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LARISSA VIEIRA MAZZUCCO

**EVALUATION OF THE IMMUNE RESPONSE OF CATS TO EXPERIMENTAL
VACCINES AGAINST *Toxoplasma gondii***



LAGES

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade do Estado de Santa Catarina (CAV/UDESC) como requisito para obtenção do título de mestre em Ciência Animal.

Orientador: Prof. Dr. Andreas Lazaros Chrysafidis

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“C'est le temps que tu as perdu pour ta rose
qui fait ta rose si importante”

Antoine de Saint-Exupéry, Le Petit Prince

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RESUMO

Toxoplasma gondii é um protozoário zoonótico de distribuição mundial, sendo os felinos domésticos os únicos hospedeiros definitivos capazes de eliminar oocistos no ambiente, desempenhando papel central na transmissão da toxoplasmose. Dessa forma, o desenvolvimento de vacinas capazes de reduzir a eliminação de oocistos representa uma estratégia promissora para o controle da doença sob a perspectiva de Saúde Única. Este estudo teve como objetivo avaliar a imunogenicidade e a eficácia de uma vacina experimental composta por taquizoítos inativados de *T. gondii* associados a nanopartículas poliméricas lipídicas, administrada pelas vias subcutânea e intranasal em gatos domésticos. Foram conduzidos dois experimentos independentes utilizando as cepas VEG e ME49 para o desafio experimental. A resposta imune humoral foi avaliada por sorologia (IgG), a resposta mucosal pela quantificação de IgA fecal e a resposta celular por ELISPOT para detecção de células produtoras de IFN- γ . A eficácia da vacinação foi determinada pela quantificação diária da eliminação de oocistos nas fezes após o desafio. A vacinação por via subcutânea induziu resposta humoral mais precoce e maiores títulos de IgG antes do desafio, enquanto a administração intranasal promoveu resposta celular mais intensa em um dos experimentos, caracterizada por maior produção de IFN- γ . Entretanto, não foram observadas diferenças significativas entre os grupos quanto à produção de IgA fecal ou à eliminação de oocistos após a infecção experimental. Os resultados demonstram que a formulação vacinal foi capaz de induzir respostas imunes distintas de acordo com a via de administração, porém essas respostas não foram suficientes para reduzir significativamente a excreção de oocistos nas condições experimentais avaliadas. Os achados contribuem para o avanço do conhecimento sobre vacinas contra *T. gondii* em felinos e reforçam a necessidade de novas estratégias que promovam imunidade intestinal mais efetiva, visando interromper a transmissão ambiental do parasito.

Palavras-chave: Imunogenicidade. Oocistos. Saúde Única. Vacina.

ABSTRACT

Toxoplasma gondii is a globally distributed zoonotic protozoan, and domestic cats are the only definitive hosts capable of shedding environmentally resistant oocysts, playing a central role in the transmission of toxoplasmosis. Therefore, the development of vaccines capable of reducing oocyst shedding represents a promising strategy for disease control within the One Health framework. This study aimed to evaluate the immunogenicity and efficacy of an experimental vaccine composed of inactivated *T. gondii* tachyzoites associated with polymeric lipid nanoparticles, administered by the subcutaneous or intranasal route in domestic cats. Two independent experiments were conducted using the VEG and *T. gondii* ME49 strains for the challenge. Humoral immune responses were evaluated by serological detection of IgG antibodies, mucosal immunity by quantification of fecal IgA, and cellular immunity by IFN- γ ELISPOT assay. Vaccine efficacy was assessed by daily quantification of oocyst shedding in feces following challenge. Subcutaneous vaccination induced an earlier humoral immune response and higher IgG antibody titers before challenge, whereas intranasal administration elicited a stronger cellular immune response in one of the experiments, characterized by increased IFN- γ production. However, no significant differences were observed among the groups regarding fecal IgA production or oocyst shedding following challenge. These findings demonstrate that the vaccine formulation induced distinct immune responses according to the route of administration; however, these responses were not sufficient to significantly reduce oocyst shedding under the experimental conditions evaluated. The results contribute to the advancement of knowledge on vaccines against *T. gondii* in felids and highlight the need for new strategies capable of inducing more effective intestinal immunity to interrupt environmental transmission of the parasite.

Keywords: Immunogenicity. Oocysts. One Health.

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LIST OF ABBREVIATIONS AND ACRONYMS

ATCC	American Type Culture Collection
AUC	Area Under the Curve
CAV	Centro de Ciências Agroveterinárias
CD4 ⁺	Cluster of differentiation 4-positive T lymphocytes
CD8 ⁺	Cluster of differentiation 8-positive T lymphocytes
CO ₂	Carbon dioxide
CT	Control group
D0-D98	Experimental Days
df	Degrees of freedom
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot assay
<i>F. catus</i>	<i>Felis catus</i>
FeLV	Feline leukemia virus
FIV	Feline immunodeficiency virus
g	Relative centrifugal force
GRA12	Dense granule antigen 12
GTMA	Glycidyltrimethylammonium chloride
HCV	Veterinary Teaching Hospital
HRP	Horseradish peroxidase
IFAT	Indirect Immunofluorescence Antibody Test
IFN- γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-12	Interleukin-12
IN	Intranasal
LADETES	Laboratório de Desenvolvimento de Tecnologias em Saúde
LAPAR	Laboratório de Parasitologia e Doenças Parasitárias
log ₁₀	Logarithm base 10
ME49	<i>T. gondii</i> ME49 strain of <i>Toxoplasma gondii</i>
mRNA	Messenger ribonucleic acid
NK	Natural killer
NPLs	Polymeric lipid nanoparticles
OOPG	Oocysts per gram of feces
p	p-value
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PIP	Inactivated <i>Toxoplasma gondii</i> preparation
RPMI	Roswell Park Memorial Institute medium
rROP2	Recombinant rhoptry protein 2
SC	Subcutaneous
SFU	Spot-forming units
SINAN	Sistema de Informação de Agravos de Notificação
SOPs	Standard Operating Procedures

<i>T. gondii</i>	<i>Toxoplasma gondii</i>
Th1	T-helper type 1
TMB	Tetramethylbenzidine
UEA1	Animal Experimentation Unit 1
UEL	Universidade Estadual de Londrina
UDESC	Universidade do Estado de Santa Catarina
VEG	<i>T. gondii</i> VEG strain of <i>Toxoplasma gondii</i>
VXN-Toxo	Vaxinano Experimental <i>Toxoplasma gondii</i> vaccine

SYMBOLS LIST

–	Negative
%	Percentage
+	Positive
×	Multiplication
°C	Degrees Celsius
γ	Greek letter gamma
μg	Microgram
μL	Microliter
mg	Milligram
mL	Milliliter
ng/mL	Nanogram per milliliter
R^2	Coefficient of determination

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1 INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan with worldwide distribution and the causative agent of toxoplasmosis, a zoonotic disease of major public and veterinary health importance. It is estimated that approximately one-third of the global population has been exposed to the parasite (Dubey, 2009). Felids, particularly domestic cats (*Felis catus*), are the only definitive hosts capable of producing and shedding environmentally resistant oocysts, thereby serving as the primary reservoir and key drivers for the environmental dissemination of the parasite (Innes et al., 2019).

The life cycle of *T. gondii* comprises both sexual and asexual stages. Sexual reproduction occurs exclusively in the intestinal epithelium of felids, whereas asexual multiplication takes place in a wide range of intermediate hosts, including mammals and birds (Tenter; Heckeroth; Weiss, 2000). Challenge occurs mainly through the ingestion of tissue cysts in raw or undercooked meat, ingestion of sporulated oocysts contaminating food, water, or soil, or by congenital transmission (Robert-Gangneux; Dardé, 2012). Due to their robust wall structure, sporulated oocysts remain highly resistant to environmental stressors and can persist in water bodies for years, constituting a major source of challenge for humans and animals (Dubey, 2009).

Because domestic cats are the only hosts capable of completing the sexual cycle of *T. gondii*, reducing oocyst shedding has been recognized as one of the most promising strategies to decrease environmental contamination and interrupt parasite transmission. Consequently, vaccination has emerged as an important preventive approach, particularly when capable of limiting intestinal parasite replication and subsequent oocyst excretion.

Several vaccine platforms have been investigated against *T. gondii*, including recombinant proteins, DNA and mRNA vaccines, and nanoparticle-based antigen delivery systems (Ducournau et al., 2020; Fasquelle et al., 2023). Among these, nanoparticles with a polysaccharide core and lipid shell (NPLs) have attracted considerable interest because they improve antigen stability, enhance antigen uptake by antigen-presenting cells, and promote efficient immune stimulation (Thakur; Foged, 2020). In addition, the route of vaccine

administration plays an important role in shaping the immune response. While subcutaneous immunization mainly induces systemic immunity, intranasal vaccination has the potential to stimulate both systemic and mucosal immune responses, including secretory IgA production, which may be particularly relevant for pathogens such as *T. gondii* that actively invade and replicate within the intestinal mucosal epithelium (Cardona; Moncada; Gómez-Marin, 2015; Yang et al., 2025).

Despite these advances, no commercial vaccine is currently available for domestic cats to control intestinal replication or prevent oocyst shedding, and information regarding the immunogenicity and efficacy of nanoparticle-based vaccines in this species remains limited. In particular, the influence of different administration routes on humoral, cellular, and mucosal immune responses, as well as their relationship with oocyst shedding following challenge with different *T. gondii* strains, has not been fully elucidated. Addressing these knowledge gaps is essential for the development of effective vaccination strategies capable of reducing environmental contamination and limiting parasite transmission within the One Health framework.

Accordingly, the present study aimed to evaluate the immunogenicity and efficacy of an inactivated *T. gondii* vaccine formulated with polymeric lipid nanoparticles and administered via the subcutaneous or intranasal route in domestic cats experimentally challenged with two distinct *T. gondii* strains (VEG and ME49). Humoral, cellular, and mucosal immune responses, as well as oocyst shedding following challenge, were evaluated.

2 REVIEW

2.1 BIOLOGY OF *Toxoplasma gondii*

An obligate intracellular protozoan of the phylum Apicomplexa (class Conoidasida, subclass Coccidia, order Eucoccidiorida, suborder Eimeriorina, family Sarcocystidae), *T. gondii* is the etiological agent of toxoplasmosis, a globally distributed zoonotic disease of major importance to public and veterinary health (Dubey, 2009; Tenter; Heckeroth; Weiss, 2000). This parasite has a facultative heteroxenous life cycle in which sexual reproduction occurs exclusively in the intestinal epithelium of felids, the definitive hosts, whereas asexual multiplication takes place in a wide range of intermediate hosts, including mammals and birds (Tenter; Heckeroth; Weiss, 2000).

One of the most remarkable biological characteristics of *T. gondii* is its low host-cell specificity, allowing it to infect virtually any nucleated cell of warm-blooded animals. Three infective stages have been identified throughout its life cycle: tachyzoites, bradyzoites, and sporozoites (Tenter; Heckeroth; Weiss, 2000). Tachyzoites are the rapidly multiplying forms responsible for the acute phase of challenge, whereas bradyzoites are enclosed within tissue cysts and establish chronic challenge. Sporozoites are contained within sporulated oocysts shed by definitive hosts (Tenter; Heckeroth; Weiss, 2000; Weiss; Dubey, 2009).

Transmission may occur through several routes. The most common route of challenge in humans and animals is the ingestion of tissue cysts containing bradyzoites in raw or undercooked meat. Challenge may also occur through the ingestion of sporulated oocysts contaminating water, soil, fruits, or vegetables, as well as through congenital transmission when tachyzoites cross the placental barrier and infect the developing fetus (Robert-Gangneux; Dardé, 2012; Tenter; Heckeroth; Weiss, 2000). Less common routes include the ingestion of unpasteurized milk containing viable tachyzoites, organ transplantation, blood transfusion, and laboratory accidents (Robert-Gangneux; Dardé, 2012; Tenter; Heckeroth; Weiss, 2000).

In felids, sexual reproduction occurs within the intestinal epithelium, leading to the production and excretion of millions of unsporulated oocysts. Following primary challenge, sexual reproduction (gametogony) occurs within the

intestinal epithelium, leading to the production and excretion of millions of unsporulated oocysts in the feces. The pre-patent period depends strictly on the infective stage ingested, ranging from 3 to 5 days after tissue cyst consumption to over 19 days following oocyst or tachyzoite ingestion (Dubey, 2009). Once released into the environment, oocysts undergo sporulation within two to four days, producing two sporocysts, each containing four sporozoites, thereby becoming highly resistant and infective to a wide range of warm-blooded hosts (Dubey, 2009; Tenter; Heckeroth; Weiss, 2000).

After ingestion of sporulated oocysts or tissue cysts, parasites are released during digestion and invade the intestinal epithelium, where they differentiate into tachyzoites. These proliferative forms disseminate through the bloodstream and lymphatic system, infecting numerous organs and tissues, including skeletal and cardiac muscles, the central nervous system, retina, placenta, liver, lungs, and kidneys (Dubey, 2009; Dubey; Lindsay; Speer, 1998; Tenter; Heckeroth; Weiss, 2000). Intracellular multiplication of tachyzoites results in host-cell lysis and induces a marked inflammatory response, with disease severity depending on parasite virulence and host immune competence (Dubey, 2009; Innes, 2010; Robert-Gangneux; Dardé, 2012).

As the host immune response develops, tachyzoites differentiate into bradyzoites, which become enclosed within tissue cysts and remain viable throughout the lifetime of the host, establishing chronic challenge (Dubey; Lindsay; Speer, 1998). In immunocompetent individuals, these cysts generally remain latent. However, in immunocompromised hosts, bradyzoites may reactivate and reconvert into tachyzoites, resulting in severe clinical manifestations associated with reactivated toxoplasmosis (Dubey, 2009; Robert-Gangneux; Dardé, 2012).

The remarkable environmental resistance of sporulated oocysts, together with the lifelong persistence of tissue cysts in intermediate hosts and the broad host range of *T. gondii*, contributes to the successful maintenance of its life cycle in nature. These biological characteristics explain the widespread distribution of the parasite and underscore its importance as one of the most successful zoonotic protozoa affecting both humans and animals (Dubey, 2009; Innes et al., 2019).

2.2 EPIDEMIOLOGY AND PUBLIC HEALTH IMPORTANCE

Toxoplasmosis is one of the most widespread zoonotic parasitic diseases, affecting humans and a wide variety of warm-blooded animals worldwide. Approximately one-third of the global population has been exposed to *T. gondii*, although seroprevalence varies considerably among geographic regions according to climatic conditions, sanitation, dietary habits, socioeconomic factors, and cultural practices (Dubey, 2009; Robert-Gangneux; Dardé, 2012).

A systematic review and meta-analysis estimated a global anti-*T. gondii* IgG seroprevalence of 32.9% among pregnant women, with higher prevalence reported in Latin America, Africa, the Middle East, and parts of Europe and Asia (Bigna et al., 2020). These differences are largely associated with environmental conditions that favor oocyst survival, as well as regional differences in food consumption and hygiene practices.

In immunocompetent individuals, challenge is usually asymptomatic or results in mild clinical manifestations. However, immunocompromised patients are at risk of severe disease due to reactivation of latent tissue cysts, particularly in HIV-infected individuals or patients undergoing immunosuppressive therapy, leading to life-threatening encephalitis, pneumonitis, myocarditis, and ocular toxoplasmosis (Montoya; Liesenfeld, 2004; Robert-Gangneux; Dardé, 2012).

Congenital toxoplasmosis remains one of the most serious outcomes of the disease. Primary maternal challenge during pregnancy may result in miscarriage, stillbirth, or permanent neurological and ocular sequelae. Although the probability of vertical transmission increases with gestational age, fetal damage is generally more severe when challenge occurs during early pregnancy (Robert-Gangneux; Dardé, 2012)

Beyond its impact on human health, toxoplasmosis also causes important economic losses in livestock production, particularly in sheep and goats, where challenge is associated with abortion, neonatal mortality, and reduced reproductive performance (Innes et al., 2019). In Brazil, the high seroprevalence of challenge, the remarkable genetic diversity of circulating *T. gondii* strains, and the continued occurrence of gestational toxoplasmosis reinforce the importance of surveillance and preventive strategies (BRASIL, 2025; Pena et al., 2008; Shwab et al., 2018).

Considering the close interaction among humans, animals, and the environment, toxoplasmosis is recognized as a One Health disease. Because felids are the only definitive hosts capable of shedding environmentally resistant oocysts, strategies aimed at reducing oocyst excretion by domestic cats may substantially decrease environmental contamination and interrupt parasite transmission to humans and other animals (Innes et al., 2019).

2.3 ROLE OF FELIDS IN THE EPIDEMIOLOGY

The epidemiological importance of felids stems from their exclusive role in the sexual cycle of *T. gondii*, which results in the production and shedding of environmentally resistant oocysts. This unique characteristic places domestic and wild felids at the center of the parasite transmission cycle, making them the main source of environmental contamination (Dubey, 2009; Innes et al., 2019; Tenter; Heckeroth; Weiss, 2000).

Following primary challenge, sexual reproduction occurs in the intestinal epithelium, resulting in the excretion of millions of unsporulated oocysts in the feces. Following the pre-patent phase, oocyst shedding usually lasts for one to two weeks. Despite this short shedding window, the large number of oocysts released makes cats the principal contributors to environmental dissemination of *T. gondii* (Dubey, 2001, 2009).

Once excreted, oocysts sporulate within one to five days and become highly resistant to environmental conditions. Their persistence in soil and water for extended periods facilitates transmission to a wide range of intermediate hosts, including livestock, wildlife, and humans (Dubey, 2009; Jones; Dubey, 2018).

Environmental contamination with sporulated oocysts is one of the major sources of *T. gondii* challenge. Humans and animals become infected mainly through the consumption of contaminated food or water or by accidental ingestion of contaminated soil. In livestock, challenge is commonly associated with grazing on contaminated pastures (Innes et al., 2019; Jones; Dubey, 2018).

Domestic cats are infected primarily through predation of infected rodents, birds, and other intermediate hosts harboring tissue cysts. Consequently, free-roaming cats are more likely to become infected and shed oocysts than animals

maintained indoors and fed commercial diets. Importantly, direct contact with cats is not considered the main source of human challenge; rather, exposure to sporulated oocysts in the environment represents the greatest risk (Jones; Dubey, 2018).

Because felids are the only hosts capable of shedding oocysts, strategies aimed at reducing or preventing oocyst excretion are considered essential for interrupting the transmission cycle of *T. gondii*. In this context, vaccination of domestic cats has emerged as a promising approach to reduce environmental contamination and support toxoplasmosis control within the One Health framework (Innes et al., 2019).

2.4 EXPERIMENTAL STRAINS

Considerable genetic diversity has been described among *T. gondii* isolates, resulting in differences in virulence, tissue tropism, cyst-forming capacity, and host immune modulation. In Europe and North America, the parasite population is predominantly composed of the three clonal lineages (Types I, II, and III), whereas South America displays a much greater genetic diversity, with numerous atypical genotypes associated with distinct epidemiological and clinical characteristics of toxoplasmosis (Dubey et al., 2012; Pena et al., 2008).

Among the experimental strains most frequently used in research are ME49 (ATCC 50861), a Type II strain, and VEG (ATCC 50840), a Type III strain. The *T. gondii* ME49 strain is characterized by low virulence in murine models, a high capacity for tissue cyst formation, and the establishment of chronic challenge, making it widely used in studies investigating immunology, pathogenesis, and vaccine efficacy (Howe; Sibley, 1995; Saeij; Boyle; Boothroyd, 2005).

In contrast, the *T. gondii* VEG strain has been extensively employed in studies involving felids because of its high infectivity and efficient oocyst production following challenge. Dubey (2001) demonstrated that cats fed isolated bradyzoites of the *T. gondii* VEG strain shed large numbers of oocysts, confirming its high capacity to complete the sexual cycle in the definitive host and establishing this strain as an important experimental model for studies of oocyst

shedding. Subsequently, Garcia et al. (2007) used the *T. gondii* VEG strain to evaluate an intranasal vaccine based on rhoptry proteins and demonstrated a significant reduction in oocyst shedding in experimentally infected cats.

The use of different *T. gondii* strains in vaccine studies enables the evaluation of protective efficacy against isolates with distinct biological characteristics. In the present study, the use of the tissue cyst-forming *T. gondii* ME49 strain together with the *T. gondii* VEG strain, which is widely employed in oocyst shedding studies, allowed a broader assessment of vaccine-induced immune responses and protective efficacy under different challenge conditions.

2.5 IMMUNE RESPONSE AGAINST *TOXOPLASMA GONDII*

Challenge with *T. gondii* elicits a complex immune response involving both innate and adaptive immunity. Although the host immune response is generally unable to eliminate the parasite completely, it plays a fundamental role in controlling tachyzoite replication, limiting parasite dissemination, and promoting the establishment of chronic challenge characterized by tissue cyst formation. Protection against *T. gondii* relies predominantly on a T helper 1 (Th1)-type immune response, in which interferon-gamma (IFN- γ) is considered the key cytokine responsible for parasite control (Lakhrif et al., 2018; Innes et al., 2019; Mévélec; Lakhrif; Dimier-Poisson, 2020).

2.5.1 Innate immunity

Innate immunity constitutes the first line of defense against *T. gondii*. Following challenge, dendritic cells and macrophages recognize parasite-derived molecules and produce interleukin-12 (IL-12), which promotes the activation of natural killer (NK) cells and stimulates the early production of IFN- γ . This cytokine activates macrophages and enhances their ability to restrict intracellular parasite replication, playing a central role in the early control of challenge (Howe; Sibley, 1995; Yarovsky, 2014).

Experimental studies in cats have demonstrated the importance of this early cellular response. Yin et al. (2015) reported that peripheral blood mononuclear cells (PBMCs) produced antigen-specific IFN- γ shortly after challenge, before the detection of specific antibodies. These findings indicate that

cellular immunity is activated before the humoral response and highlight IFN- γ as an important marker of protective immunity against *T. gondii*.

2.5.2 Adaptive immunity

Adaptive immunity is essential for maintaining long-term control of *T. gondii* challenge. CD4⁺ T lymphocytes coordinate the immune response through cytokine production, whereas CD8⁺ T lymphocytes eliminate infected cells through cytotoxic mechanisms and produce IFN- γ . Together, these cell populations contribute to controlling parasite replication and maintaining chronic challenge (Innes et al., 2019; Mévélec; Lakhrif; Dimier-Poisson, 2020).

The humoral immune response also contributes to parasite control. Following challenge, immunoglobulin M (IgM) is produced during the early phase, followed by immunoglobulin G (IgG), which provides long-lasting immunity and is widely used as a serological marker of previous exposure and vaccine-induced immune responses (Montoya; Liesenfeld, 2004; Robert-Gangneux; Dardé, 2012).

Because *T. gondii* challenge is acquired primarily through the oral route, mucosal immunity also plays an important protective role. Secretory immunoglobulin A (IgA) is the predominant antibody at mucosal surfaces and contributes to limiting parasite adhesion and invasion of the intestinal epithelium. Although its protective role is not yet fully understood, experimental studies suggest that effective mucosal immune responses may reduce intestinal parasite replication and consequently decrease oocyst shedding by felids (Cardona; Moncada; Gómez-Marin, 2015; Mévélec; Lakhrif; Dimier-Poisson, 2020).

Importantly, protection against oocyst shedding may depend not only on the magnitude of systemic immune responses but also on the recognition of parasite stages that develop within the feline intestine. Oral immunization with tissue cysts or bradyzoites of the attenuated T-263 strain prevented subsequent oocyst shedding, whereas the administration of tachyzoites failed to confer equivalent protection despite inducing a marked antibody response. These findings suggest that exposure to bradyzoite- and intestinal-stage antigens may be necessary to generate effective local immunity against the sexual development of *T. gondii* in cats (Arranz-Solís; Saeij, 2022).

Knowledge of these immune mechanisms has guided the development of novel vaccination strategies against *T. gondii*. An ideal vaccine should induce a robust Th1-type immune response characterized by sustained IFN- γ production, together with the generation of specific antibodies, particularly systemic IgG and mucosal IgA, thereby limiting parasite replication and reducing oocyst shedding by domestic cats (Innes et al., 2019; Mévélec; Lakhfir; Dimier-Poisson, 2020).

2.6 VACCINES AGAINST *TOXOPLASMA GONDII*

The development of vaccines against *T. gondii* has been considered one of the most promising strategies for controlling toxoplasmosis. Although sanitary measures and food safety practices contribute to reducing parasite transmission, they are insufficient to interrupt its life cycle. Therefore, vaccination has emerged as an important approach to reduce challenge in animals and, consequently, human exposure, particularly within the One Health framework (Innes et al., 2019).

Over the past decades, several vaccine platforms have been investigated, including live-attenuated vaccines, recombinant proteins, DNA vaccines, mRNA vaccines, and nanoparticle-based delivery systems (Ducournau et al., 2020; Fasquelle et al., 2023). However, the only commercially available vaccine is Toxovax®, a live-attenuated S48 strain licensed exclusively for use in sheep (Verma; Khanna, 2013).

Live-attenuated vaccines generally induce broader and longer-lasting immune responses because they better mimic natural challenge and expose the host to a wider antigenic repertoire. However, concerns regarding residual virulence, genetic stability, and potential reversion to virulence limit their application. In contrast, inactivated, recombinant, and nanoparticle-based vaccines are safer and easier to standardize, but often provide only partial protection (Arranz-Solís; Saeij, 2022; Qadeer et al., 2026).

Among the experimental approaches evaluated in cats, Garcia et al. (2007) demonstrated that intranasal immunization with rhoptry proteins significantly reduced oocyst shedding. Similarly, Zulpo et al. (2017) reported reduced oocyst excretion following immunization with the recombinant rROP2 protein, while Yang et al. (2025) showed that immunization with the recombinant GRA12 protein

induced both cellular and humoral immune responses and reduced the intensity and duration of oocyst shedding after challenge. Xie et al. (2026) evaluated a live-attenuated *T. gondii* Pru Δ pp2a-c mutant and reported a 94.5% reduction in total oocyst shedding, although five of six vaccinated cats still shed oocysts

More recently, Brito et al. (2026) demonstrated that intranasal immunization with *T. gondii* membrane proteins combined with CpG induced systemic IgG, intestinal IgA, and IFN- γ -producing T-cell responses, while partially reducing parasite burden in mice

In parallel, nanoparticle-based vaccine platforms have attracted increasing interest. Studies by Dimier-Poisson et al. (2015), Ducournau et al. (2017, 2020), and Fasquelle et al. (2023) demonstrated that nanoparticles composed of a maltodextrin core and an anionic phospholipid shell (NPLs) enhance antigen delivery, promote protective immune responses, and reduce parasite burden in different experimental models, highlighting their potential as vaccine delivery systems against *T. gondii*.

2.7 LIPID NANOPARTICLES AS ANTIGEN DELIVERY SYSTEMS

Advances in nanotechnology have promoted the development of novel antigen delivery systems to improve vaccine efficacy. Among these, nanoparticles have attracted considerable attention because they protect antigens from degradation, enhance their uptake by antigen-presenting cells, and improve both cellular and humoral immune responses (Thakur; Foged, 2020). Among the different nanoparticle platforms, Nanoparticles with a Polysaccharide Core and Lipid Shell (NPLs), composed of a maltodextrin core coated with an anionic phospholipid shell, have emerged as promising carriers for *T. gondii* antigens. These nanoparticles exhibit high protein-loading capacity, good biocompatibility, low toxicity, and efficient antigen delivery, making them attractive candidates for vaccine development (Fasquelle et al., 2023).

The potential of this platform has been demonstrated in several experimental models. Dimier-Poisson et al. (2015) reported that NPL-based vaccines induced protective immune responses and reduced parasite burden and brain cyst formation in mice. Using the same platform, Ducournau et al. (2017) demonstrated protection against congenital toxoplasmosis in pregnant rats, while

Ducournau et al. (2020) showed that intranasal immunization of sheep elicited a strong Th1 immune response and significantly reduced fetal challenge. These successful outcomes in rodent and ovine models provided the scientific basis for evaluating the NPL platform as a candidate vaccine against *T. gondii* in the feline definitive host in the present study (Dimier-Poisson et al., 2015; Ducournau et al., 2017, 2020). More recently, a large-scale retrospective study of VNX-Toxo vaccination in captive wildlife reported no vaccine-associated adverse events among 784 vaccinated animals representing 58 species, supporting the favorable tolerability of this inactivated nanoparticle-based vaccine (Scuotto et al., 2025). Overall, the available evidence supports NPLs as a promising vaccine platform and justifies their use in the vaccine formulation evaluated in the present study. In addition to the physicochemical properties of NPLs, the route of administration may directly influence vaccine performance. Intranasal immunization can induce both systemic and mucosal responses, including serum IgG, intestinal IgA, and antigen-specific T-cell responses Brito et al. (2026), whereas parenteral routes predominantly induce systemic immunity. Therefore, evaluating different administration routes may contribute to optimizing the immunogenicity and protective efficacy of NPL-based vaccines against *T. gondii* (Mévélec; Lakhri; Dimier-Poisson, 2020; Thakur; Foged, 2020).

However, vaccine performance may depend not only on the administration route but also on the antigenic composition and parasite stages represented in the formulation. Tachyzoite-based vaccines may not adequately represent antigens expressed during bradyzoite differentiation and intestinal development. Therefore, future NPL formulations could incorporate multistage antigens to broaden protection (Arranz-Solís; Saeij, 2022; Qadeer et al., 2026).

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OBJECTIVES

2.8 GENERAL

To evaluate the immunogenicity and protective efficacy of the VXN-Toxo vaccine administered by the subcutaneous and intranasal routes in domestic cats experimentally challenged with the VEG and *T. gondii* ME49 strains of *T. gondii*.

2.9 SPECIFIC OBJECTIVES

- To evaluate the humoral (serum IgG), cellular (IFN- γ production by PBMCs), and mucosal (fecal IgA) immune responses elicited by the VXN-Toxo vaccine.
- To evaluate the effect of vaccination on oocyst shedding following challenge with the VEG and *T. gondii* ME49 strains of *T. gondii*.
- To compare the immunogenicity and efficacy of the VXN-Toxo vaccine administered by the subcutaneous (SC) and intranasal (IN) routes.

3 MANUSCRIPT

HUMORAL, CELLULAR, AND MUCOSAL IMMUNE RESPONSES AND OOCYST SHEDDING IN DOMESTIC CATS IMMUNIZED WITH AN INACTIVATED *TOXOPLASMA GONDII* VACCINE DELIVERED BY POLYMERIC LIPID NANOPARTICLES

3.1 ABSTRACT

Background/Objectives: *Toxoplasma gondii* is a globally distributed zoonotic protozoan, and domestic cats are the only definitive hosts capable of shedding environmentally resistant oocysts. This study evaluated the immunogenicity and protective efficacy of an experimental vaccine composed of inactivated *T. gondii* tachyzoites associated with polymeric lipid nanoparticles (NPLs), administered by subcutaneous (SC) or intranasal (IN) routes in domestic cats. Methods: Eighteen seronegative cats, evaluated across two independent experiments, were allocated into control, SC-vaccinated, and IN-vaccinated groups. Animals were immunized following a prime–boost schedule and challenged with either the VEG or ME49 strain of *T. gondii*. Humoral, mucosal, and cellular immune responses were assessed by IFAT, fecal IgA ELISA, and IFN- γ ELISpot, respectively. Vaccine efficacy was evaluated through oocyst shedding after challenge. Results: Vaccination induced distinct immune profiles according to the administration route. The SC route promoted earlier systemic IgG responses, whereas the IN route induced higher IFN- γ production. Fecal IgA levels remained low and did not differ significantly among groups. Following the challenge, all animals showed increased IgG titers and IFN- γ responses. Despite the induction of humoral and cellular immunity, neither vaccination strategy significantly reduced oocyst shedding, shedding duration, or total oocyst output. Conclusions: The NPL-based vaccine was immunogenic in domestic cats, inducing measurable systemic and cellular immune responses. However, these responses were insufficient to significantly reduce oocyst shedding following challenge with the VEG or ME49 strains. These findings highlight the challenges of inducing protective intestinal immunity against *T. gondii* in its definitive host and support further optimization of nanoparticle-based vaccination strategies.

Keywords: feline toxoplasmosis; clinical safety; oocyst shedding; IFN- γ ELISpot; secretory IgA; intranasal immunization.

3.2 INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan with worldwide distribution and is the causative agent of toxoplasmosis, a zoonotic disease of major public and veterinary health importance. It is estimated that approximately one-third of the global population has been exposed to the parasite (Dubey, 2009). Felids, particularly domestic cats (*Felis catus*), are the only definitive hosts capable of producing and shedding environmentally resistant oocysts, thereby playing a central role in the transmission and maintenance of the parasite in nature (Innes et al., 2019).

This parasite exhibits a complex life cycle characterized by both sexual and asexual stages. Sexual reproduction occurs exclusively within the intestinal epithelium of felids, whereas asexual multiplication takes place in a wide range of intermediate hosts, including mammals and birds (Tenter; Heckeroth; Weiss, 2000). Three infective stages have been identified: tachyzoites, bradyzoites, and sporozoites. Challenge may occur through the ingestion of tissue cysts containing bradyzoites in raw or under-cooked meat, ingestion of sporulated oocysts contaminating water, soil, or food, or through congenital transmission when tachyzoites cross the placental barrier to infect the developing fetus (Robert-Gangneux; Dardé, 2012; Tenter; Heckeroth; Weiss, 2000). After sporulation, the highly resistant and infectious oocysts shed by felids contribute significantly to the persistence of the parasite in animal and human populations (Dubey, 2009; Innes et al., 2019).

Reducing oocyst shedding by domestic cats is considered a key strategy for limiting environmental contamination and interrupting the transmission of *T. gondii*. In this context, intranasal vaccination has attracted considerable interest due to its ability to induce both mucosal and systemic immune responses, potentially restricting intestinal parasite replication and oocyst excretion (Garcia et al., 2007; Innes et al., 2019; Zhang et al., 2022; Zulpo et al., 2012, 2017).

Despite of its potential, no vaccine is currently licensed for use in domestic cats. The only commercially available vaccine, Toxovax®, is approved

exclusively for sheep and is based on a live attenuated strain (Verma; Khanna, 2013). This limitation highlights the need for the development of safe and effective vaccine strategies specifically designed for felines.

Several vaccine platforms have been investigated against *T. gondii*, including recombinant proteins, DNA vaccines, mRNA-based approaches, multi-epitope constructs, exosomes, and nanoparticle-based delivery systems (Ducournau et al., 2020; Fasquelle et al., 2023). Among these, polymeric lipid nanoparticles have emerged as promising antigen carriers due to their ability to improve antigen stability, enhance cellular uptake, and promote sustained immune stimulation (Thakur; Foged, 2020).

Previous studies in mice and sheep have demonstrated that NPL-based vaccines can induce protective immune responses against *T. gondii*, reducing parasite burden and improving protection following challenge (Ducournau et al., 2017). Maltodextrin nanoparticles associated with anionic phospholipids have shown promise as vaccine delivery systems against *T. gondii*, inducing protective immune responses and reducing parasite burden, cyst formation, and congenital transmission in experimental models (Dimier-Poisson et al., 2015; Ducournau et al., 2017, 2020).

In addition to vaccine composition, the route of administration plays a critical role in shaping the immune response. Subcutaneous immunization is generally associated with the induction of systemic humoral immunity, whereas intranasal administration may stimulate both systemic and mucosal immune responses, including the production of secretory IgA and the activation of cellular immune mechanisms (Cardona; Moncada; Gómez-Marin, 2015; Yang et al., 2025). These characteristics are particularly relevant for *T. gondii*, whose life cycle in felids involves intestinal stages responsible for oocyst production and shedding.

Moreover, distinct *T. gondii* strains may differ in virulence, tissue tropism, and host immune modulation, highlighting the importance of evaluating vaccine performance under different challenge conditions. Despite recent advances in vaccine development, limited information is available regarding the immunogenicity of nanoparticle-based vaccines in domestic cats, as well as the influence of different administration routes on humoral, cellular, and mucosal

immune responses. Furthermore, the relationship between vaccine-induced immunity and the reduction of oocyst shedding remains incompletely understood.

Considering that different *T. gondii* strains exhibit distinct biological and immunological characteristics, evaluating vaccine candidates under diverse challenge conditions is essential to better comprehend their protective efficacy. To address these gaps, this study aimed to evaluate the immunogenicity and efficacy of an inactivated *T. gondii* vaccine delivered by polymeric lipid nanoparticles via subcutaneous or intranasal routes in cats challenged with two distinct strains (VEG and ME49).

3.3 MATERIAL AND METHODS

3.3.1 ETHICS STATEMENT

This study was approved by the Ethics Committee on Animal Use of Santa Catarina State University (CEUA-CAV-UDESC; protocol number 7783280224) and was conducted in accordance with a formal collaboration agreement established between Vaxinano, Lille, France, and the Laboratory for the Development of Health Technologies Parasitology at the Agroveterinary Sciences Center, Santa Catarina State University (LADETES-CAV-UDESC), Lages, Santa Catarina, Brazil. All experimental procedures and animal handling practices were performed in compliance with the local standard operating procedures (SOPs) and animal welfare regulations.

3.3.2 STUDY DESIGN AND ANIMALS

The study was carried out in two independent experiments. Experiment 1 included 10 cats and used the *T. gondii* VEG strain of *T. gondii* for the infectious challenge, whereas Experiment 2 included 8 cats and used the *T. gondii* ME49 strain.

A total of 18 mixed-breed domestic cats (*Felis catus*), aged between 10 and 14 weeks at enrollment, were allocated in the study. All cats underwent clinical examinations and were considered healthy by a veterinarian. Blood samples were collected for serological testing, and only animals seronegative for *T. gondii*, feline immunodeficiency virus (FIV), and feline leukemia virus (FeLV)

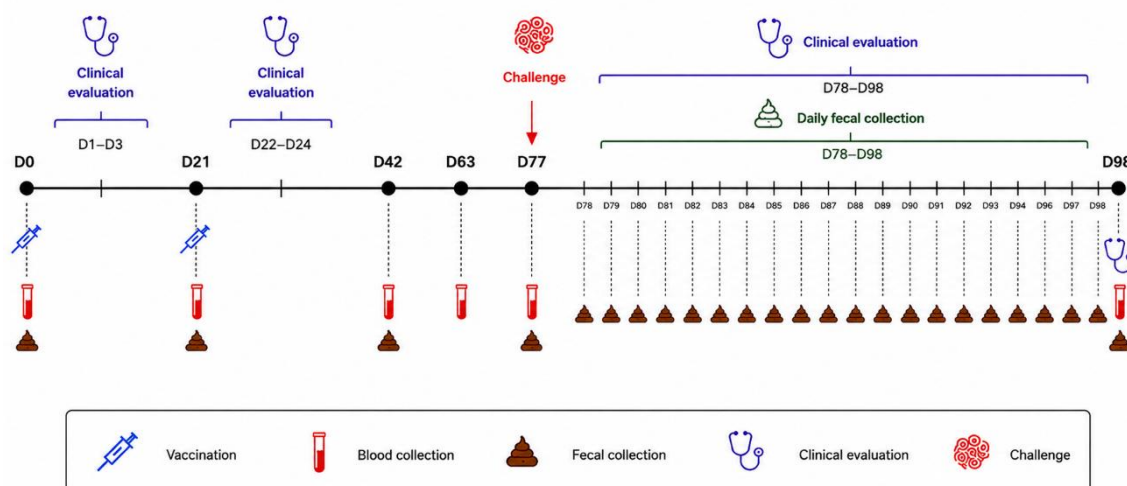
were included. Prior to study start, all animals underwent standard protocols for internal and external parasite control.

Cats were randomly allocated into three experimental groups: control (CT), subcutaneously vaccinated (SC), and intranasally vaccinated (IN). In Experiment 1, the number of individuals per group were CT (n = 3), SC (n = 3), and IN (n = 4), whereas in Experiment 2, group sizes were CT (n = 2), SC (n = 3), and IN (n = 3). A schematic representation of the experimental timeline, including vaccination, clinical monitoring, challenge challenge, and blood and fecal sample collection, is presented in Figure 1.

To minimize potential observer bias, researchers responsible for sample processing, laboratory analyses, and outcome assessment were blinded to the experimental group allocation throughout the study. Group identities were disclosed only after completion of the analyses.

The animals were housed at the Animal Experimentation Unit 1 (UEA1) under controlled environmental conditions, including a temperature of approximately 20 °C, natural photoperiod, ad libitum access to water, commercial feline diet, and environmental enrichment. Cats were maintained in collective enclosures with standardized management and supervised periods of free movement, except during oocyst shedding periods, for avoiding environmental contamination with oocysts, in accordance with biosecurity protocols.

Figure 1. Experimental timeline showing vaccination, clinical evaluations, challenge challenge with *T. gondii*, and blood and fecal sample collection throughout the study period.



Prepared by the author with the assistance of ChatGPT (OpenAI), 2026.

3.3.3 VACCINE FORMULATION

The vaccine formulation used in this study was provided by Vaxinano (France). The vaccine (VXN-Toxo, lot 17092024) consisted of inactivated *T. gondii* tachyzoites associated with cationic polymeric lipid nanoparticles (NPLs), which acted as an adjuvant. The parasites were produced in Vero cell cultures, purified by centrifugation and successive washes in phosphate-buffered saline (PBS), and subsequently inactivated through repeated freeze–thaw cycles to preserve antigenic integrity (Fasquelle et al., 2023).

The NPLs were prepared as previously described from maltodextrin solubilized in 2M NaOH, followed by the addition of epichlorohydrin and glycidyltrimethylammonium chloride (GTMA), neutralization with acetic acid, high-pressure homogenization, and purification by tangential flow ultrafiltration. The final vaccine formulation was prepared by homogenizing NPLs with inactivated *T. gondii* tachyzoites at a 3:1 ratio (w/w) in distilled water and stored at 4 °C until administration (Fasquelle et al., 2023). Each vaccine dose contained 50 µg of total *T. gondii* protein antigen associated with NPLs. Control animals received phosphate-buffered saline (PBS) as placebo.

3.3.4 IMMUNIZATION PROTOCOL

Animals were immunized following a prime–boost schedule on D0 and D21. Cats assigned to SC group received the vaccine in the interscapular region

(200 μ L per dose), using a sterile syringe and needle after local antisepsis with 0.5% alcoholic chlorhexidine. Cats assigned to IN group received 50 μ L of the vaccine, consisting of approximately 25 μ L into each nostril, using 0.5 mL Pasteur pipettes. Control animals (CT) received placebo (PBS) via both subcutaneous and intranasal routes according to the same schedule and handling procedures used for the vaccinated groups.

In order to facilitate handling and minimize stress, animals received gabapentin (100 mg/animal) before vaccine administration. To assess vaccine safety, all animals were clinically monitored after vaccination by measuring heart rate, respiratory rate, and body temperature, as well as by evaluating the presence of local reactions at the injection site and nostrils. These parameters were recorded at 2, 4, 8, 12, 24, and 48 h post-vaccination.

3.3.4 PARASITE CHALLENGE

Two independent challenge experiments were performed using distinct *T. gondii* strains. In Experiment 1, cats were challenged with *T. gondii* VEG strain (Type III). In Experiment 2, cats were challenged with *T. gondii* ME49 strain (Type II). Challenges were performed on Day 77.

Both inocula were produced by the Protozoology Laboratory of the State University of Londrina (UEL), Paraná, Brazil. Mice were infected intraperitoneally with *T. gondii* tachyzoites, and the brains were harvested after 60 days of challenge. The entire organ was homogenized in 1 mL of sterile 0.9% saline solution, and a 25 μ L aliquot of the homogenate was examined microscopically for tissue cyst counting. Based on the number of cysts observed, the concentration of the suspension was calculated and adjusted to obtain the required challenge dose (Zulpo et al., 2012). The cyst suspensions were transported refrigerated in an insulated container from UEL to UDESC. Cats in Experiment 1 received 200 tissue cysts of *T. gondii* VEG strain, whereas cats in Experiment 2 were infected with 50 tissue cysts of *T. gondii* ME49 strain. The respective number of tissue cysts was suspended in sterile saline solution and administered orally in a final volume of 500 μ L using a nasogastric cannula. Gabapentin (100 mg/animal) was also used for minimizing the stress of the cats during the procedure.

3.3.5 BLOOD SAMPLING AND PROCESSING

Blood samples were collected from the jugular vein at predetermined experimental time points (Figure 1) using physical restraint by two to three trained operators. The animals received gabapentin (100 mg/animal) approximately 30 min before collection. Samples were distributed into EDTA tubes and tubes without anticoagulant.

The blood samples collected without anticoagulant were centrifuged at $1,860 \times g$ for 8 min (KC8, Kindly, Brazil) for sera collection, which were placed into microtubes and stored at -20°C until use in serological assays.

Peripheral blood mononuclear cells (PBMCs) were isolated immediately after EDTA-blood collection on Days 0, 21, 42, 77, and 98. The PBMCs were obtained by density gradient centrifugation using Histopaque®-1077 (Merck, Darmstadt, Germany), following the manufacturer's instructions with adaptations provided by Vaxinano (Fasquelle; Vreulx; Betbeder, 2024; Histopaque®-1077).

Cell viability was determined by trypan blue exclusion using a Neubauer hemocytometer (Fasquelle; Vreulx; Betbeder, 2024) and visualized under an optical microscope (Primo Star 1, Zeiss, China). PBMCs were cryopreserved in fetal bovine serum supplemented with 10% di-methyl sulfoxide (DMSO), placed in a controlled-rate freezing container (Corning® CoolCell™ LX Cell Freezing Container, Corning Inc., Corning, NY, USA) for 24 h and stored at -80°C (CryoCube F570n, Eppendorf, United Kingdom) until further analysis.

3.3.6 OOCYST SHEDDING EVALUATION

Fecal samples were collected daily for 21 consecutive days after experimental challenge. Samples were collected from each experimental group, pooled, homogenized, and processed for parasitological analyses.

Initially, samples were examined using a qualitative sucrose flotation technique for the detection of the onset of oocyst shedding, based on previously described methods, with adaptations (Garcia et al., 2007; Zulpo et al., 2012, 2017). Briefly, fecal material was diluted in distilled water, mixed with sucrose solution, filtered, centrifuged at $1,500 \times g$ for 4min, and a drop collected from the

meniscus was examined microscopically for the presence of oocysts. Once oocyst shedding was detected, samples were analyzed quantitatively using the McMaster technique to determine the number of oocysts per gram of feces (OPG). Samples that tested negative using the quantitative method were subsequently re-evaluated using the qualitative sucrose flotation technique (Garcia et al., 2007; Zulpo et al., 2012, 2017).

3.3.7 SEROLOGICAL EVALUATION

The humoral immune response was evaluated by determining anti-*T. gondii* IgG antibody titers using the indirect immunofluorescence antibody test (IFAT) (Remor-Sebolt et al., 2024). Serum samples were initially screened at a dilution of 1:16 in phosphate-buffered saline (PBS). Samples testing positive at this dilution were subsequently subjected to serial four-fold dilutions (1:64, 1:256, 1:1024, and higher if necessary) until a negative reaction was observed. The cutoff titer for seropositivity was established at a dilution of 1:64 and antibody titers were defined as the highest serum dilution exhibiting specific fluorescence.

3.3.8 MUCOSAL IMMUNE RESPONSE EVALUATION

For fecal IgA quantification, samples collected on Days 0 (D0), 42 (D42), and 98 (D98) were selected for analysis. Immediately after defecation, samples were processed within 30 min, an aliquot was diluted in a preservative solution containing 0.1% (w/v) sodium azide in phosphate-buffered saline (PBS; pH 7.2) and stored at -80 °C until analysis (Zulpo et al., 2012). In Experiment 1, samples from the CT (F1 and F2), SC (F3 and F4), and IN groups (F5) were analyzed. In Experiment 2, samples from CT (F17 and F19), SC (F11, F12, and F14), and IN groups (F13, F15, and F18) were analyzed.

Total fecal IgA concentrations were determined using a commercial ELISA kit for feline IgA (Cat IgA ELISA Kit, ab190547; Abcam, Cambridge, UK), according to the manufacturer's instructions. Prior to analysis, fecal samples were diluted in the sample diluent provided with the kit at a final dilution of 1:10,000 and analyzed in duplicate. The absorbance was measured at 450 nm using a microplate reader (LMR-96, Loccus, Brazil). A standard curve was generated for each assay using the standards provided with the kit and showed high linearity

($R^2 = 0.990$). IgA concentrations were calculated from the standard curve and expressed as ng/mL.

3.3.9 IFN- γ ELISPOT ASSAY

Cell-mediated immune responses were evaluated using an IFN- γ enzyme-linked immunospot (ELISpot) assay. Cryopreserved peripheral blood mononuclear cells (PBMCs) were rapidly thawed in a 37 °C water bath, transferred to pre-warmed complete RPMI-1640 medium, and centrifuged at $300 \times g$ for 10 min to remove dimethyl sulfoxide (DMSO). Cells were subsequently resuspended in RPMI medium supplemented with 10% fetal bovine serum, penicillin-streptomycin 1% (complete RPMI). Cell counts and viability were determined using a Neubauer hemocytometer and trypan blue exclusion (Fasquelle; Vreulx; Betbeder, 2024).

Due to the low number of viable cells recovered from some animals, PBMCs were pooled by group for analysis (Ketterl et al., 2015). Pooled PBMC suspensions were adjusted to 1×10^6 cells per well and cultured in triplicate (unstimulated negative controls, antigen-stimulated wells, and ConA-stimulated positive controls) in 96-well plates containing complete RPMI medium. Cells were stimulated with pure *T. gondii* antigens, the same used for vaccine preparation. Prior to use, the stock solution was diluted 1:10 in PBS, and cells were stimulated with a final concentration equivalent to 5 μ g of antigen per 1×10^6 PBMCs. Wells containing only cells and culture medium served as negative controls. Plates were incubated for 24 h at 37 °C in a humidified atmosphere FORMA Series 3 WaterJacketed CO₂ Incubator (Thermo Scientific, USA). Concanavalin A (ConA) 50ug/ml was used as a positive control.

Feline IFN- γ production was evaluated using the ELISpot Plus: Cat IFN- γ (ALP) kit (product code 3122-4APW-2; Mabtech, Sweden), according to the manufacturer's instructions.

Following stimulation, cells were transferred to the pre-coated ELISpot plates, previously washed and blocked according to the manufacturer's instructions. A total of 2×10^5 cells were added per well in a final volume of 100 μ L. Plates were incubated for an additional 24 h at 37 °C in 5% CO₂ (Fasquelle; Vreulx; Betbeder, 2024).

After incubation, plates were washed repeatedly with PBS, and IFN- γ detection was performed using a biotinylated detection antibody followed by streptavidin–alkaline phosphatase conjugate and chromogenic substrate, according to the manufacturer's recommendations. The reaction was stopped with distilled water, and plates were air-dried at room temperature.

Spot quantification was performed at the Instituto Butantan using an automated ELISpot reader (IRIS ELISpot Reader, Mabtech, Sweden). Representative wells included unstimulated negative controls, ConA-stimulated positive controls, and antigen-stimulated wells. Raw Spot Forming Unit (SFU) values obtained from antigen-stimulated wells were corrected by subtracting background responses from unstimulated control wells. Corrected values were subsequently normalized to 10^6 PBMCs to allow standardized comparisons among experimental groups and time points. Results were expressed as spot-forming units (SFU) per 10^6 PBMCs.

Raw spot-forming unit (SFU) values obtained from antigen-stimulated wells were corrected by subtracting the corresponding background responses from unstimulated negative-control wells and subsequently normalized to 10^6 PBMCs. The overall cellular immune response throughout the experimental period was additionally assessed by calculating the area under the curve (AUC) from the normalized SFU/ 10^6 PBMC data across the evaluated time points.

3.3.10 STATISTICAL ANALYSES

Statistical analyses were performed using GraphPad Prism software (version 5.01; GraphPad Software, San Diego, CA, USA) and Jamovi (version 2.6).

Data distribution was assessed for normality using the Shapiro–Wilk test. Serological data were analyzed using the Kruskal–Wallis test followed by the Dwass–Steel–Critchlow–Fligner (DSCF) post hoc multiple comparisons test in Jamovi. Longitudinal changes within each experimental group were evaluated using the Friedman test for repeated measures followed by Dunn's post hoc test. Correlations between immune response parameters and oocyst shedding were assessed using Spearman's rank correlation coefficient. Differences were considered statistically significant when $p < 0.05$.

3.3.11 USE OF ARTIFICIAL INTELLIGENCE

During the preparation of this work, the author used ChatGPT (GPT-5.6 Sol, OpenAI) to assist with the production of Figure 1, also with grammatical correction and suggestions for improvement of textual clarity, readability and structure. The first author critically reviewed, verified, and edited all AI-assisted content and assumes full responsibility for the final content of this work.

3.4 RESULTS

3.4.1 VACCINE SAFETY AND CHALLENGE OUTCOME

Some kittens sneezed after first IN vaccination using a spray device, and vaccination was repeated using 0.5 ml Pasteur pipettes. Second vaccination used only the pipettes and no reactions were detected. No other adverse reactions were observed in the SC or IN groups in the clinical evaluations post vaccination.

Animals were monitored daily throughout the study for clinical signs, particularly following challenge. In Experiment 1, one IN animal (F10) and one CT animal (F9) developed severe but self-limiting diarrhea during the oocyst shedding period, which resolved spontaneously. In Experiment 2, after the oocyst shedding phase, one CT animal (F17) developed clinical signs consistent with toxoplasmosis, including apathy, ataxic gait, reduced food intake, and progressive weight loss. Supportive treatment consisting of fluid therapy and wet food supplementation was initiated, along with sulfadiazine (15 mg/kg SID for 7 days) and continuous clinical monitoring. The animal showed gradual clinical improvement, with recovery of body weight and general health parameters within seven days of treatment.

3.4.2 HUMORAL IMMUNE RESPONSE

Experiment 1 (*T. gondii* VEG strain)

The kinetics of *T. gondii*-specific IgG titers throughout Experiment 1 are shown in Figure 2. Significant differences among groups were detected at D21 ($p = 0.011$), D42 ($p = 0.011$), D63 ($p = 0.013$), D77 ($p = 0.011$), D79 ($p = 0.011$), and D81 ($p = 0.011$). Post hoc pairwise comparisons identified significant

differences between the SC and IN groups at D21 ($p = 0.038$), D42 ($p = 0.038$), D63 ($p = 0.047$), D77 ($p = 0.038$), D79 ($p = 0.038$), and D81 ($p = 0.038$). No statistically significant differences were detected between CT and SC or between CT and IN at these time points.

No statistically significant differences were observed between Groups CT and SC at the same time points (D21, $p = 0.065$; D42, $p = 0.065$; D63, $p = 0.086$; D77, 79, and 81, $p = 0.065$). s.

Following experimental challenge at D77, an increase in IgG titers was observed across the experimental groups. Antibody levels increased during the post-challenge period, and at D98 a significant overall difference among groups was detected (Kruskal–Wallis, $p = 0.037$); however, post hoc pairwise comparisons did not identify statistically significant differences between specific groups.

Figure 2. Mean $\log_{10}$ *T. gondii*-specific IgG titers ($\pm$ SD) in cats from the group (CT), SCvaccinated group (SC), and intranasally vaccinated group (IN) during Experiment 1 (*T. gondii* VEG strain).

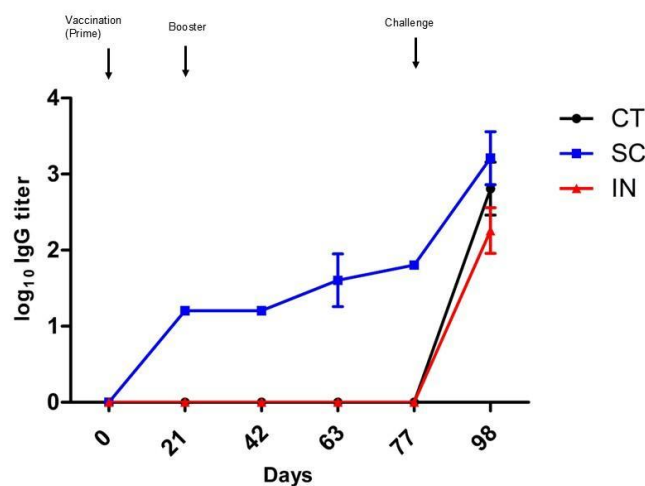


Table 1 – Individual anti-*T. gondii* IgG titers in cats from Experiment 1, challenged with *T.gondii* *T. gondii* VEG strain at D77.

Group	ID	D0	D21	D42	D63	D77	D98
CT	F1	0	0	0	0	0	1024
CT	F2	0	0	0	0	0	1024
CT	F9	0	0	0	0	0	256
SC	F3	0	16	16	16	64	1024
SC	F4	0	16	16	64	64	1024
SC	F8	0	16	16	64	64	4096
IN	F5	0	0	0	0	0	256
IN	F6	0	0	0	0	0	256
IN	F7	0	0	0	0	0	256
IN	F10	0	0	0	0	0	64

Experiment 2 (*T. gondii* ME49 strain)

The humoral immune response was evaluated by quantifying anti-*T. gondii* IgG titers at six predefined time points (Figures 1 and 3). Until D21, IgG titers remained at baseline levels in all experimental groups, indicating the absence of a detectable humoral response at these early time points.

From D42 onward, the onset of the humoral response was observed in the SC group, with animals F11 and F12 seroconverting at this time point. In contrast, SC-F14 did not present detectable IgG titers at D42 and seroconverted only after experimental challenge at D77.

Although Group SC consistently exhibited higher mean titers throughout the experimental period, statistical comparisons among groups did not reveal

significant differences at any of the analyzed time points (Kruskal–Wallis test: D42, $p = 0.364$; D63, $p = 0.143$; D77, $p = 0.143$; D98, $p = 0.059$).

Figure 3. Mean $\log_{10}$ *T. gondii*-specific IgG titers ($\pm$ SD) in cats from the group (CT), SC vaccinated group (SC), and intranasally vaccinated group (IN) during Experiment 2 (*T. gondii* ME49 strain).

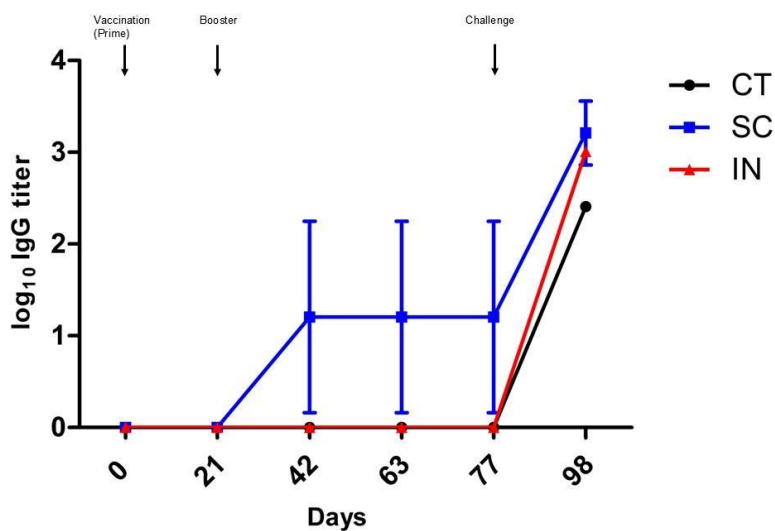


Table 2 – Individual anti-*Toxoplasma gondii* IgG titers in cats from Experiment 2 challenged with the ME49 strain at D77.

Group	ID	D0	D21	D42	D63	D77	D98
CT	F17	0	0	0	0	0	256
CT	F19	0	0	0	0	0	256
SC	F11	0	0	16	64	64	4096
SC	F12	0	0	16	64	64	1024
SC	F14	0	0	0	0	0	1024
IN	F13	0	0	0	0	0	1024
IN	F15	0	0	0	0	0	1024
IN	F18	0	0	0	0	0	1024

3.4.3 MUCOSAL IMMUNE RESPONSE

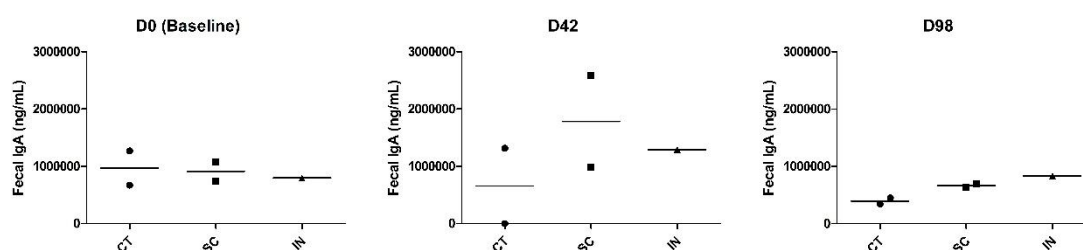
Experiment 1 (*T. gondii* VEG strain)

The distribution of fecal IgA concentrations among the experimental groups throughout Experiment 1 is shown in Figure 4. No significant differences were observed among groups at baseline (D0; Kruskal–Wallis test, $p = 1.000$), and this pattern remained unchanged at D42 ($p = 0.819$) and D98 ($p = 0.223$). These findings indicate that fecal IgA concentrations were comparable among groups throughout the experimental period.

Longitudinal analysis within each group was performed using the Friedman test. No significant temporal variation in fecal IgA concentrations was observed in either the group (CT; $p = 0.500$) or the SC vaccinated group (SC; $p = 0.500$).

Overall, the results did not reveal statistically significant differences in fecal IgA concentrations either among experimental groups or across sampling time points.

Figure 4. Distribution of fecal IgA concentrations (ng/mL) in control (CT), SC vaccinated (SC), and intranasally vaccinated (IN) cats at D0, D42, and D98 during Experiment 1 (*T. gondii* VEG strain).



Source: by the author, 2026.

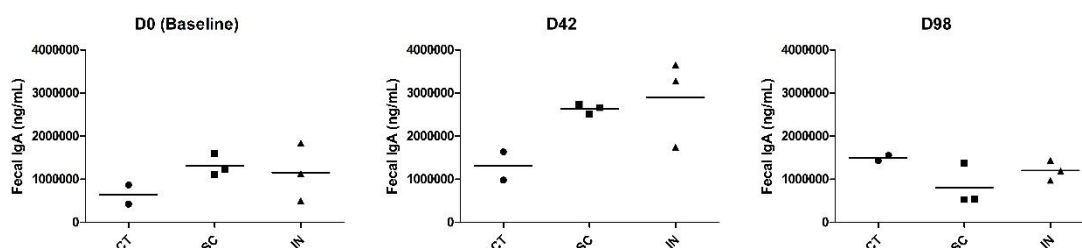
Experiment 2 (*T. gondii* ME49 strain)

The distribution of fecal IgA concentrations in Experiment 2 is presented in Figure 5. At baseline (D0), no statistically significant differences were observed among the experimental groups (Kruskal–Wallis test, $p = 0.236$), indicating comparable initial IgA levels. Similarly, no significant differences were detected at D42 ($p = 0.119$) or D98 ($p = 0.151$).

Longitudinal analysis within each group was performed using the Friedman test. No significant temporal variation in fecal IgA concentrations was observed in the group (CT; $p = 0.500$), the SC vaccinated group (SC; $p = 0.194$), or the intranasally vaccinated group (IN; $p = 0.194$).

Overall, the results did not reveal statistically significant differences in fecal IgA concentrations either among experimental groups or across sampling time points.

Figure 5. Distribution of fecal IgA concentrations (log10) in (CT), SC(SC), and intranasally vaccinated (IN) cats at D0, D42, and D98 during Experiment 2 (*T. gondii* ME49 strain).



Source: by the author, 2026.

3.4.4 CELLULAR IMMUNE RESPONSE

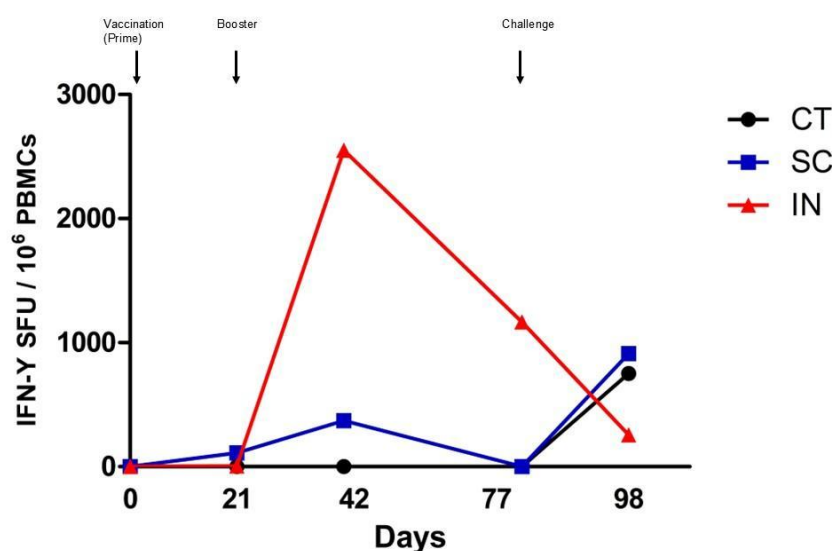
Experiment 1 (*T. gondii* VEG strain)

At baseline (D0) and on D21, all experimental groups exhibited absent or near-basal levels of cellular activation. Following immunization, the intranasally vaccinated group (IN) displayed the most pronounced cellular response, reaching a peak of 2550 SFU/ 10^6 PBMCs on D42. Thereafter, the response gradually declined, reaching 255 SFU/ 10^6 PBMCs on D98. In contrast, the SC vaccinated group (SC) exhibited a lower peak response, reaching 370 SFU/ 10^6 PBMCs on D42. At the final evaluation (D98), increased SFU values were observed in both SC and CT group, suggesting possible nonspecific activation at this time point.

To assess the overall magnitude and persistence of the cellular response throughout the experimental period, the AUC analysis was performed. Group IN showed the highest cumulative response (AUC = 106,803), corresponding to a 13.5-fold increase relative to the group (AUC = 7,875) and a 4.8-fold increase

relative to the SC vaccinated group (AUC = 22,225). Overall, according to AUC analysis, intranasal immunization induced a stronger and more sustained IFN- γ response than subcutaneous vaccination in the majority of Experiment 1 (Figure 6).

Figure 6. Longitudinal kinetics of the cellular immune response evaluated by IFN- γ ELISpot in feline PBMCs during Experiment 1 (*T. gondii* VEG strain).



Source: by the author, 2026.

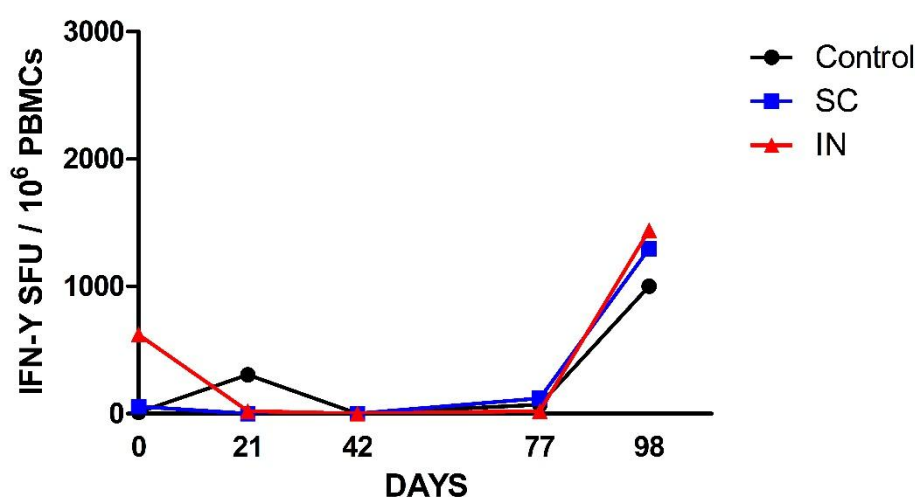
Experiment 2 (*T. gondii* ME49 strain)

Distinct baseline levels of cellular activation were observed among groups. At D0, the group (IN) exhibited the highest frequency of cytokine-secreting cells, reaching approximately 620 SFU/10⁶ PBMCs, whereas the remaining groups showed low basal responses.

During the experimental period, a transient increase in cellular activity was observed in the group CT at D21, reaching approximately 305 SFU/10⁶ PBMCs. Subsequently, all groups exhibited low or near-basal responses until D77. At D98, a marked increase in IFN- γ -producing cells was detected in all groups, reaching approximately 1000 SFU/10⁶ PBMCs in CT, 1295 SFU/10⁶ PBMCs in SC, and 1440 SFU/10⁶ PBMCs in IN.

To evaluate the overall magnitude and persistence of the cellular response throughout the experimental period, area under the curve (AUC) analysis was performed. Group IN exhibited the highest cumulative response (AUC = 22,610), followed by CT (AUC = 18,970) and SC (AUC = 17,535). Overall, the intranasally vaccinated group displayed the highest cumulative IFN- γ response during Experiment 2 (Figure 7).

Figure 7. Longitudinal kinetics of the cellular immune response evaluated by IFN- γ ELISpot in feline PBMCs during Experiment 2 (*T. gondii* ME49 strain).



Source: by the author, 2026.

3.4.5 OOCYST SHEDDING

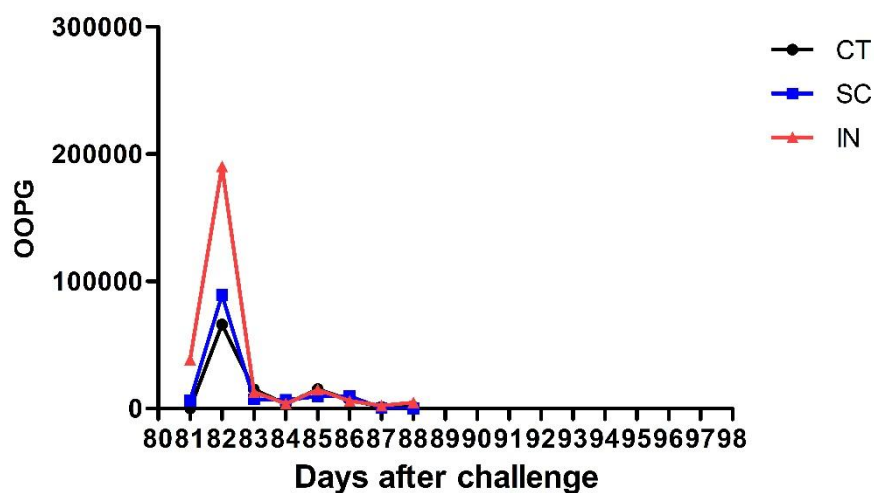
Experiment 1 (*T. gondii* VEG strain)

The kinetics of oocyst shedding following challenge in Experiment 1 is presented in Figure 8. Oocyst excretion was detected in all experimental groups after challenge, reaching maximum values of 66,000 OOPG in the CT group, 89,300 OOPG in the SC group, and 190,400 OOPG in the IN group. Following peak excretion, oocyst shedding progressively declined until complete cessation in all groups.

The duration of oocyst shedding was 7 days in CT and 8 days in both vaccinated groups (SC and IN). Although numerical differences were observed in peak oocyst excretion and shedding duration, statistical analysis revealed no significant differences among the experimental groups (Kruskal–Wallis test: $\chi^2 =$

0.679, $df = 2$, $p = 0.712$). Furthermore, pairwise comparisons did not identify significant differences between any group combinations ($p > 0.05$).

Figure 8. Kinetics of oocyst shedding, expressed as oocysts per gram of feces (OOPG), in cats from the control (CT), subcutaneously vaccinated (SC), and intranasally vaccinated (IN) groups from D77 (challenge) to D98 during Experiment 1 (*T. gondii* VEG strain).



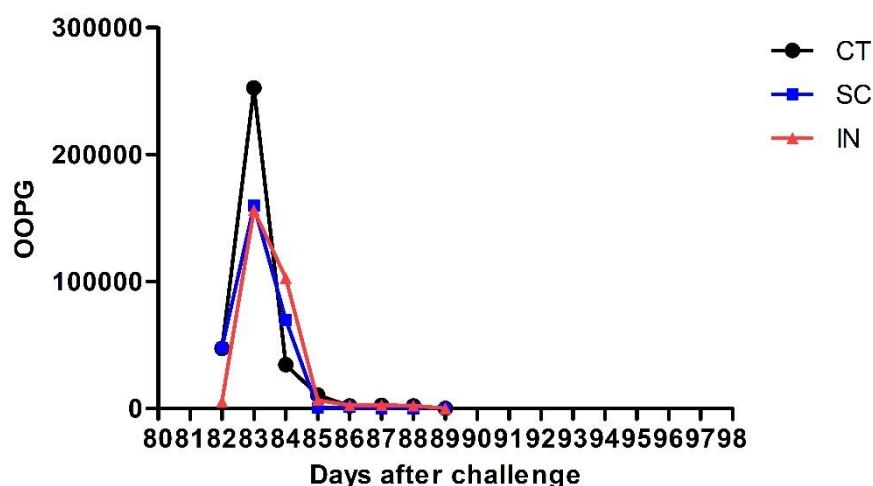
Source: the author, 2026.

Experiment 2 (*T. gondii* ME49 strain)

The kinetics of oocyst shedding following challenge challenge in Experiment 2 are presented in Figure 9. Oocyst excretion was detected in all experimental groups beginning at D82, with peak shedding occurring at D83. Maximum excretion values reached 252,500 OOPG in the CT group, 159,950 OOPG in the SC group, and 156,250 OOPG in the IN group. Thereafter, oocyst shedding progressively declined until complete cessation.

The duration of oocyst shedding was 7 days in CT, 6 days in SC, and 8 days in IN. Although numerical differences were observed in both peak oocyst excretion and shedding duration, statistical analysis revealed no significant differences among the experimental groups (Kruskal–Wallis test: $\chi^2 = 1.72$, $df = 2$, $p = 0.424$). Similarly, pair-wise comparisons did not identify significant differences between any group combinations ($p > 0.05$).

Figure 9. Kinetics of oocyst shedding expressed as oocysts per gram of feces (OOPG) in cats from the (CT), SC vaccinated (SC), and intranasally vaccinated (IN) groups during Experiment 2 (*T. gondii* ME49 strain).



Source: by the author, 2026.

3.5 DISCUSSION

The present study evaluated the immunogenicity, safety, and protective potential of a nanostructured vaccine against *T. gondii* in cats administered via subcutaneous and intranasal routes. Overall, both vaccination strategies were well tolerated and were associated with systemic, mucosal, and cellular immune responses, although some of these responses did not differ significantly from those observed in the control group. Subcutaneous immunization promoted earlier seroconversion and higher anti-*T. gondii* IgG titers during the pre-challenge period, whereas intranasal vaccination was associated with a more pronounced IFN- γ response, particularly in Experiment 1.

Given the central role of IFN- γ in host resistance against *T. gondii*, the elevated frequencies of IFN-producing cells detected following antigen stimulation suggest the induction of antigen-specific cellular immunity (Fasquelle; Vreulx; Betbeder, 2024). In contrast, fecal IgA concentrations did not differ significantly among groups throughout the study. Despite the induction of measurable immune responses, neither vaccination strategy resulted in a significant reduction in oocyst shedding following challenge with the VEG or ME49 *T. gondii* strains. The systemic humoral response differed according to the

route of administration, with a more evident effect in Experiment 1 (*T. gondii* VEG strain), in which significant differences were observed between the vaccinated groups. In Experiment 2 (*T. gondii* ME49 strain), differences among groups were less pronounced and did not reach statistical significance. Overall, these findings suggest that the route of administration may influence the humoral response, although this effect was not consistent across both experiments.

Following challenge, a marked increase in IgG titers was observed in all groups, reflecting the strong antigenic stimulation induced by the parasite. Consequently, the differences observed during the vaccination phase became less evident in the post-challenge period, indicating that challenge could induce seroconversion or boost pre-existing immunity regardless of the vaccination protocol. Similar findings were reported by Schreiber et al. (2021), who demonstrated a rapid increase and long-term persistence of anti-*T. gondii* IgG antibodies in experimentally infected cats.

The results obtained in the present study are consistent with previous findings in cats. A significant increase in serum IgG levels was reported in cats immunized SC with the recombinant GRA12 protein formulated with ISA 201 adjuvant, highlighting the ability of this route to induce a robust systemic humoral response (Yang et al., 2025). In contrast, an intranasal vaccine based on the recombinant ROP2 protein demonstrated that mucosal immunization may induce specific immune responses, although not necessarily high systemic IgG levels (Zulpo et al., 2017). Furthermore, recent reviews have emphasized that mucosal vaccination tends to drive part of the immune response toward mucosal tissues and cellular immune mechanisms, whereas parenteral immunization generally promotes stronger systemic antibody responses (Brito et al., 2023; Yang et al., 2025).

Despite the induction of a specific humoral response, no significant reduction in oocyst shedding was observed in either experiment. This finding supports previous observations indicating that serum IgG levels, although valuable markers of immunogenicity and antigen recognition, are not necessarily associated with protection against oocyst excretion in felines (Zulpo et al., 2017). Therefore, effective protection against *T. gondii* is likely dependent on the combined action of humoral, cellular, and mucosal immune mechanisms,

highlighting the importance of an integrated evaluation of these responses when assessing vaccine candidates for the definitive host (Fasquelle; Vreulx; Betbeder, 2024).

Cell-mediated immunity represents an important component of protection against *T. gondii*, and IFN- γ is widely used as a marker of Th1-type immune responses in vaccine studies (Brito et al., 2023). In the present study, intranasal immunization was associated with the strongest cellular response, particularly in Experiment 1, where Group IN exhibited the highest SFU and AUC values. Although this pattern was less pronounced in Experiment 2, the intranasal route also generated the highest cumulative cellular response. Similar studies have demonstrated that antigen-specific stimulation with *T. gondii* antigens induces IFN- γ secretion by immune cells, while nanoparticle-based vaccine formulations promote strong antigen-specific cellular responses, supporting the use of IFN- γ as a marker of vaccine-induced immunity against *T. gondii* (Ducournau et al., 2023; Fasquelle; Vreulx; Betbeder, 2024).

These findings are in agreement with previous studies on nanoparticle-based vaccines against *T. gondii*, indicating that intranasal immunization is capable of inducing cellular immune responses characterized by IFN- γ production and the activation of Th1-associated immune mechanisms (Dimier-Poisson et al., 2015; Ducournau et al., 2020; Fasquelle et al., 2023; Vargas-Montes et al., 2025). It has been reported that lipidated maltodextrin nanoparticles efficiently deliver *T. gondii* antigens to immune cells and promote antigen-specific IFN- γ responses without requiring intrinsic immuno-modulatory activity, thereby facilitating the development of Th1-associated cellular immunity (Thakur; Foged, 2020; Vargas-Montes et al., 2025).

Following the challenge, an increase in the frequency of IFN- γ -producing cells was observed in all groups, including the controls, indicating a strong immune stimulation triggered by challenge. Increased IFN- γ production has also been reported in cats immunized with the recombinant GRA12 protein (Yang et al., 2025).

Despite the cellular response observed in intranasally immunized animals, no significant reduction in oocyst shedding was detected following experimental challenge. This finding suggests that systemic IFN- γ responses alone may be

insufficient to control the sexual replication of *T. gondii* in the feline intestinal epithelium and that additional local immune mechanisms may be required. The high biological efficiency of *T. gondii* in its definitive host, in which infection with only a few bradyzoites can result in the shedding of millions of oocysts, may further contribute to the difficulty of achieving effective control of parasite transmission through vaccination (Dubey, 2001).

Secretory immunoglobulin A (IgA) is a crucial component of mucosal immunity, serving as the first line of defense at epithelial surfaces by limiting pathogen adherence and invasion. Similarly, previous studies using nanoparticle-based mucosal vaccines against *T. gondii* reported that mucosal antibody responses were modest when compared with the magnitude of the cellular immune response, suggesting that nanoparticle formulations may preferentially promote Th1-associated immunity rather than robust IgA production (Ducournau et al., 2023).

In contrast, Brito et al. (2026) demonstrated that intranasal immunization with *T. gondii* membrane proteins combined with CpG induced intestinal IgA, serum IgG, and IFN- γ -producing CD4⁺ and CD8⁺ memory T cells in mice, together with a partial reduction in parasite burden. These differences suggest that antigen composition and adjuvant selection may influence the magnitude of the mucosal response.

In the present study, fecal IgA concentrations did not differ significantly among experimental groups, and these responses were not accompanied by a significant reduction in oocyst shedding. Similar findings have been reported in previous studies evaluating mucosal vaccination strategies against *T. gondii* in cats. Intranasal immunization with rhoptry proteins induced only modest intestinal IgA responses despite partial protection against oocyst shedding (Zulpo et al., 2012). Furthermore, reductions in oocyst excretion were not directly associated with fecal IgA levels, suggesting that IgA alone may not adequately reflect the protective mechanisms operating in the feline intestinal mucosa (Zulpo et al., 2017).

The absence of significant differences in fecal IgA levels observed in the present study may also be related to the high biological variability of this marker. Recent studies have shown that fecal IgA concentrations in cats can be

influenced by individual factors, stress, and intestinal homeostasis, contributing to substantial inter-animal variation (Serres-Corral et al., 2025; Yang et al., 2025).

Taken together, these results suggest that fecal IgA was not a sensitive marker of vaccine-induced immunity under the conditions evaluated. Furthermore, the lack of association between fecal IgA levels and oocyst shedding reinforces the hypothesis that protection against the sexual stages of *T. gondii* in the feline intestine depends on the interaction of multiple local immune mechanisms rather than on mucosal antibody responses alone.

Oocyst shedding is the most relevant epidemiological parameter for evaluating vaccine efficacy against *T. gondii* in felids, as cats are the definitive hosts responsible for environmental dissemination of the parasite. In the present study, humoral and cellular immune responses were detected in vaccinated animals; however, these responses were not accompanied by a significant reduction in oocyst shedding, as no significant differences in OOPG values were observed among the experimental groups. In the present study, the immune responses observed in vaccinated animals were not accompanied by a significant reduction in oocyst shedding. In contrast, previous studies using different vaccination strategies have reported substantial reductions in oocyst excretion. Intranasal immunization with rhoptry proteins resulted in reductions of approximately 89% and 86.7% (Garcia et al., 2007; Zulpo et al., 2017), while immunization with recombinant rGRA12 significantly reduced both the duration and total number of oocysts shed (Yang et al., 2025). More recently, Xie et al. (2026) reported a 94.5% reduction in total oocyst shedding following vaccination with the live-attenuated PruΔpp2a-c mutant, although five of six vaccinated animals still shed oocysts. These contrasting outcomes may reflect differences in vaccine composition, antigenic repertoire, and the magnitude or type of immune response elicited, highlighting the need to better define the immune mechanisms associated with effective control of the sexual stages of *T. gondii* in cats.

Some limitations of the present study should be considered when interpreting the results. The relatively small number of animals per group and the pooling of PBMC samples for ELISpot analysis limited the assessment of individual variability and precluded inferential statistical analyses of cellular immune responses. Nevertheless, the study provides valuable information

regarding the immunogenicity and biological performance of a nanoparticle-based vaccine platform evaluated directly in the definitive host of *T. gondii*.

The absence of protection against oocyst shedding highlights the biological challenge of inducing effective intestinal immunity in the definitive host. Future studies should investigate whether modifications to the NPL platform, including alternative adjuvant formulations, additional booster immunizations, or the incorporation of stage-specific recombinant antigens, could improve local immune responses in the feline intestine and enhance protection against parasite transmission. Such approaches may contribute to the development of vaccination strategies capable of targeting the intestinal stages of the parasite more effectively and ultimately reduce environmental contamination.

3.6 CONCLUSIONS

The nanoparticle-based vaccine formulations evaluated in this study were safe and capable of inducing detectable immune responses in domestic cats. Subcutaneous immunization promoted a stronger systemic humoral response, characterized by higher IgG titers, whereas intranasal administration elicited a more pronounced cellular response, as evidenced by increased IFN- γ production.

The absence of significant reductions in oocyst shedding, despite measurable immune activation, underscores the complexity of interrupting parasite transmission in felids, the definitive host of *T. gondii*, in which the parasite exhibits highly efficient intestinal replication and oocyst production. Overall, the results demonstrate the immunogenic potential and safety of the nanoparticle-based vaccine formulation evaluated in this study. These findings highlight the need for further investigations focused on optimizing antigen composition, adjuvant selection, and immunization strategies to enhance protective efficacy and achieve more effective control of oocyst shedding in cats.

3.7 PATENTS

The vaccine formulation evaluated in this study is under the intellectual property of Vaxinano.

4 FINAL CONSIDERATIONS

This dissertation evaluated the immunogenicity and efficacy of an experimental vaccine composed of inactivated *Toxoplasma gondii* tachyzoites associated with polymeric lipid nanoparticles, administered to domestic cats by the subcutaneous and intranasal routes. Considering the role of felids in oocyst shedding and in maintaining the epidemiological cycle of the parasite, the development of vaccination strategies capable of reducing environmental contamination represents an important approach to toxoplasmosis control from a One Health perspective.

The results demonstrated that the vaccine formulation induced distinct immune responses depending on the route of administration. Subcutaneous immunization promoted an earlier humoral response and higher IgG titers before the challenge, whereas intranasal administration induced a stronger cellular response in one of the experiments, as evidenced by increased IFN- γ production. However, neither vaccination strategy significantly reduced oocyst shedding after challenge with the *T. gondii* ME49 strains and VEG strains.

Although the observed protective efficacy was limited, the findings provide important information on the immune response of felids to a vaccine platform based on polymeric lipid nanoparticles. The results reinforce the complexity of inducing effective intestinal immunity in cats and indicate that the activation of systemic immune responses alone may not be sufficient to interfere with the enteric phase of the *T. gondii* life cycle.

The main limitations of the study included the small number of animals per group, resulting from ethical and operational constraints inherent to experimental studies involving felids, as well as individual biological variability and the differences observed between the two experiments. Nevertheless, the use of two parasite strains allowed a broader evaluation of the vaccine-induced response under different challenge conditions.

Finally, this study contributes to advancing knowledge of vaccination strategies against *T. gondii* in domestic cats and provides a basis for the development of new vaccine formulations. Future studies should prioritize the optimization of mucosal immunity, including the evaluation of novel antigens,

adjuvants, and delivery systems, as well as different immunization regimens capable of enhancing intestinal protection and consistently reducing oocyst shedding, thereby contributing to toxoplasmosis control under a One Health approach.

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