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INTRODUCTION

Toxoplasmosis is a zoonotic parasitic disease caused by the protozoan *Toxoplasma gondii*. Up to a third of the global human population is estimated to carry a *T. gondii* infection, which can result in severe complications in immunocompromised individuals and pregnant women [1 , 2 , 3]. Transmission to humans occurs mainly (i) through ingestion of undercooked meat containing tissue cysts with bradyzoites, (ii) ingestion of vegetables, fruit and water contaminated by sporulated oocysts from cat stool or (iii) by congenital transmission from mother to fœtus by tachyzoites [4-7]. As the definitive host (DH) of *T. gondii*, the Felidae family (and mostly cats) play a crucial role in spreading the parasite in the environment through the release of oocysts in their faeces, which remain infectious, once sporulated, for an extended period of time [8].

In 1999, a 3-year vaccination trial of stray cats living around a swine farm, using a mutant strain of *T. gondii* (T263), showed a clear correlation between the reduction of oocysts in the environment and the decrease of *T. gondii* seroprevalence in finishing pigs [9]. This experiment showed for the first time the potential to target cats for vaccination against *T. gondii* to reduce meat contamination and indirectly protect humans. Another live attenuated vaccine, based on tachyzoites of the mutant S48 strain, is commercially available in Europe and New-Zealand (OVILIS® ToxoVax by MSD Animal Health) but only used in sheep, to prevent *T. gondii* induced abortion [2] and economic losses. Other types of vaccines have been tested in cats (eg irradiated or genetically modified strains, antigenic vaccines) [10, 11] but some of these trials were challenging to apply due to environmental and/or practical constraints, or did not prevent oocyst excretions by cats. Nevertheless, targeting animals to protect human health should still be considered as shown by the

** Abbreviations : DH : definitive host, IH : intermediate host*

successful achievement of rabies eradication in most European countries by oral vaccination of wild carnivores [12 , 13]. As such, a vaccine targeting cats to prevent environmental contamination by reducing *T. gondii* oocyst excretion, is of strong interest to limit human and livestock contamination [14].

Another live attenuated vaccine strain of *T. gondii,* called Mic1-3KO, deleted for *MIC1* and *MIC3* genes (encoding for two microneme proteins Mic1 and Mic3, respectively) was developed in 2005 [15]. This double gene deletion reduces the ability of the parasite to penetrate host cells and prevents any reversion to virulence. This is an important difference with the S48 strain which is attenuated after multiple culture passages [2, 16] and could potentially revert to a virulent strain. The Mic1-3KO strain has been previously successfully tested as a preventive vaccine in mice [17] and sheep [18]. Due to its efficiency as a vaccine candidate in these animal species, we tested it as a subcutaneous vaccine for cats. The aims of this 1st trial were to assess the immunogenicity of the Mic1-3KO vaccine strain in cats, and analyse protection of the animals against challenge with a wild type *T. gondii* strain. In addition, we assessed Th1 and Th2 serum cytokines following vaccination and challenge, to further investigate the feline immune response induced by the Mic1-3KO vaccine-candidate. Indeed, most of the mechanisms for the cell invasion capacity of the parasite, as well as host immune responses, have been elucidated using *in vitro* and murine *in vivo* models, intermediate hosts (IH) of the parasite [19 , 20 , 21], but very few have focused on the DH immune response [22, 23]. Therefore, vaccination studies are a useful opportunity to assess *in vivo* potential correlates of immune protection to *T. gondii* in the feline host.

As mass vaccination strategies for feral and stray cats require an orally administrable vaccine, we also developed an oral formulation to allow mucosal vaccination of cats with the Mic1-3KO strain and followed the same host parameters.

MATERIALS AND METHODS

Animals & ethic statement

Specific pathogen free domestic short-haired female cats, aged 4 months at the start of each trial, were obtained from Isoquimen (Barcelona, Spain). They were housed according to European laws and guidelines (Directive 2010/63/EU) on animal welfare (agreements 21/01/13-2 and #9859-2017050516182349v4).

Toxoplasma gondii strains

The vaccine-candidate strain Mic1-3KO, is a live attenuated strain of *T. gondii* (patent WO2005/072754, [15]) from genotype I. The challenge was performed using tissue-cysts of the wild-type 76K strain of *T. gondii* (genotype II). Tachyzoites of the Mic1-3KO strain and of the same strain expressing the Green Fluorescent Protein (Mic1-3KO-GFP) were cultured in HFF (Human Foreskin Fibroblast) cells, at 37°C and 5% CO_2. All *T. gondii* strains used in this study were provided by Vitamfero (France). HFF Cells were maintained in complete culture medium composed of DMEM, 4mM Glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin (all from Lonza, Belgium) supplemented with 10% of fetal calf serum (Gibco, Thermo Fisher Scientific, France). The culture medium was replaced 24h before harvesting of the parasites prior to administration to the animals. Parasite counting was done using Kova-slides (Kova-International, USA) under the microscope [24, 25] and the concentration of Mic1-3KO tachyzoites was calculated in order to administer the desired quantity by subcutaneous injection or orally.

In vitro tests of different gastro-resistant formulas

For oral administration of the Mic1-3KO strain, preliminary *in vitro* experimental testing of different oral formulations were performed. Briefly, 10^7 tachyzoites/ml of the Mic1-3KO-GFP were included in either water, DMEM as a positive control, or different formula (F1 to F4, suppl. material & methods, table S1) then diluted 1/5 in a pH4 solution (VWR, France) and incubated at 38,5°C for 4h. After incubation, the parasites were fixed with 1% Paraformaldehyde (Euromedex, France) for 30min at room temperature. After two washes with 1X PBS (Gibco, France), the percentage of viable tachyzoites was assessed by flow cytometry on a FACS Canto2 (BD Biosciences, USA) using the GFP Mean Fluorescence intensities of the parasites identified on morphologic parameters. In parallel, after 4h of incubation, 100µl of the tachyzoites-containing formula were added to confluent HFF cells in a 12-wells plate and culture at 37°C, 5% CO_2. Presence of intracellular parasites was checked daily using an inverted microscope. The formula that showed the highest survival and virulence of the tachyzoites, was then chosen for the oral vaccine.

Vaccination protocols

For the subcutaneous (s.c.) vaccination protocol, a first group of 4 cats, called “2 injections”, received a first injection s.c. of 200µl serum-free DMEM containing 10^5 tachyzoites of the Mic1-3KO strain at day 0 (D0), and an identical boost injection at day 35 (D35). The group called “1 injection” (n=4 cats), received the same dose of the vaccine-candidate only at D35. S.c. injections were done in the back of the neck, using a 1mL syringe and a 25G x 5/8 needle (Beckton Dickinson, France). Both groups and one control cat (called “No vaccine”) were challenged orally with 1200 murine-brain tissue cysts containing bradyzoites of the 76K

97 strain at day 97 (D97) and animals were followed up to 131 days from the 1st vaccine
98 injection.
99 For the oral vaccination protocol, 10^5 of the Mic1-3KO tachyzoites were mixed in 400µl of F1
100 and given orally to the "Vaccinated" batch. This group of cats received a second oral
101 administration of 10^8 tachyzoites of Mic1-3KO strain in 400µl of F1 at D60. At D122, the
102 "Vaccinated" and "Non vaccinated" batches (n=4 cats/group) were challenged orally with
103 1200 bradyzoites-containing cysts of the 76K strain and animals were followed up to 161
104 days after the 1st vaccine administration.
105 Blood samples were collected weekly throughout both protocols, after intramuscular
106 tranquilization of the animals (15mg/kg ketamine+1mg/kg diazepam). After 20min of clotting
107 at room temperature, tubes were centrifuged for 10min at 2000rpm. Serum was collected
108 and frozen at -80°C until further analyses.
109
110 **Anti-*T. gondii* IgM and IgG measurement by indirect ELISA**
111 Serum anti-*T. gondii* antibodies were measured by an in-house indirect ELISA. Briefly, wells
112 were coated either with 10µg/ml of *T. gondii* antigens (76K strain , Vitamfero, France), or
113 with 1X PBS only. After three washes with 1X PBS+0.05% Montanox20 (SEPPIC, France),
114 plates were blocked with 1X PBS +1% BSA (Sigma, USA), washed again and plated with sera
115 diluted at 1/200 in 1X PBS. After overnight incubation at 4°C, plates were washed and either
116 anti cat-IgM or IgG coupled to horseradish peroxydase (Bio-Rad antibodies, France) were
117 added, at the recommended dilution in 1X PBS. After 1h at 37°C, and three washes with 1X
118 PBS+0.05% Montanox20, signal was revealed using TMB solution (Bio-Rad, France). Reaction
119 was stopped with H_3PO_4 1M solution (Merck, Germany). Plates were read at 450nm with the
120 MultiskanFC (Thermo Scientific, USA) and analysed with SkanIt Research Edition 4.1

software. OD values from PBS background binding were subtracted from the signal obtained with *T. gondii* specific antibodies.

Serum cytokine measurement by ELISA

Serum cytokines were measured with commercial sandwich ELISA kits specific for feline IL-2, IFNγ, IL-4, and IL-5 (Bio-Techne, USA), following manufacturer's instructions. Briefly, plates were coated overnight with capture antibody at room temperature, then washed three times with wash buffer (1X PBS +0.05% Montanox20). After blocking with 1X PBS+1% BSA, plates were washed again, sera diluted 1/50 in 1X PBS and standards were added in wells according to manufacturer's instructions. After 2h, incubation plates were washed and coated with specific biotinylated detection antibody, for 2h at room temperature. After three washes, streptavidin coupled to horseradish peroxidase, diluted in 1X PBS as recommended, was added to each well for 20min at room temperature. Plates were then washed, and reaction was revealed using TMB solution. Reaction was stopped with H_3PO_4 1M solution and plates were read at 450nm and 540nm for background noise, according to manufacturer instructions, on the MultiskanFC reader and analysed with SkanIt Research Edition 4.1 software.

Oocyst identification from cat faeces

At D-7 before vaccination, and daily from D0 of both vaccination trials, cat fecal samples were collected in plastic collection cups with screw lids, according to the manual of OIE for fecal samples collection (OIE Terrestrial Manual 2013). Each sample was stored at 4°C for a maximum of 3 days after collection, for oocysts purification and analysis. Sucrose flotation technique was used to purify oocysts as described previously [24 , 25]. 10µl of each purified

sample were examined under Kova (Kova International, Netherlands). Light microscope (DMI 4000B, Leica, Germany) with ×200 magnification and blue ultra-fluorescent (UV) light was used for oocysts detection taking advantage of autofluorescence of *T. gondii* oocysts [26].

Multilocus nested PCR-RFLP genotyping of oocysts

Purified *T. gondii* oocysts were previously diluted to a concentration of 10^5 oocysts/mL and subjected to three freeze-thawing cycles at -80°C/RT, prior to DNA extraction. During the last two thaws, the tubes were sonicated to destroy the resistant wall of the oocysts. The DNA was then extracted using the Mini Kit QIAamp DNA (Qiagen, USA), following the tissue protocol. Strain typing was performed using genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico as described previously [27, 28]. PCR products were digested with specific restriction enzymes and patterns were revealed on a 2.5 agarose gel, using GelDoc XR and analysed with Quantity One 1-D software (Bio-Rad, France).

RESULTS

Antibody production and animal protection after subcutaneous vaccination

The first vaccination trial with Mic1-3KO was performed by subcutaneous injection. Seven days after the 1st injection of the vaccine, both groups of cats displayed an increase in specific anti-*T. gondii* IgM (Figure 1A) followed by a high production of serum anti-*T. gondii* IgG, reaching a plateau 35 days post injection (Figure 1B). The 2nd injection of the vaccine strain in the “2 injections” group did not show any booster effect in IgG production after the 2nd injection. In the control cat, the administration of the challenge strain also induced *T. gondii* specific IgM production firstly, followed by specific IgG, reflecting the infectivity of the

murine brain-tissue cysts of the 76K strain. However, despite high IgG serum levels before D97, the challenge induced the production of new specific IgM in vaccinated cats, but had a minimal impact on IgG serum levels which remained elevated. Interestingly, the levels of IgM produced after the challenge in the control cat were much higher than in the vaccinated cats, and comparable to levels produced at the time of first vaccination of the "2 injections" batch.

We then assessed if the Mic1-3KO strain could protect cats from natural infection and oocyst production by daily analysis of collected cat faeces. No oocysts were found in the faeces before challenge with the wild-type strain (data not shown). However, after administration of the tissue cysts of the challenge strain, all cats shed oocysts with the earliest excretion seen on day four post infection (Figure 1C). The period of oocyst shedding was from day 4 to day 13 as expected from litterature (suppl. figure S2, A).

Figure 1D shows the genotyping by multilocus nested PCR-RFLP, of the oocysts shed by each cat included in the trial, compared to reference strains (suppl. material & methods, table S2), on three of the tested loci : C29-2, SAG1 and BTUB. All cats shed oocysts of genotype II strain exclusively, corresponding to the genotype of the challenge strain, as confirmed by the "No vaccine" cat which could only shed genotype II oocysts.

Design of a gastro-resistant vaccination formula for oral vaccination

For oral administration of the Mic1-3KO vaccine-candidate, we had to develop different gastro-resistant formulas to administer live tachyzoites to the feline intestines. Indeed, this strain is only available as tachyzoites, which are highly sensitive to acidic pH of the stomach [8]. For this, we took advantage of the Mic1-3KO-GFP, which allowed monitoring of the parasites by flow cytometry for *in vitro* assays prior to cat administration of the Mic1-3KO

strain. Mic1-3KO-GFP parasites were formulated in different gastro-resistant solutions and incubated in conditions mimicking intestinal passage in felines. Viability of the parasites was assessed by counting the percentage of GFP+ tachyzoites after such treatments (Figure 2A and 2B) using flow cytometry. Whereas about 80% of the parasite did not survive in F4, nor in distilled water, 3 out of 4 formulas used (F1 to F3) were able to keep 75% of the parasites alive after treatment. To confirm viability and infectivity of the remaining GFP+ parasites, they were also plated onto monolayers of HFF cells after treatment, and the presence of tachyzoites within cells was monitored daily post-infection (Figure 2C). The F1 formula was selected for the oral vaccination trial as it allowed the best parasite viability and infectivity.

Antibody production and animal protection after oral vaccination

Once the formula selected, cats were orally vaccinated with same amount of tachyzoites as for the subcutaneous vaccination trial at D0, and then with a higher quantity of tachyzoites at D60 in the same formula, to boost antibody production. Murine-brain tissue cysts of the 76K strain was orally given to all cats, 60 days after the last vaccination. IgM levels showed a high background before vaccination in both batches, however anti-*T. gondii* IgM levels in serum of vaccinated cats increased after the 2nd oral administration of the vaccine strain (Figure 3A), quickly followed by an increase in anti-*T. gondii* specific IgG (Figure 3B). The peak of specific IgG production was reached 28 days after the 2nd administration showing similar kinetics of IgG production following the subcutaneous injection, and decreased slightly after 28 days. Like in the s.c. vaccination trial, the challenge strain given orally induced a boost in *T. gondii* specific IgM and IgG production in the vaccinated group. The non-vaccinated group induced anti-*T. gondii* IgM and IgG after the inoculation with the challenge strain (Figure 3A and 3B).

Daily analysis of the cat faeces after oral vaccination with Mic1-3KO showed no oocysts found before challenge with the wild-type strain (data not shown), as in the s.c. vaccination trial. However, after administration of the murine brain-tissue cysts of the challenge strain, all cats started to shed oocysts from four days post challenge up to thirteen days, similar to the s.c. vaccine experiment (Figure 3C). We also genotyped the oocysts shed by multi-locus nested PCR-RFLP. As in Figure 1, three of the tested loci are presented in Figure 3D. The genotype II was determined for oocysts shed upon challenge in the oral vaccination trial (Figure 3D, samples 1 to 4, corresponding to oocysts found in faeces of two cats in each sample), as compared to reference strains.

As *T. gondii* is a parasite mainly transmitted through ingestion, we aimed to see if *T. gondii* specific IgA were produced and detectable in serum. Slight increases of serum IgA were measured sporadically after subcutaneous injection of the vaccine strain (suppl. figure S1, A) in both vaccinated groups and also after challenge. However, we did not detect any IgA production in the serum of the cat who only received the challenge strain. Similarly, no induction of IgA production was seen after oral administration of Mic1-3KO tachyzoites (suppl. figure S1, B), despite an apparent two fold increase in anti-*T. gondii* IgA production upon challenge in the vaccinated group as compared to the non-vaccinated group. The limited number of animals included in the trial and high individual variation regarding the IgA measurements limit our ability to make conclusions about this increase.

Cytokine production induced by Mic1-3KO vaccination

To define a possible Th1 or Th2 signature upon vaccination with Mic1-3KO, cytokine production was also studied throughout both vaccination trials (Figure 4), in the vaccinated

241 groups only. Since there was no boost effect seen in antibody production after a 2nd
242 subcutaneous injection of the vaccine, results shown in Figure 4A and 4B are focused on the
243 "1 injection" group. IL-2 and IFNγ levels in cat serum were relatively low and the
244 concentration of both cytokines remained stable throughout the subcutaneous vaccination
245 trial, under 20ng/ml (Figure 4A). As opposed to these Th1 cytokines, IL-4 levels were high in
246 serum of cats with an average range of 77,5ng/ml ± 3,6 and IL-5 concentrations were much
247 lower (23,7 ng/ml ± 0,8). However no changes were seen upon vaccination or challenge in
248 these Th2 cytokines (Figure 4B). Throughout the oral vaccination, as for the s.c. trial, no
249 significant changes in Th1 or Th2 serum cytokines were observed (Figure 4C and 4D). Taken
250 together, these results show no variation in cytokines classically associated with Th1 or Th2
251 immune signatures, after vaccination or challenge.
252
253 **DISCUSSION**
254 The aim of this study was to assess the immunogenicity of a new vaccine candidate against
255 *T. gondii* in cats and evaluate the protection of the vaccinated animals against the wild-type
256 parasite. This new vaccine candidate strain differs from the ones tested previously [29] as it
257 is a double-knock out for two genes *MIC1* and *MIC3*, therefore greatly limiting the possibility
258 to revert to any potential virulence [15]. The Mic1-3KO strain has been successfully used as a
259 prophylactic vaccine in mice and in sheep [17, 18]. In both murine and ovine vaccination
260 trials, the Mic1-3KO strain was administrated subcutaneously. Hence, we set up a first
261 vaccination assay in cats, with the same administration route. Specific *T. gondii* IgG could be
262 measured as soon as 10 days post-injection, reaching a plateau one month later. A second
263 injection of the vaccine did not improve this IgG production, indicating that the vaccine
264 strain is immunogenic from the 1st injection, which supports a vaccination protocol.

However, despite induction of high levels of anti-*T. gondii* IgG by the vaccine candidate Mic1-3KO, subcutaneous vaccination did not prevent sexual replication of the challenge strain in the cats' intestines and oocyst excretion.

Recent work on vaccination against infectious diseases such as SIV/HIV or against cancer, suggested that immunization route influences the efficiency of the vaccine [30-32], i.e. to protect mucosa from a foodborne pathogen, the vaccine would be more efficient if given by the mucosal route. These observations, as well as the results of s.c. vaccination experiment, led us to conclude that the subcutaneous administration of the Mic1-3KO strain to cats did not result in any local protection at the mucosa level, despite inducing specific IgG production. Moreover, an oral vaccine would be more suitable for mass vaccination of feral and stray cats, living around farms, so we performed a second vaccination trial, giving the Mic1-3KO orally to cats. However, the Mic1-3KO vaccine strain is only available as a tachyzoite stage, which are very sensitive to acidic pH. Therefore, to administer the Mic1-3KO vaccine-candidate orally, we tested different gastro-resistant formulas *in vitro*, to select the formula allowing the optimal viability of infectious tachyzoites. All formulas tested contained guar gum, which is commonly tested for colon-targeted drug delivery systems [33], allowing the drug to resist intestinal passage and maintain its efficiency for a local effect [34]. Presence of carboxymethylcellulose (CMC) and sodium bicarbonate were also required to help GFP-Mic1-3KO tachyzoites to survive the acidic pH *in vitro* treatment. The main useful characteristic of CMC for our study is that it is insoluble in low pH media but dissolves at a higher, near neutral pH of the distal gut [35], thus protecting tachyzoites through the feline stomach and duodenum. Sodium bicarbonate served as a buffering agent, to help tachyzoites resist an acidic pH, and chicken aroma gave some palatability for the cats. After the 1st oral administration of the Mic1-3KO strain, no IgG production was

observed in any of vaccinated animals. This observation raised the question of whether sufficient tachyzoites survived in the stomach of cats, as opposed to our *in vitro* studies, and thus were not seen by the immune system. Hence, a second administration of the vaccine strain was performed at day 60 with a higher quantity of tachyzoites. The 2nd oral administration induced a boost and allowed the production of a high IgG titer which increased upon challenge in the vaccinated group. Taken together, these results show that the Mic1-3KO is highly immunogenic for cats, whether given subcutaneously or orally. We were also able to develop an oral formula preserving a majority of viable vaccine-candidates and inducing antibodies against *T. gondii* antigens. This confirms that their mucosal immune system responded to the vaccine-candidate antigens. Whether the live Mic1-3KO tachyzoites entered the mucosa at the pharygeal-buccal level or at the duodenum level, as suggested by Dubey [36, 37], was not possible to investigate in our oral vaccination trial.

As *T. gondii* is an intestinal parasite, we aimed to look at mucosal protection by monitoring IgA after vaccination and challenge in both trials. Faeces IgA from cats are possible to measure [38], however, in our hands, the heterogeneity of the diluted samples and the high background obtained with our in-house ELISA with these samples, did not allow us to measure locally produced anti-*T. gondii* IgA (data not shown). We thus looked at serum anti-*T. gondii* IgA. Subcutaneous vaccination did not induce a high level of serum IgA but challenge with the wild-type *T. gondii* strain slightly increased the concentration of specific IgA. Since this increased IgA production is more evident in the animals which received only one injection of the vaccine, it confirms the needlessness of the recall injection. In contrast, the oral vaccination did not induce serum specific *T. gondii* IgA at detectable levels, however interestingly, when challenged with the wild-type *T. gondii*, the vaccinated cats produced anti-*T. gondii* IgA in a higher titer than the positive control animals, reflecting a possible

313 improved protection at the mucosal level, as compared to the vaccine strain when given
314 orally.
315 Despite high titers of specific anti-*T. gondii* IgG induced by the Mic1-3KO vaccine strain, upon
316 challenge with a virulent wild-type *T. gondii*, surprisingly all cat shed oocysts, regardless of
317 the vaccination route of the Mic1-3KO. One possible explanation is the different genotypes
318 between the two strains. Indeed, Mic1-3KO has been obtained from a genotype I strain,
319 whereas the challenge was done using the 76K strain, which is of genotype II. These two
320 genotypes display different antigens [39], even if it has been shown that the Mic1-3KO strain
321 behaves like a genotype II strain in the mouse model [40], and is protective in vaccinated
322 mice and sheep challenged with a genotype II strain [17, 18], that is mostly found in Europe
323 [41, 42]. Therefore, it is possible that some antigens of the genotype II wild type strain were
324 new to the vaccinated animals and helped the parasite to evade anti-*T. gondii* IgG induced
325 by the Mic1-3KO strain. The follow-up of IgM production in both trials could support this
326 hypothesis. Indeed, IgM titers in the serum increased and then subsided as expected. In the
327 oral vaccination trial, cats showed some anti-*T. gondii* IgM prior to vaccination or challenge,
328 which we did not see in animals of the s.c. vaccination trial. These cross-reactive IgMs are
329 quite common in humans and justify why diagnostic serologies need to be done on both IgM
330 and IgG to confirm an acute infection [43, 44]. However, after the challenge, we observed an
331 increase of IgM production in both trials. The second increase could be the sign of a primary
332 immune response against wild-type *T. gondii* antigens, specific to genotype II, which are not
333 present on the Mic1-3KO cell membrane. This hypothesis is supported by the recent
334 publication of Ramakrishnan *et al.*, where they vaccinated cats with a live attenuated
335 HAP2KO genotype II strain, obtained with CRISPR-Cas9 technology. Cats vaccinated with
336 either oocysts or tissue cysts from HAP2KO parasites were protected against challenge with

337 a wild type strain of *T. gondii* of the same genotype [11]. Another possibility for the different
338 outcomes in our experiments is that animals were vaccinated with tachyzoites, for which
339 cats are the least susceptible species. Indeed, several thousands of tachyzoites are required
340 to infect cats whereas only one bradyzoite or one oocyst are enough [8 , 45]. The parasite
341 stage can also influence the cat's seroconversion [46]. Therefore, cats in our vaccination
342 trials might have responded less intensely to the Mic1-3KO strain, in spite of high IgG
343 production.
344 To study the safety of the vaccine strain and the risk of infectivity, faeces were collected and
345 analyzed on a daily basis, throughout the length of both trials. None of the vaccinated cats
346 shed oocysts upon vaccination only. Genotyping of the oocysts shed after challenge in both
347 the subcutaneous and oral trials, showed that the oocysts were of genotype II, the same as
348 the oocysts excreted by cats which were only challenged (with genotype II 76K strain). These
349 genotyping analyses demonstrate that the Mic1-3KO is safe, cannot form oocysts and does
350 not recombine with the challenge strain in the lumen of the cat intestine. This could be due
351 to the fact that the vaccine was given two months before the challenge with the wild-type
352 strain of *T. gondii*, which prevented any genetic cross in the intestines of the cats, as for such
353 a cross to occur, a cat needs to be simultaneously infected with the different genotypes [39 ,
354 47].
355 The host immune response to *T. gondii* infection has been mainly studied in the IH murine
356 model which develops a Th1-type response with production of IL-12 by inflammatory
357 monocytes and subsequent IFNγ production by NK cells and T cells. For cats, which can be
358 both DH and IH, the immune response against *T. gondii* infection has been poorly studied.
359 Therefore, we measured IFNγ and IL-2 in for Th1 responses, and IL-4 and IL-5 for Th2
360 responses in both vaccination studies. We did not observe any significant changes in the

concentrations of Th1 and Th2 cytokines in cat serum, either after vaccination or challenge. As effector functions of cytokines are mostly local, it would have been interesting to isolate mesenteric lymph nodes and perform *ex vivo* stimulation and cell proliferation assays to compare vaccinated and challenged immune responses, but this was not ethically possible to do it in these vaccination trials.

CONCLUSION

The proven efficacy of the Mic1-3KO strain in mice and sheep, supported its use as a promising vaccine candidate to block the parasite life cycle in its definitive host, in order to prevent environmental and livestock contaminations. The vaccination trials we performed showed good safety of this vaccine candidate strain in cats with no side-effects observed and a good tolerance. Moreover, the Mic1-3KO strain did not sexually reproduce in the cats, suggesting that it is not able to disseminate through cat faeces. Interestingly, this vaccine candidate is highly immunogenic in cats, regardless of the vaccination route, as in other species tested. However, antibodies produced upon vaccination were not able to stop a wild-type strain of *T. gondii* to sexually reproduce in the cat intestine and to shed oocysts, illustrating the unique host-pathogen interactions specific to this host. Future research addressing the relationship between the cat intestinal mucosa and *T. gondii* are now required to identify new targets and improve this vaccination strategy such as adding the delta-6-desaturase enzyme, as suggested by a very recent publication on feline intestinal organoids [48]. Blocking the *T. gondii* life cycle in its definitive host to protect animal and human health would be completely in line with the OneHealth approach [49].

AUTHORSHIP

D.L.R. designed and performed the experiments and analysed the datas. V.Dj performed the experiments and analysed the datas. S. M. provided vaccination and challenge strains of *T. gondii*. V.Do. and S.P. provided technical and scientific help in designing the oral formulation. A.C.L and C.S performed and analysed PCR-RFLP genotyping experiments. C. C., D.V., Y.V., A.G.C. and F.B. provided technical support. E.S. and R.B. partipated to the study design. D.L.R. wrote the paper with the help of V.Dj. and comments from I.V. and R.B. All authors have read and approved the manuscript and attest they meet the ICMJE criteria for authorship.

CONFLICT-OF-INTEREST DISCLOSURE :

Animals included in the subcutaneous vaccination trial were bought by Vitamfero, of which S.M. and E.S are former employees. E.S. is now an independent consultant and S.M. is currently unemployed. The authors confirm that there are no financial or personal interest, or belief that could affect their objectivity in reporting on the results obtained in this study.

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525

526 **FIGURE LEGENDS**

527 **Figure 1 : Anti-*T. gondii* antibody production and protection of animals after subcutaneous**
528 **vaccination**

529 (A) IgM production after s.c. injection of Mic1-3KO vaccine strain (D0 and D35, blue arrows)
530 and oral challenge with type II *T. gondii* strain (D95, red arrow). (B) IgG production after s.c.
531 injection of Mic1-3KO vaccine strain (D0 and D35, blue arrows) and oral challenge with type
532 II *T. gondii* strain (D95, red arrow). *n = 4 cats/batch (except "no vaccine" control n=1), errors*
533 *bars : SEM.* (C) Presence of oocysts found in cat faeces upon challenge in the s.c. vaccination
534 trial. *(note: no oocysts were found in animal faeces prior to challenge)*. (D) Genotyping of the
535 oocysts shed after the s.c. vaccination trial. Three loci of the ones tested by the PCR-RFLP are
536 shown (C29-2, SAG1 and BTUB). Genotype of the reference strains here (GT1, PTG, CTG) are

listed in suppl. Table S2. Faeces were identified for each cat, therefore each well represent oocysts shed by one cat, either from the “2 injections” or from the “1 injection” batch.

Figure 2 : Development of the gastro-resistant formulation of Mic1-3KO strain for oral vaccination

(A) Flow cytometry analysis of Mic1-3KO-GFP included in formula n°1 (F1) after 4h at 38,5°C in a pH4 solution. Left panel shows the formula without (w/o) Mic1-3KO-GFP strain. Right panel shows the GFP positive viable parasites after treatment (blue gate). The grey population present on both panels represents the aroma included in F1. (B) Quantification of live GFP positive viable parasites by flow cytometry after treatment. (C) Inverted bright field microscopy images of HFF cell monolayers, at D2 and D6 post treatment with Mic1-3KO-GFP strain included in F1, post *in vitro* treatment. *Bar = 10µm.*

Figure 3 : Anti-*T. gondii* antibody production and protection of animals after oral vaccination

(A) IgM production after oral administration of Mic1-3KO vaccine strain (D0 and D60, blue arrows) and oral challenge with type II *T. gondii* strain (D125, red arrow). (B) IgG production after oral vaccination of the Mic1-3KO vaccine strain (D0 and D60, black arrows) and oral challenge with type II *T. gondii* strain (D125, red arrow). *n = 4 cats/batch, errors bars: SEM.* (C) Presence of oocysts found in cat faeces upon challenge in the oral vaccination trial *(note : no oocysts were found in animal faeces prior to challenge)*. (D) Genotyping of the oocysts shed after the oral vaccination trial. Faeces were pooled for 2 cats (samples 1 to 4), sample 1 and 2 belonging to the vaccinated animals and sample 3 and 4 to the non vaccinated cats. In between are presented references strains which genotypes are listed in suppl. Table S2

(MAS, TgCatBr5, TgCatBr64, TgToucan). Three loci of the ones tested by the PCR-RFLP are shown (C29-2, SAG1 and BTUB).

Figure 4 : Cytokine production after subcutaneous and oral vaccination

Serum concentration of Th1 (A) and Th2 (B) cytokines throughout the s.c. vaccination trial of the "1 injection" batch. Serum concentration of Th1 (C) and Th2 (D) cytokines throughout the oral vaccination trial of the "vaccinated" batch, corresponding to the "1 injection batch" of the s.c. vaccination trial. IL-2, IFNγ, IL-4 and IL-5 concentrations are expressed in ng/ml (pg/ml x 1000). *n = 4 cats/batch, errors bars: SEM.*

SUPPLEMENTARY MATERIAL

Supplementary material and methods contains production of *T.gondii* total antigen extract and anti-*T. gondii* IgA measurement by indirect ELISA. It also provides supplementary table S1 (composition of gastro-resistant formulas tested) and table S2 (PCR-RFLP genotypes of T. gondii reference strains used it the manuscript. **Supplementary Figure S1** shows specific anti-*T. gondii* IgA production after vaccination and oral challenge and **Supplementary Figure S2 shows** quantification of oocysts shed by cats vaccinated or not with the Mic1-3KO strain.

SUPPLEMENTARY FIGURES LEGENDS

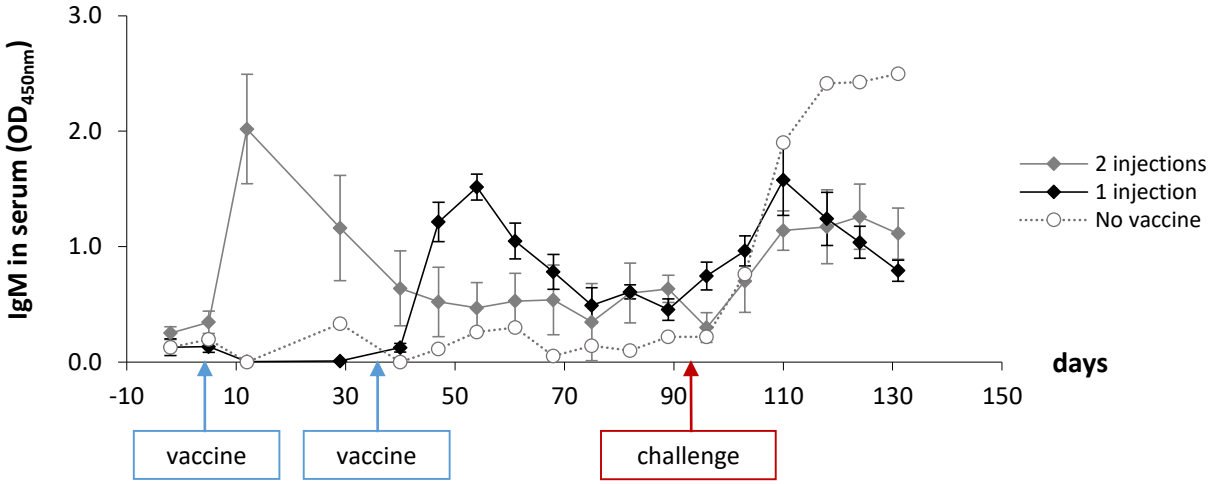
Supplementary Figure S1 : Specific anti-*T. gondii* IgA production after vaccination and oral challenge

(A) IgA production after s.c. injection of Mic1-3KO vaccine strain (D0 and D35, blue arrows) and oral challenge with type II *T. gondii* strain (D95, red arrow). *n = 4 cats/batch (except "no Vaccine" control, n=1), errors bars: SEM.* (B) IgA production after oral vaccination of the

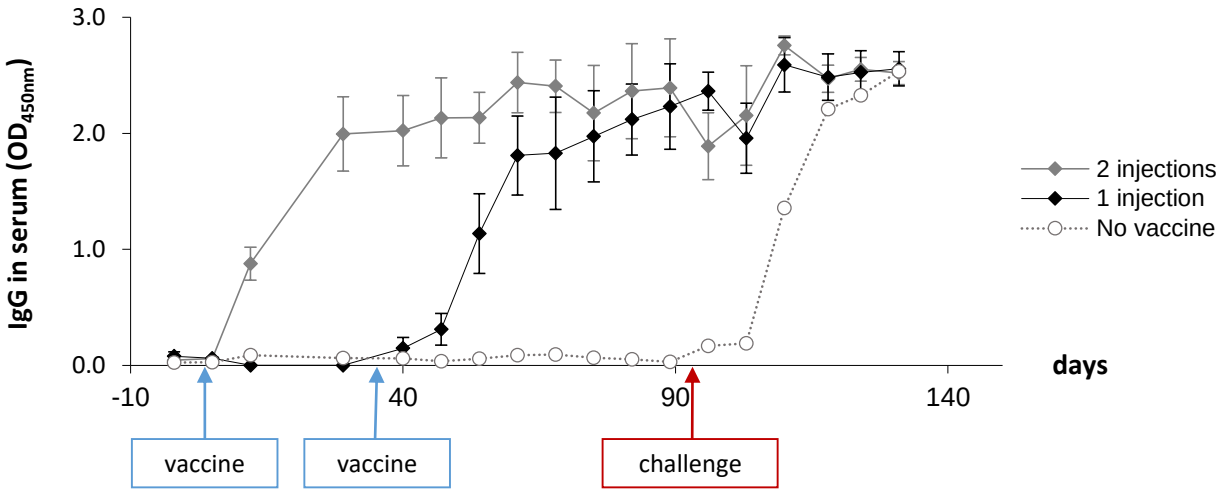
584 Mic1-3KO vaccine strain (D0 and D60, blue arrows) and oral challenge with type II *T. gondii*
585 strain (D125, red arrow). *n = 4 cats/batch, errors bars: SEM.*
586
587 **Supplementary Figure S2 : quantification of oocysts shed by cats vaccinated or not with**
588 **the Mic1-3KO strain.**
589 (A) Average of oocysts per gram of faeces shed by cats after subcutaneous vaccination and
590 oral challenge (*n = 4 cats/batch ; exept the no vaccine control, n=1)* (B) Average of oocysts
591 per gram of faeces shed by cats after oral vaccination and oral challenge (*n = 4 cats/batch).*
592

Figure 1 : anti-*T. gondii* antibodies production and protection of animals after subcutaneous vaccination

A



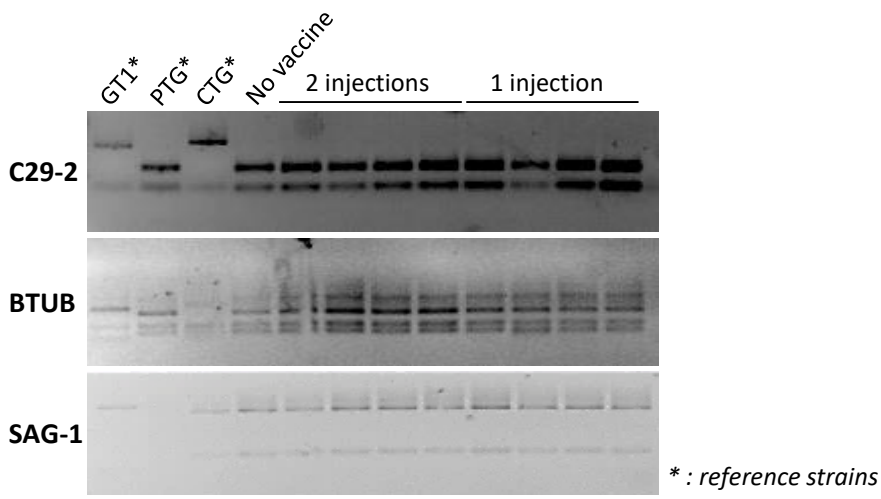
B



C

Days post challenge	1	2	3	4	5	7	7	8	9	10	11	12	13	14	15	
No vaccine				■	■	■	■	■	■	■	■	■	■			
1 injection					■	■	■	■	■	■	■	■	■			-
2 injections					■	■	■	■	■	■	■	■	■			+

D



** : reference strains*

Figure 2 : development of the gastro-resistant formulation of Mic1-3KO strain for oral vaccination

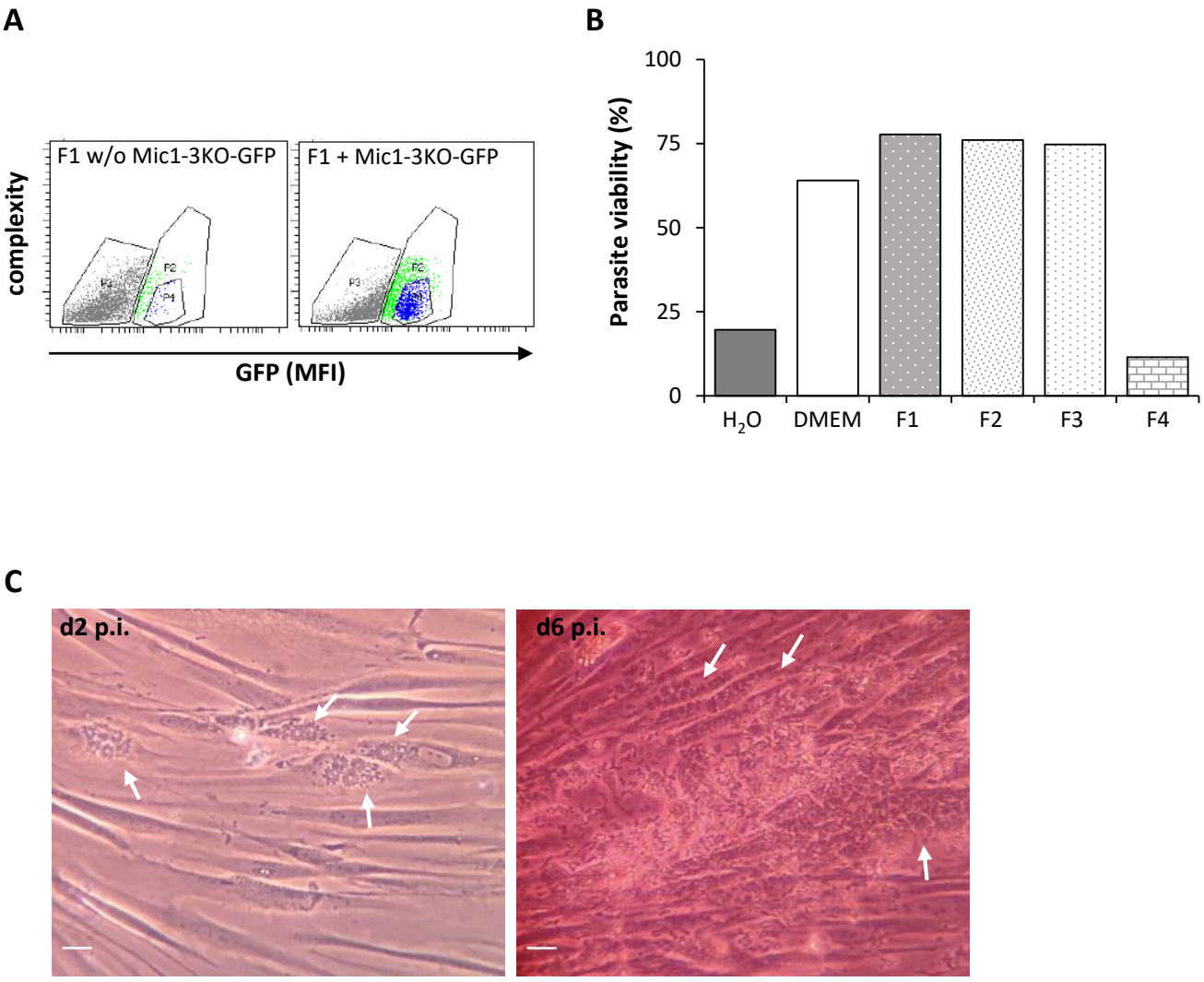
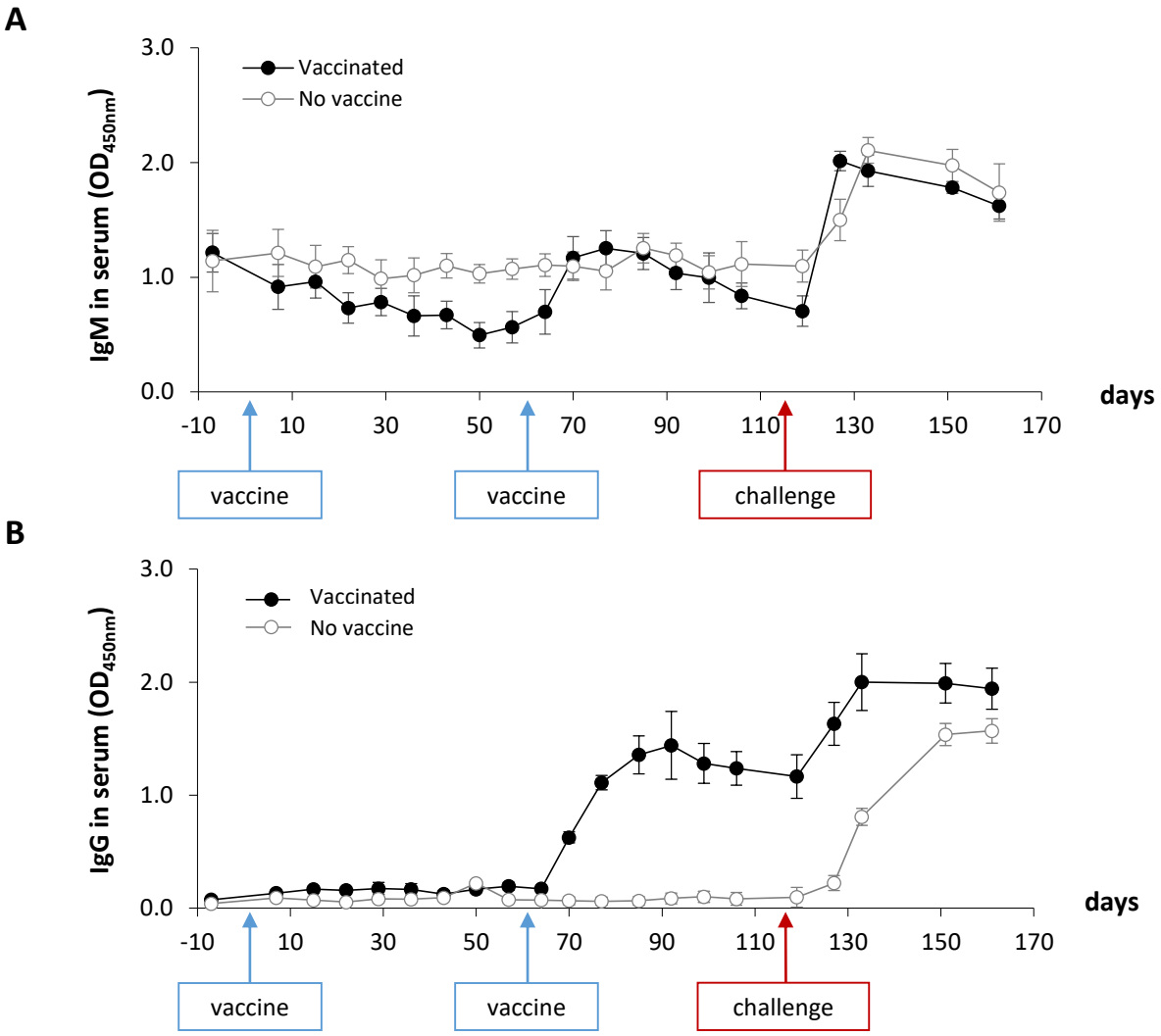


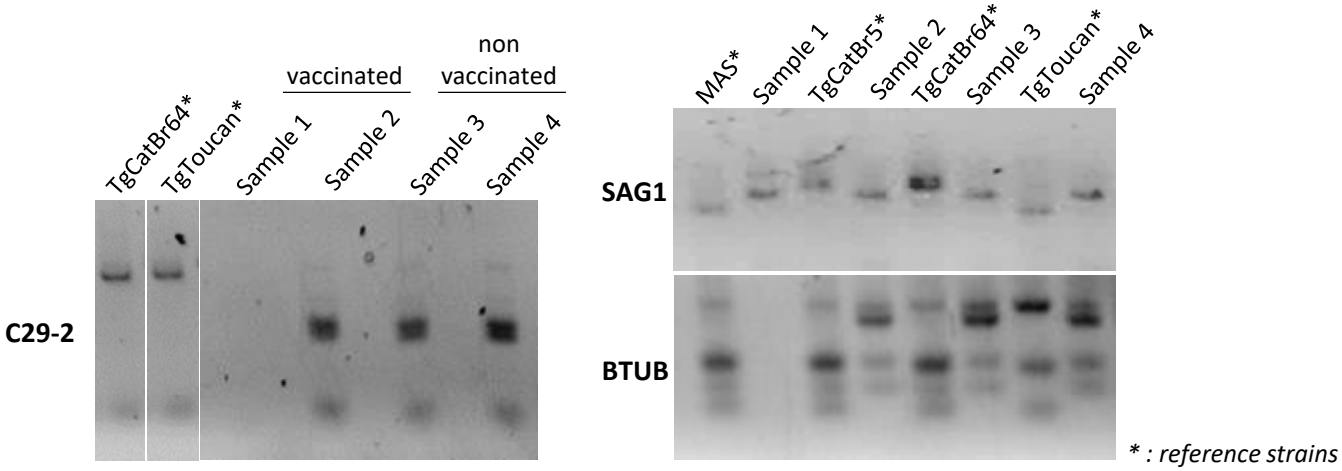
Figure 3 : anti-*T. gondii* antibodies production and protection of animals after oral vaccination



C

Days post challenge	1	2	3	4	5	7	7	8	9	10	11	12	13	14	15	
vaccinated																-
non vaccinated																+

D



** : reference strains*

Figure 4 : Cytokine production after subcutaneous and oral vaccination