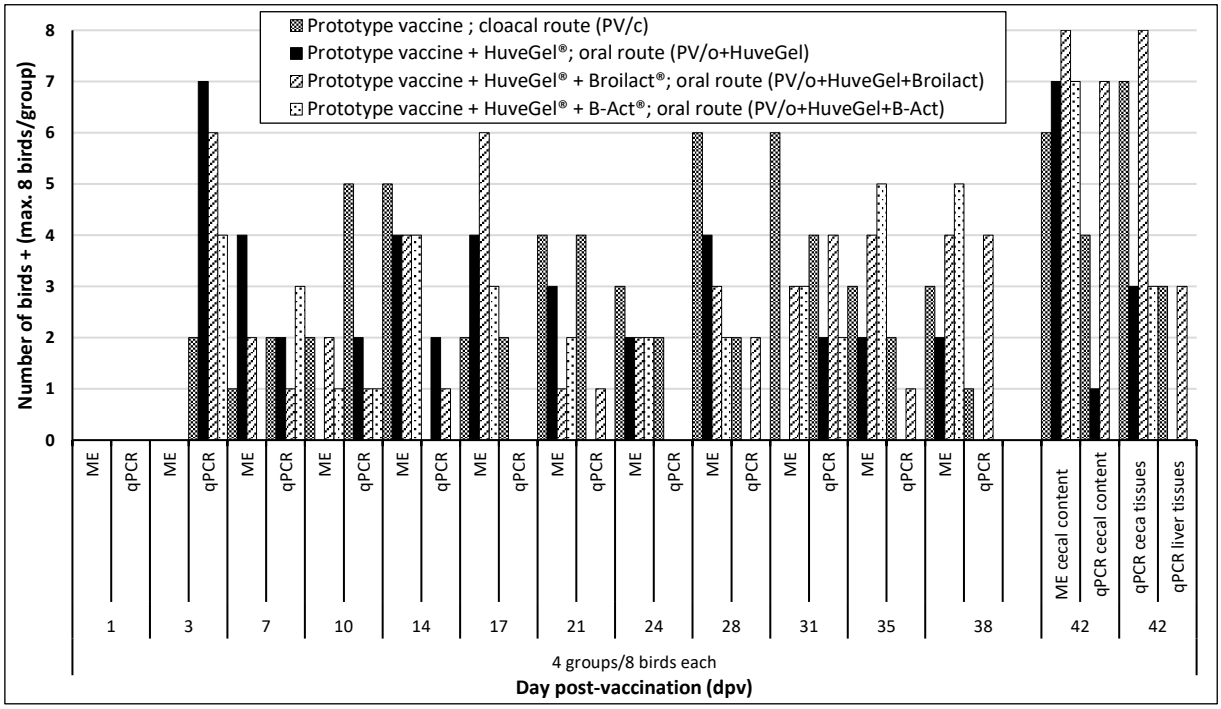


Figure 2a: Number of birds whose cloacal swabs, cecal contents, and organ tissue samples were positive for the presence of histomonads in experiment I. The presence of histomonads was performed using microscopic examination of isolated parasites and confirmed using qPCR. The number of tested birds was 8 birds/group until the euthanasia of all birds at 42 dpv.

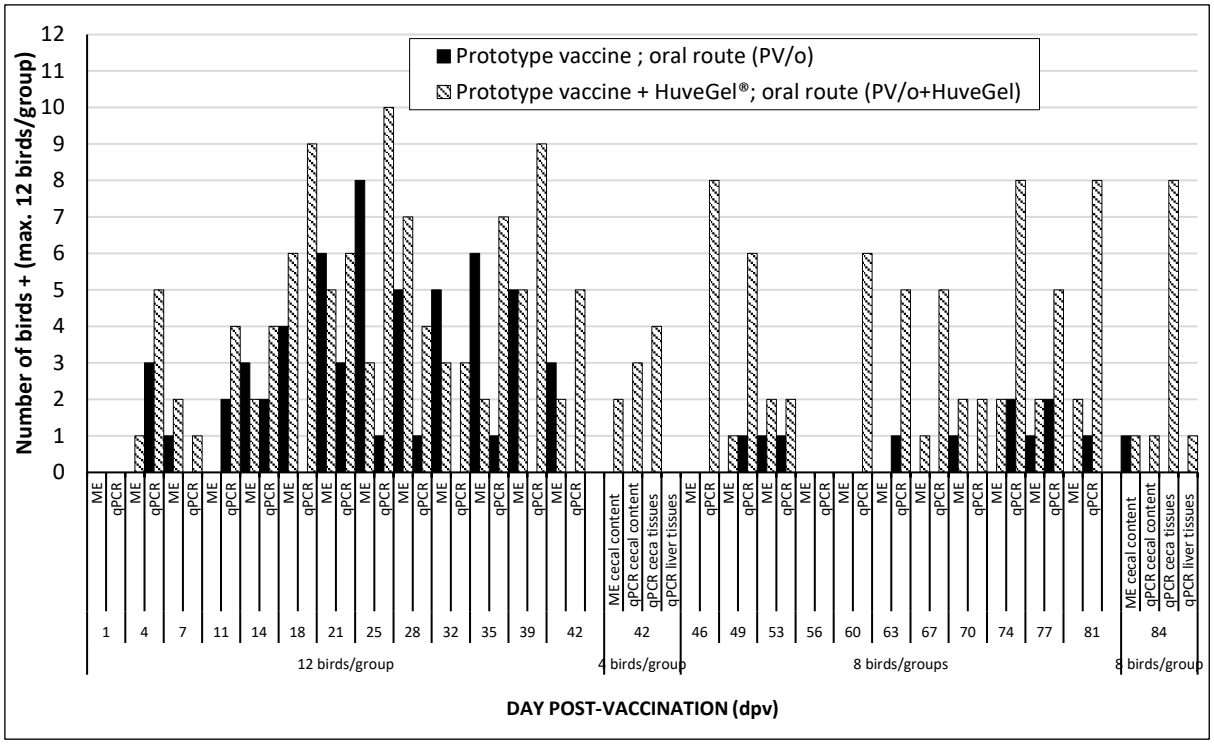


Figure 2b: Number of birds whose cloacal swabs, cecal contents, and organ tissue samples were positive for the presence of histomonads in experiment II. The number of tested birds was 12 birds/group from 1 to 42 dpv and 8 birds/group from 46 to 84 dpv after the euthanasia of 4 birds from each group at 42 dpv. The presence of histomonads was performed using microscopic examination of isolated parasites and confirmed using qPCR.

4.4. Humoral immune response

Experiment I: Except for 2 out of 8 vaccinated birds in the PV/c group and 1 out of 8 vaccinated birds in the PV/o+HuveGel+B-Act group, all serum samples collected from vaccinated birds on 28 dpv, showed levels of IgG antibodies against *Histomonas meleagridis* lower than the cutoff value (cutoff = 0.450) and, therefore, were regarded as seronegative. At 42 dpv, the PV/o+HuveGel and PV/o+HuveGel+B-Act groups remained unchanged, i.e. 8 out of 8 vaccinated birds in the PV/o+HuveGel group and 7 out of 8 birds in the PV/o+HuveGel+B-Act group remained seronegative, while 4 out of 8 vaccinated birds in the PV/c group and 3 out of 8 in the PV/o+HuveGel+Broilact group had levels of IgG antibodies against *Histomonas meleagridis* above the cutoff, which represents an increase of 25% and 37.5% compared to 28 dpv in these two groups respectively (Figure 3a).

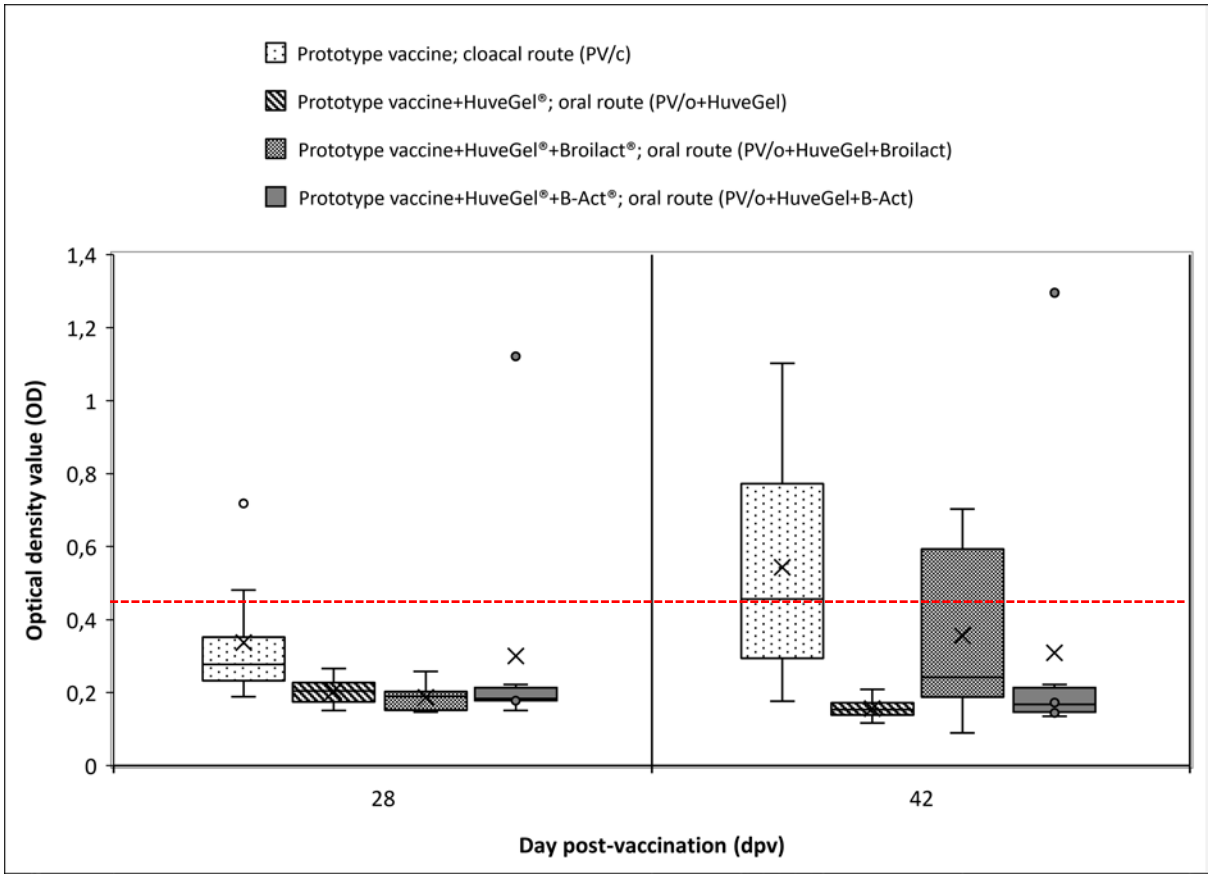


Figure 3a: Kinetic of circulating anti-histomonas IgG antibodies in each group of experiment I at 28 and 42 dpv using indirect sandwich ELISA. The *Histomonas* prototype vaccine was administered to day-old turkeys. The length of each boxplot corresponds to the interquartile (IQ) range, with the upper quartile of the box representing the 75th percentile and the lower quartile the 25th percentile. The horizontal line in the box indicates the median value. Outlier values are shown as dots. The dotted horizontal line extending across the graph marks the cutoff between histomonas-seropositive and seronegative, sera with an optical density (OD) ≥ 0.450 are considered seropositive. The marks (X) represent the average values of the OD obtained from all animals of the same group.

Experiment II: At 7 dpv, 6 out of 8 sera samples collected from vaccinated birds in the PV/o and PV/o+HuveGel groups and 8 out of 10 sera samples taken from birds of the NC group showed levels of IgG antibodies against *Histomonas meleagridis* above the cutoff value (cutoff = 0.450) and therefore were regarded as seropositive. At 28 dpv, the number of seropositive birds decreases in the three groups with 2 out of 6 in the PV/o and PV/o+HuveGel groups each and 0 out of 10 in the NC group. At 56 dpv, an increasing number of seropositive birds in both vaccinated groups compared to 28 dpv was observed, reaching 4 out of 8 seropositive birds in the PV/o group and 8 out of 8 in the PV/o+HuveGel group. At 82 dpv, the number of seropositive birds in the PV/o group increased further to 5 out of 8 seropositive birds, while the PV/o+HuveGel group remained unchanged from 56 dpv with 8 out of 8 still seropositive birds (Figure 3b).

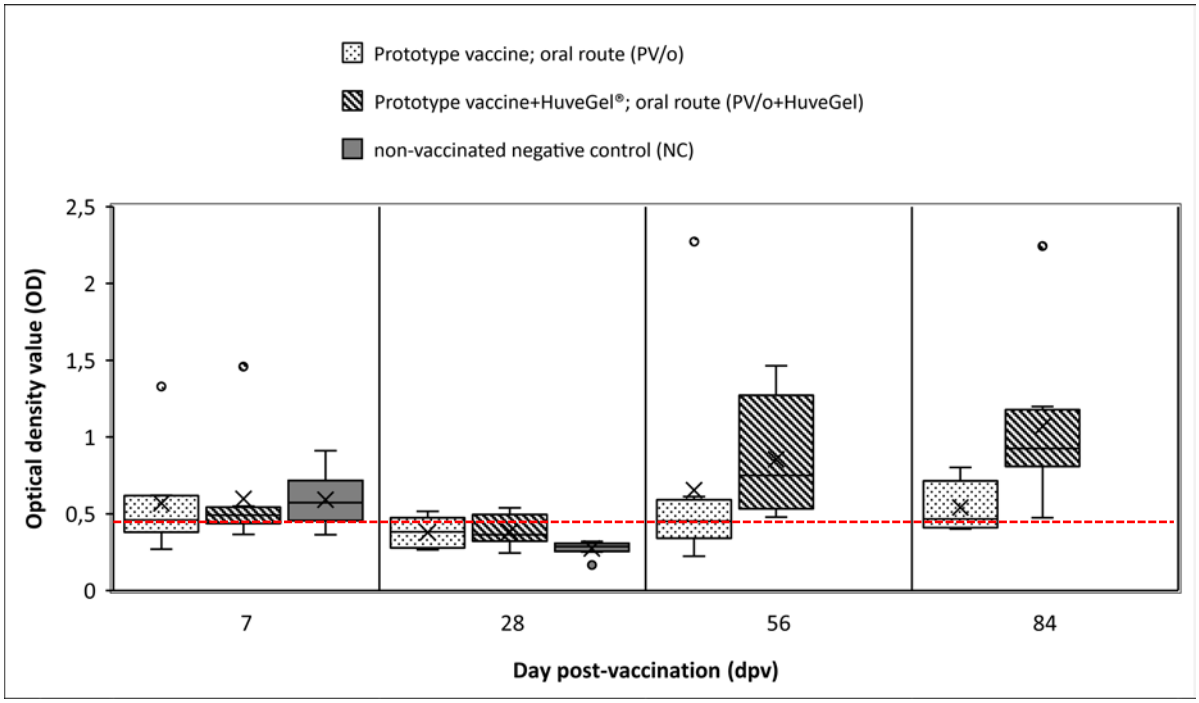


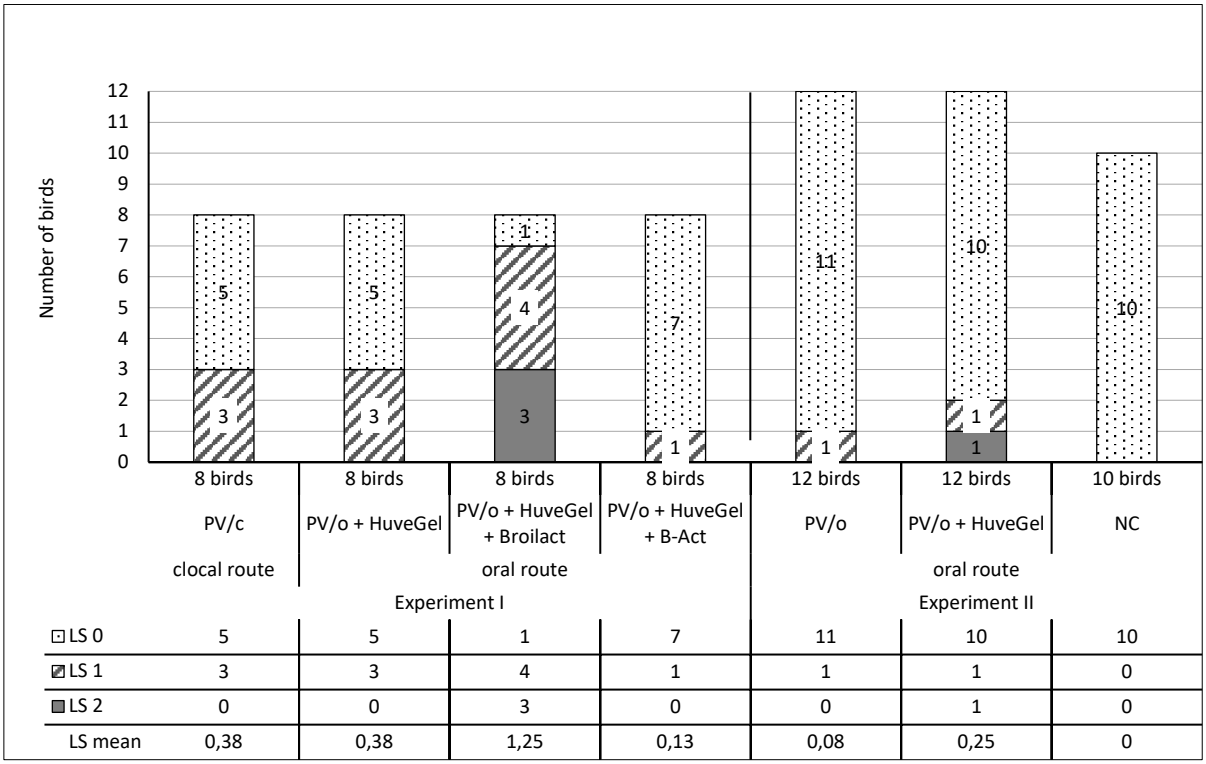
Figure 3b: Kinetic of circulating anti-histomonas IgG antibodies in each group of experiment II at 7, 28, 56, and 84 dpv using indirect sandwich ELISA. The *Histomonas* prototype vaccine was administered to day-old turkeys. The length of each boxplot corresponds to the interquartile (IQ) range, with the upper quartile of the box representing the 75th percentile and the lower quartile the 25th percentile. The horizontal line in the box indicates the median value. Outlier values are shown as dots. The dotted horizontal line extending across the graph marks the cutoff between histomonas-seropositive and seronegative, where sera with an optical density (OD) ≥ 0.450 are considered seropositive. The marks (X) represent the average values of the OD obtained from all animals of the same group.

4.5. Gross lesions

In both experiments, no gross lesions were observed in the liver of vaccinated birds during the postmortem examination. On the other hand, 17 out of 56 vaccinated birds showed gross lesions in the cecum. These cecal gross lesions concerned all the vaccinated groups (Figure 4b). In experiment I, the PV/o+HuveGel+Broilact group was the group in which the most lesions were observed, affecting 7 out of 8 vaccinated birds, followed by the PV/c and PV/o+HuveGel groups with 3 out of 8 vaccinated birds each. The PV/o+HuveGel+B-Act group was the group in which the fewest cecal lesions were observed with 1 out of 8 vaccinated birds. In experiment II, the PV/o+HuveGel group showed 2 out of 12 cecal lesions against 1 out of 12 in the PV/o group.

In both experiments, 13 out of 17 (76%) cecal lesions were scored with the lowest score of 1 (LS 1) while 4 out of 17 (24%) cecal lesions had a lesion score of 2 (LS 2). None of the birds had lesions scored 3 or 4 and no lesions were found in the livers and ceca of the non-

vaccinated birds. The number of gross cecal lesions observed in vaccinated birds of each group and their lesion score is shown in Figure 4a.

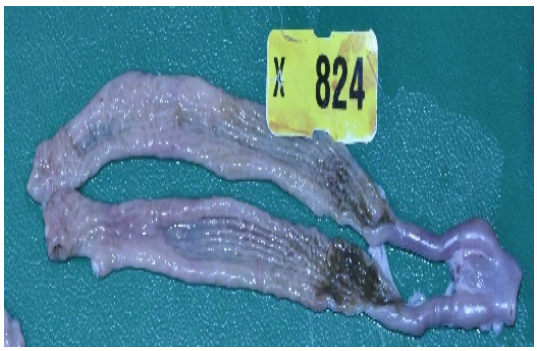


LS 0	5	5	1	7	11	10	10
LS 1	3	3	4	1	1	1	0
LS 2	0	0	3	0	0	1	0
LS mean	0,38	0,38	1,25	0,13	0,08	0,25	0

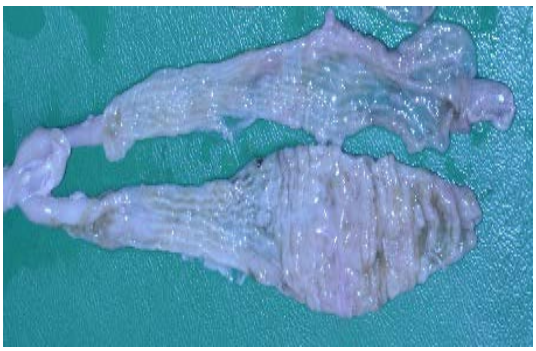
Figure 4a: Number of gross cecal lesions observed in vaccinated birds during postmortem examination. According to their severity, cecal lesions were classified using a lesion scoring system ranging from 0 to 4. The most severe cecal lesion (LS 2) and the highest mean lesion score (1.25) were observed in the PV/o+HuveGel+Broilact group.

Cecal lesion score 0

Cecal lesion score 1



Cecal lesion score 2



Liver lesion score 0

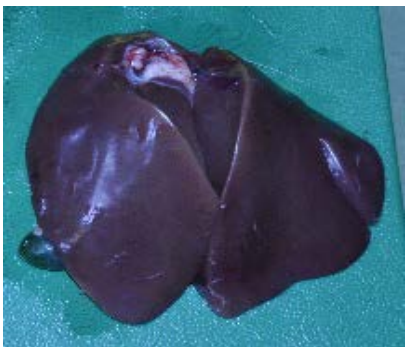


Figure 4b: Example of some lesion scores observed in some vaccinated turkeys.

4.6. Detection of *Histomonas* DNA from organ samples

All liver tissue samples of vaccinated birds were tested negative for the presence of *Histomonas* DNA, except for 3 out of 8 birds (3/8) in the PV/c group, 3 out of 8 birds (3/8) in the PV/o+HuveGel+Broilact group (in experiment I), and 1 out of 12 birds (1/8) in the PV/o+HuveGel group (in experiment II).

In experiment I, *Histomonas* DNA was detected in the ceca of 7 out of 8 birds in the PV/c group, 3 out of 8 birds in the PV/o+HuveGel group, 8 out of 8 birds in the PV/o+HuveGel+Broilact group, and 3 out of 8 birds in the PV/o+HuveGel+B-Act group. In experiment II, *Histomonas* DNA was detected in 12 out of 12 birds in the PV/o+HuveGel group, while no *Histomonas* DNA could be detected in the PV/o group. No gross liver or cecal lesions were observed in the non-vaccinated birds of the negative control group.

4.7. Localization and distribution of histomonads in host organs

Immunohistochemical staining revealed histomonads in 8 out of 8 cecal samples collected from the vaccinated birds in the PV/c group and 7 out of 8 cecal samples in the PV/o+HuveGel+Broilact group in experiment I, and in 11 out of 12 cecal tissue samples from birds of PV/o+HuveGel group in experiment II. On the other hand, no histomonads could be observed in the cecal tissue samples collected from the vaccinated birds in the PV/o+HuveGel and PV/o+HuveGel+B-Act groups in experiment I and the PV/o group in experiment II.

In cecal samples where histomonads were detected, histomonads were observed exclusively in the *lamina propria* of the ceca without tissue invasion (Figure 5).

No histomonads could be detected in liver tissue samples collected from the vaccinated birds in both experiments. Also, no histomonads could be detected in any of the cecal and liver tissue samples collected from the negative control group.

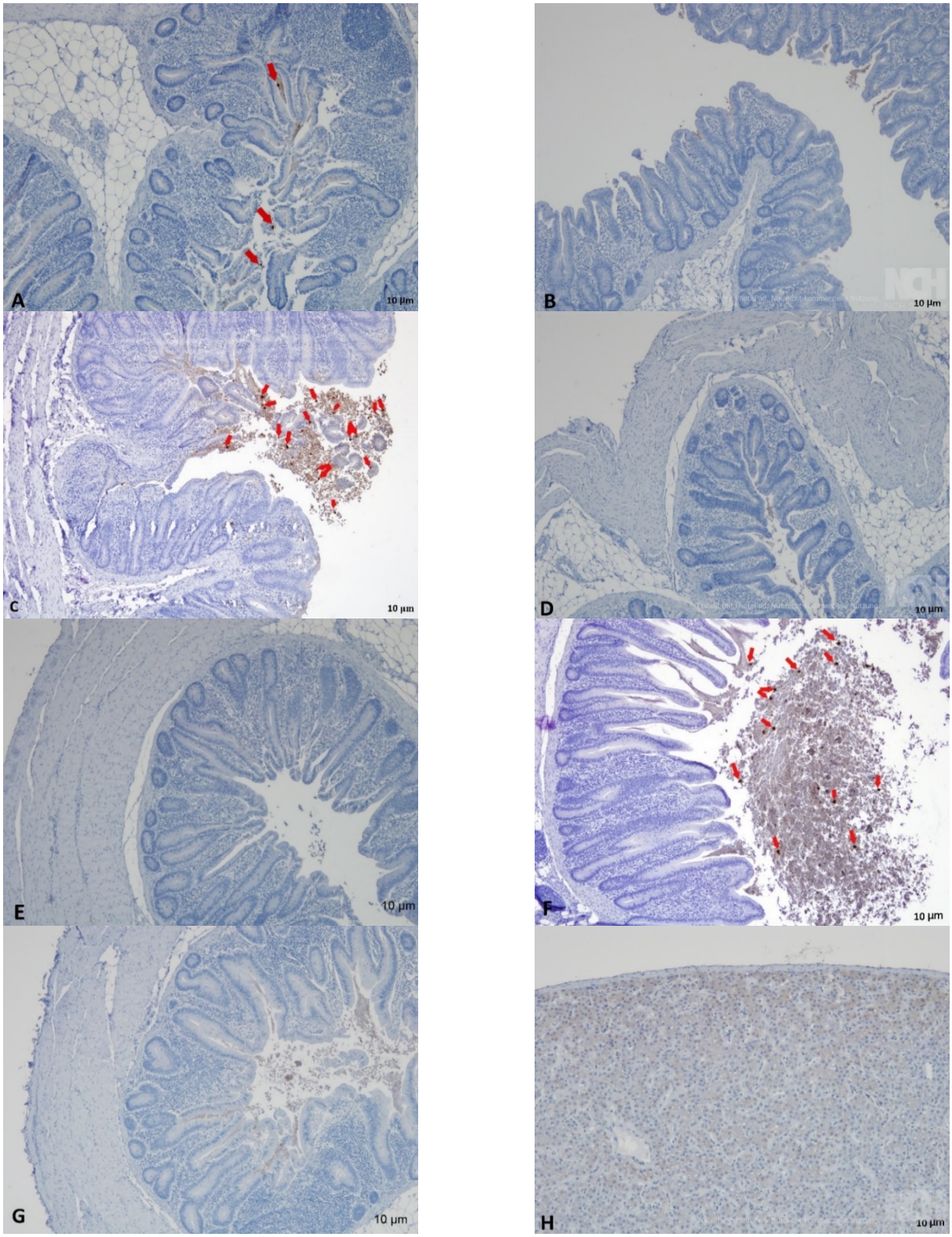


Figure 5: Immunohistochemical examination of cecal tissue samples from groups PV/c (**A**); PV/o+HuveGel in experiment I (**B**); PV/o+HuveGel+Broilact (**C**); PV/o+HuveGel+B-Act (**D**); PV/o (**E**); PV/o+HuveGel in experiment II (**F**); NC (**G**) and liver tissue samples in the NC (**H**). Histomonads appear as dark round cells in tissue sections (red arrows).

Table 4: Cecal lesion scores and detection of histomonads in cecal and liver samples using PCR and IHC in each group of experiments I and II.

Experiment I						
Groups	Bird N	Ceca			Liver	
		LS	qPCR	IHC	qPCR	IHC
Prototype vaccine alone; Cloacal route	801♂	0	–	+	–	–
	802♀	1	+	+	+	–
	803♂	0	+	+	–	–
	804♀	0	+	+	–	–
	805♂	0	+	+	+	–
	806♀	0	+	+	–	–
	807♂	1	+	+	–	–
	808♀	1	+	+	+	–
Prototype vaccine + HuveGel®; Oral route	809♂	0	+	–	–	–
	810♀	1	–	–	–	–
	811♂	0	–	–	–	–
	812♀	0	+	–	–	–
	813♂	1	–	–	–	–
	814♀	0	+	–	–	–
	815♂	0	–	–	–	–
	816♀	1	–	–	–	–
Prototype vaccine + HuveGel®+ Broilact®; Oral route	817♂	2	+	+	–	–
	818♀	0	+	+	+	–
	819♂	2	+	+	–	–
	820♀	1	+	+	+	–
	821♂	2	+	+	–	–
	822♀	1	+	+	–	–
	823♂	1	+	–	–	–
	824♀	1	+	+	+	–
Prototype vaccine + HuveGel®+ B-Act®; Oral route	825♂	1	–	–	–	–
	826♀	0	+	–	–	–
	827♂	0	+	–	–	–
	828♀	0	–	–	–	–
	829♂	0	+	–	–	–
	830♀	0	–	–	–	–
	831♂	0	–	–	–	–
	832♀	0	–	–	–	–

Experiment II						
Groups	Bird N	Ceca			Liver	
		LS	qPCR	IHC	qPCR	IHC
Negative Control	551♂	0	–	–	–	–
	552♀	0	–	–	–	–
	553♂	0	–	–	–	–
	554♀	0	–	–	–	–
	555♂	0	–	–	–	–
	556♀	0	–	–	–	–
	557♂	0	–	–	–	–
	558♀	0	–	–	–	–
	559♂	0	–	–	–	–
	560♀	0	–	–	–	–
Prototype vaccine alone; Oral route	561♂	0	–	–	–	–
	562♀	0	–	–	–	–
	563♂	0	–	–	–	–
	564♀	0	–	–	–	–
	565♂	0	–	–	–	–
	566♀	0	–	–	–	–
	567♂	0	–	–	–	–
	568♀	0	–	–	–	–
	569♂	0	–	–	–	–
	570♀	0	–	–	–	–
	571♂	0	–	–	–	–
	572♀	1	–	–	–	–
Prototype vaccine + HuveGel®; Oral route	573♂	0	+	+	–	–
	574♀	0	+	+	–	–
	575♂	0	+	+	–	–
	576♀	0	+	+	–	–
	577♂	1	+	+	–	–
	578♀	0	+	+	–	–
	579♂	0	+	+	+	–
	580♀	0	+	+	–	–
	581♂	2	+	–	–	–
	582♀	0	+	+	–	–
	583♂	0	+	+	–	–
	584♀	0	+	+	–	–

LS: cecal lesion score; IHC: Immunohistochemistry
Minus sign (−) = negative result; Plus sign (+) = positive result

5. DISCUSSION

Successful vaccination with a gut mucosal vaccine such as the attenuated histomonosis vaccine involves, in addition to an efficacious vaccine, an effective application, as well as birds with a healthy intestinal tract colonized by normal pathogen-free microbiota. Experimental studies have demonstrated the protective effect of an attenuated vaccine based on a clonal culture of *H. meleagridis* in turkeys against a virulent challenge (Hess et al., 2008; Liebhart et al., 2010; Sulejmanovic et al., 2013; Sulejmanovic et al., 2016; Mitra et al., 2017). However, so far, little attention has been given to other factors influencing the outcome of vaccination aside from the vaccine itself.

It is well-known that unprotected histomonads are not resistant to the acidic environment of the gizzard and crop (Lund, 1956; Gerhold et al., 2010; Lotfi et al., 2012), thus, to ensure that enough attenuated histomonads overcome the acidic environment of the digestive tract and reach the cecal mucosa, the histomonosis vaccine necessitate an individual administration via either intracloacal route or intraoral route after a feed and water withdrawal for around 6 hours (Hess et al., 2015).

In recent years, a new delivery method that consists in embedding vaccines in an edible diluent gel has been used in hatcheries for the oral deliverance of some live vaccines such as coccidiosis and salmonella vaccines. This new method aims to overcome the digestive destruction of the vaccine and therefore to safeguard its immunogenic action (Dasgupta and Lee, 2000; Ritzi et al., 2016; Albanese et al., 2018). This method showed an increase in intestinal vaccine uptake and therefore vaccine effectiveness (Jenkins et al., 2012).

The gut microbiota starts to establish at hatch, therefore, the earlier the introduction of beneficial microorganisms (immunobiotics), the more effective their establishment in the digestive tract (Timmerman et al., 2006; Torok et al., 2008). Several studies demonstrated that oral inoculation of chicks with cecal bacterial flora from adult hens can increase their resistance to diverse infections (Rantala and Nurmi, 1973; Milbradt et al., 2014; Varmuzova et al., 2016). This has led to the introduction of supplementation of day-old birds with cultures of immunobiotics such as direct-fed-microbials (DFMs). Several studies have demonstrated that modulation of the initial gut microbiota through the administration of DFMs can improve the mucosal immune response and therefore improve vaccine response and enhance chicks' defenses (Dalloul et al., 2003; Farnell et al., 2003; Koenen et al., 2004; Haghighi et al., 2005; Stringfellow et al., 2011; Waititu et al., 2014).

Based on these data, the vaccine uptake of the *Histomonas* vaccine could be improved by the use of a diluent gel and/or the addition of DFMs.

This study evaluated the effects of the diluent gel HuveGel® (HuvePharma N.V., Antwerp, Belgium) and the two DFMs, Broilact® (Orion Corporation, Espoo, Finland) and B-Act® (HuvePharma N.V., Antwerp, Belgium) on the vaccine uptake of the vaccine against histomonosis administered via the oral route to day-old turkeys.

During the timeframe of both experiments, no adverse reactions following vaccination were observed in all groups. There were no histomonosis-related mortalities and no vaccinated bird showed clinical signs related to histomonosis, indicating that the *Histomonas* prototype vaccine had a sufficient degree of attenuation and remained safe for day-old birds. These findings are consistent with earlier studies reporting that attenuated histomonads based on clonal culture, administered to day-old turkeys via the oral route (Liebhart and Hess, 2009; Liebhart et al., 2010) or to 14-day-old turkeys via the cloacal route (Hess et al., 2008) were safe and effective in preventing histomonosis.

A decrease in body weight gain is a common clinical sign of histomonosis in turkeys (Tyzzer et al., 1921). In this study, no difference in terms of body weight between the vaccinated groups was observed, regardless of the route of vaccination, the vaccine preparation, and the kind of DFM used. Similar results had been reported in earlier studies indicating that vaccination with attenuated histomonads does not affect the body weight gain of turkeys (Liebhart et al., 2010; Sulejmanovic et al., 2016; Beer et al., 2022), while infections with virulent histomonads lead in all instances to a reduced body weight gain in turkeys as well as chickens (Hu and McDougald, 2001; Hafez et al., 2010; Liebhart et al., 2010).

Microscopic and PCR examinations of cloacal swab cultures showed intermittent shedding of histomonads through droppings in almost all vaccinated birds. In both experiments, histomonads shedding started at the 3rd to 4th dpv and continued until the birds were euthanized indicating the success of vaccine uptake. These results are in agreement with those of other earlier studies which indicated that the inoculation of attenuated avirulent (Liebhart et al., 2011) or non-attenuated virulent histomonads (Liebhart and Hess, 2009) via both routes of administration, i.e., oral and cloacal, leads to intermittent shedding of the histomonads through the droppings of birds.

However, some differences were observed between the vaccinated groups. The shedding of histomonads was significantly higher in the birds vaccinated via the cloacal route (PV/c) in experiment I than in the birds vaccinated via the oral route (PV/o) in experiment II (100% vs. 83% of birds), which indicates that the cloacal route is more effective than the oral. Indeed, the

cloacal route has been described as being highly effective in inducing histomonosis (Hauck and Hafez, 2013).

Among the 2 groups which received a DFM in experiment I, 100% of the vaccinated birds supplemented with Broilact® shed histomonads (at least once) in their droppings versus 75% of those supplemented with B-Act®. This suggests that the additive Broilact® enhanced vaccine uptake. In experiment I, it was difficult to establish whether HuveGel® influenced vaccine uptake or not, given that all groups vaccinated via the oral route received the *Histomonas* vaccine co-administered with HuveGel® and there was no comparison group with the *Histomonas* vaccine alone via the oral route, hence the implementation of experiment II. In this latter, 100% of the birds whose *Histomonas* vaccine was co-administered with HuveGel® shed histomonads in their droppings versus 83% of whose *Histomonas* vaccine was administered alone. This suggests that HuveGel® improved vaccine uptake.

Assessment of the immune response to *Histomonas* vaccine antigens at the end of both experiments showed that the IgG response was significantly higher when birds were vaccinated via the cloacal than the oral route, when birds were supplemented with Broilact® than B-Act®, and when the *Histomonas* vaccine was co-administered with HuveGel®. These results also demonstrate a positive correlation between the prevalence of histomonad shedding in droppings and IgG antibody seropositivity rates because these were the same groups with the highest prevalence of histomonad shedding in their droppings.

In both experiments, no gross lesions were observed in the liver of vaccinated birds. On the other hand, about 30% of vaccinated birds showed gross lesions in the cecum with a lesion score of 1 (LS 1) to 2 (LS 2). These results are in agreement with those obtained in recent studies which reported the occurrence of some liver and cecal lesions with a low lesion score following vaccination of 28-old-day turkeys with an attenuated vaccine based on the clonal culture of *H. meleagridis* administered via the combination of oral and cloacal routes (Mitra et al., 2017). Two other studies reported a total absence of liver and cecal lesions in day-old turkeys vaccinated with attenuated histomonads via the oral route without challenge (Liebhart et al., 2011). Moreover, hemalum eosin staining of cecal tissue samples from turkeys did not reveal distinct inflammation or necrosis due to attenuated histomonads (Liebhart et al., 2011).

The occurrence of these slight cecal lesions indicates that the attenuated *Histomonas* used as a prototype vaccine lost its virulence but has retained a slight adhesiveness and invasiveness on the cecal mucosa. Tyzzer (1934) vaccinated turkeys with attenuated histomonads via the cloacal route and observed the occurrence of some cecal lesions. Based on histological

evidence, Tyzzer concluded that the immunizing action is due to a slight but non-progressive invasion of the cecal mucosa by the parasite which appears very rarely (Tyzzer, 1934).

Although cecal lesions were observed in both experiments in all vaccinated birds these results should be interpreted with caution. Indeed, the group supplemented with Broilact® was the group in which almost all birds (>87%) showed cecal lesions and whose mean cecal lesion score was the highest (LS 1.25). In the other groups, only a limited number of birds (max. 37.5%) showed cecal lesions and the mean cecal lesion score was very low (max. LS 0.38). It was also interesting to note that in all birds with cecal lesions in the groups whose *Histomonas* vaccine was co-administered with HuveGel® alone (PV/o+HuveGel) or with B-Act® (PV/o+HuveGel+B-Act) in experiment I and whose *Histomonas* vaccine was administered alone (PV/o) in experiment II, PCR and IHC on the cecal tissue samples did not confirm the presence of histomonads. This could be explained by the low sensitivity of the methods used.

Adminstration of day-old chicks with Broilact® modulates the initial cecal microbiota and reduces the incidence in the ceca of some pathogens such as *E. coli*, *S. enteritidis*, *S. typhimurium*, *C. perfringens*, and *Campylobacter jejuni* (Hakkinen and Schneitz, 1996; Schneitz and Nuotio, 1992; Elwinger and al., 1992; Hakkinen and Schneitz, 1999). Considering the relationship between *H. meleagridis* and the cecal bacteria and the interaction to induce histomonosis (Franker and Doll, 1964; Springer et al., 1970; Kemp, 1974; Ganas et al., 2012), the actual results suggest an influence of the bacteria contained in Broilact® on the *Histomonas* vaccine. Based on these results, it would be of great interest to conduct further trials with a larger number of birds to confirm or refute these findings and to determine if a combination "histomonosis vaccine–Broilact®" is efficacious against a challenge.

A significant correlation between PCR and IHC was observed when comparing the results of cecal samples collected during both experiments. PCR revealed the presence of *Histomonas* DNA in almost all birds vaccinated via the cloacal route (88%), those supplemented with Broilact® (100%), and those whose vaccine was co-administered with HuveGel® in experiment II (100%). Results from IHC supported PCR findings. In the other groups, PCR demonstrated the presence of *Histomonas* DNA in a limited number of vaccinated birds (max. 37%) while using IHC, no presence of histomonads could be detected. These results are in agreement with those reported in earlier studies indicating the presence of histomonads in the cecal tissues of turkeys vaccinated with attenuated histomonads without or with a subsequent challenge (Liebhart et al., 2011; Hess et al., 2008; Sulejmanovic et al., 2013). Interestingly, inconsistencies appear when comparing the results of cecal tissue samples observed in the two experiments from the two groups whose vaccine was co-administered with HuveGel®

without DFMs. Whereas in experiment I, a limited presence of histomonads (37% using PCR and 0% using IHC) was noticed in the relevant group, the group in experiment II had shown the presence of histomonads in almost all the birds (100% using PCR and 92% using IHC). As the vaccine and its application were identical in both groups, this difference could be attributed solely to the quality of day-old turkeys and/or the composition of their initial microbiota.

Using IHC, no histomonads in the liver of vaccinated birds could be detected which is in agreement with earlier results published by Sulejmanovic et al. (2016). However, PCR revealed the presence of *Histomonas* DNA in a limited number of liver samples from the groups: vaccinated via the cloacal route (37.5%), supplemented with Broilact® (37.5%), and those who got the vaccine co-administered with HuveGel® in experiment II (8.3%). This indicates the spread of *Histomonas* DNA from the ceca to the liver in these groups. In the other groups, no *Histomonas* DNA could be detected in the liver. Previous studies with 1- or 14-day-old turkeys vaccinated with an attenuated *Histomonas* vaccine noticed the absence of *Histomonas* DNA in the liver and other organs and their restriction to the ceca (Hess et al., 2008; Liebhart et al., 2011), while infection with virulent strains of histomonads always led to the spread to internal organs, primarily the liver (Hess et al., 2008; Liebhart et al., 2008; Singh et al., 2008; Liebhart and Hess, 2009). However, Mitra et al. (2017), reported the presence of some liver lesions with a low lesion score in 28-day-old turkeys vaccinated with an attenuated vaccine based on clonal cultures of histomonads without challenge. The detection of *Histomonas* DNA in the liver of vaccinated birds could indicate that some *Histomonas* DNA might be taken up by macrophages or other immune cells and transported to the liver. According to earlier studies, attenuated histomonads do not lose all their invasiveness on the cecal mucosa and therefore their spreadness to other organs (Tyzzer, 1934; Tyzzer, 1936; Lund et al., 1966; Lund et al., 1967*)*, albeit such attenuated cultures of histomonads are much less characterized than the clonal culture used in the actual study.

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7. LIST OF FIGURES AND TABLES

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