A six-months, long acting, one-shot injectable formulation of Ivermectin as a complementary malaria vector control tool to target zoophagic Anopheles : laboratory and model-based proofs of concept.

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Summary:

Context: In the current context of residual plasmodium transmission where zoophagic proclivities of Anopheles intervene, we propose to treat peridomestic animals using the endectocide Ivermectin as a complementary approach to bednets. As Ivermectin remanence with classic veterinary compounds is insufficient to induce a significant decrease in vectors' populations, we developed a long-lasting injectable formulation of ivermectin from the BEPO® technology designed to release insecticidal concentrations of the molecule for 6 months. The work reported here is a proof of concept that using this new technology could help decrease field Anopheles populations.

Methods: Eight calves were injected with Ivermectin therapeutic doses of 1.2 mg/kg body weight using 2 long lasting formulations (A and B). Efficacy of the product at killing wild derived *An. coluzzii* has been evaluated by direct-skin feeding assays from 1 to 210 days after injection (DAI). Efficacy on survival was estimated with Cox proportional hazards mixed models and Kaplan meier estimates. To predict efficacy in field-based scenarii, we used a transmission model fed with an entomological model considering different levels for the Anopheles' zoophagic preference, calves vs humans ratios, and bed net use variables.

Results: The release at mosquitocidal plasmatic concentrations of Ivermectin during 6 months is confirmed for both formulations (Hazard ratios > 1 for both formulations against their vehicle for 210 days). The Ivermectin concentration allowing to kill 90% of the mosquitoes before the extrinsic incubation period of the parasite is achieved (10 days) are 11 and 9 ng/ml for formulations A and B if the blood meal is taken before the infectious one, and 15 and 13 ng/ml if it was taken after. Modeling showed that Ivermectin treatment of calves using BEPO® technology would reduce infectious vector populations, from at least 35% for most anthropophagic Anopheles in villages where cattle to human ratio is the lowest, to more than 75% if vectors were zoophagic and calves numbers superior to humans'.

Conclusion: Our study gives the proof of concept that a long-lasting formulation of Ivermectin administered to calves could help decrease field malaria vectors' populations, which may, ultimately, have an impact at the epidemiological level.

Key Word : Anopheles, malaria, residual transmission, Ivermectin, Long lasting formulation, cattle, One-health, Burkina Faso

INTRODUCTION

Between 2000 and 2015, the estimated number of averted malaria cases was 663 millions of which 68% may be attributed to the Long-Lasting Insecticidal Nets (LLINs) and 13% to the residual insecticide spraying ([1]), making vector control by far the most efficient approach for controlling disease transmission. Despite this progress, malaria continues to represent a serious public health concern worldwide and still impairs social and economic development of endemic countries. In 2020, there were an estimated 241 millions new cases of malaria and 627 000 deaths ([2]). This represents a more than 10% increase in the number of deaths by comparison to 2019. Even if 3/4 may be attributable to the COVID crisis and the health system

saturation, it remains that malaria incidence and mortality increased globally for the first time, after having started, from 2015, to stagnate, and further to increase locally in several WHO African regions, revealing the limits of the current prevention approaches. On the vector side, among possible reasons for the lack of sustained effect, are the limited access to the LLINs and the resistance of malaria mosquitoes to the 4 classes of insecticides approved for malaria vector control, *i.e.* pyrethroids, carbamates, organophosphates and organochlorides ([3],[4]). Resistance can occur through mutations rendering the insecticide target site insensitive to the molecule, through increased metabolic detoxification processes or through structural adaptations that mitigate the effect of the insecticide ([5]). Aside from this physiological resistance, additional failure comes from behavioral features that primary and also secondary vectors display that allow them to overcome control tools and maintain or increase parasite transmission, like exophagy, zoophagy or incongruous patterns of biting ([6],[7],[8])

A specific priority to maintain efficient control of malaria transmission and to ultimately eliminate the disease is to develop novel, yet complementary, malaria control strategies. On the vector side, the WHO Global Technical Strategy for Malaria Control 2016-2030 requires the development of innovative vector control tools that can be integrated in current malaria control programs. The use of the endectocide ivermectin is viewed as such a transmission-blocking additional tool option by targeting the insect vector, and this approach is currently on the process of evaluation by the *ad hoc* WHO instances and has triggered collegial and synergistic work from researchers (WHO Malaria report, 2019, Review of endectocide based vector control tools being evaluated: https://www.who.int/vector-control/vcag/new-interventions/en/index9.html, [9]).

Ivermectin is a broad-spectrum anthelminthic medicine that was first licensed in 1981 for veterinary use ([10]). Since 1987, it has been approved for human use and widely distributed through Mass Drug Administration (MDA) campaigns all over Africa to achieve the elimination of both onchocerciasis and lymphatic filariasis ([11]). The systemic activity of ivermectin against ectoparasites, causing death of all malaria vectors species tested to date if they absorb their blood meal from ivermectin treated humans or animals ([12],[13],[14]) has triggered an interest in repurposing this molecule for malaria vector control. The concept is as simple as thrilling, because the product would render toxic what represents the cornerstone of malaria parasites transmission and what inherently constitutes a vector of plasmodium: the blood consumption. Hence, (i) the treated host delivers himself the insecticide (ii) the vectors that will bite treated hosts even just once have a great probability to die before being infectious (iii) its mode of action is different from all currently used insecticides such that physiologically resistant malaria vectors can be also targeted and resistance threat therefore mitigated (iv) it is unavoidable by any vector behavior: vectors that bite treated hosts will die regardless of their biting timing, location (indoors or outdoors), or proclivities (even zoophagic vectors could be targeted) (v) sublethal concentrations of IVM impairs fecundity, fertility and mobility of Anopheles mosquitoes [15], *a priori* mitigating the probability of appearance of the unfavorable case scenario of ivermectin resistance in vectors' populations soon after MDAs. Because it targets invertebrate-specific glutamate-gated chloride channels, and because it binds to a P-glycoprotein membrane efflux pump ([16] which corresponds to a multidrug resistant glycoprotein that prevents the molecule from crossing the blood/brain barrier ([17]), ivermectin has also an excellent safety profile even at higher than recommended dose ([18]. More than 4 billion doses have been

distributed since 1987 in the frame of the Mectizan donation program for elimination of lymphatic filariasis and onchocerciasis (https://www.merck.com/stories/mectizan/, accessed the 7th January 2022). Adverse severe effects using the therapeutic dose have been registered, but at an extremely low rate so far, in majority correlated with high parasite infection loads, and, in particular, the presence of Loa Loa co-infection ([19],[20]).

The systemic insecticide potential benefit of Ivermectin administered to humans or animals has been thoroughly demonstrated in the laboratory (e.g. [14], [12], reviewed in [13] and [9], in small-scale field trials ([21];[22]), and in different ecological settings in the fields where a reduction of the sporozoite rate has been evidenced following MDA of IVM to humans ([23], [24]). A most compelling and direct evidence of ivermectin potential at concretely avoiding malaria cases was however awaited by the WHO-MPAC at the epidemiological level. Such direct evidence has been recently obtained by Foy et al, who conducted the first randomized control trial, in Burkina Faso, showing that when added to LLIN use, repeated mass administration of single doses of Ivermectin every three weeks during a rainy season reduced malaria incidence by 20% in children aged 5 or under ([25]).

For the repurposing of this drug toward malaria control use, the limit of Ivermectin resides on the fact that the therapeutic doses and dose regimen (*i.e.* frequency of administration) that are currently approved for treating humans and animals allows reaching plasmatic concentrations above the LC50 for Anopheles mosquitoes for a too short period of time in the hosts to impact transmission. The relatively short plasma half life (about 18-56 hours in humans,[18]; upon 3 weeks for calves e.g. ([14]) does not allow, with a single dose, to maintain mosquito lethal concentrations long enough that would significantly impact malaria epidemics. Ways

to enhance and sustain this impact could be the use of higher and/or repeated doses of the current formulations of Ivermectin, or the use of new formulations where the plasmatic concentration reached after treatment could be sustained above LC50 for a longer period of time, enough to impact *Plasmodium* transmission through significant vectors densities reduction. This is the major technical gap that WHO recognized as a barrier for a wide and effective deployment so that they suggest among other proposals that a formulation releasing Ivermectin for at least a month, but preferably covering the rainy season, could be a game changer (https://cdn.who.int/media/docs/default-source/malaria/vector-control/who-ucn-gmp-2021.11-eng.pdf?sfvrsn=7454d2c7_10).

Indeed, a strategy based on the use of current formulations will suffer from strong limitations for an effective MDA perspective. Given the short half-life of the drug in humans or animals, repeated administrations will be needed, which is challenging in many aspects, for the logistics and the related costs, and for the compliance which could be substantially eroded with repeated campaigns.

Here we propose to establish the proof of concept that a prototype 6-month long acting injectable formulation of ivermectin using BEPO® technology ([26]) could stand as a complementary tool for vector resistance mitigation and residual malaria transmission management. BEPO® is an injectable *in situ* forming depot technology, based on the use of block copolymers that entrap the therapeutic molecule upon depot solidification when in contact with body fluids. The depot progressively bioresorbs while delivering the active pharmaceutical ingredient with the desired pharmacokinetics. Such ivermectin formulation technology has been previously tested for its microfilaricidal effect against Onchocerca ochengi ([27]).

The mosquitocidal effect of a long lasting BEPO® ivermectin formulation designed to release the molecule at the therapeutic dose of 0.2 mg/kg/month during at least 6 months has been tested. In Burkina Faso, direct skin feeding assays and survival experiments on wild-derived *Anopheles coluzzii* were performed at different time points following the injection of the product to local calves. To assess if such formulation could help reduce vector populations if a one-health approach was to be implemented, further modeling was used, by considering reality based scenarios taking in account calves to humans proportions, bed nets use and vectors' proclivities.

METHODS

Mosquito colony

We used the *An. coluzzii* colony established in 2008 from gravid females collected in Bama, Kou Valley (11°23'14"N, 4°24'42"W), 30 km from Bobo-Dioulasso, southwestern Burkina Faso (West Africa). To alleviate funding effects and to maintain representative genetic diversity, the colony is repeatedly replenished (every year) with F1 progeny from at least 50 wild-caught mosquito females from the same locality, after being identified for their species status by routine PCR ([28]). Potential contamination of the colony by other *Anopheles* species is routinely checked using the same technique. The mosquito colony was maintained under the standard conditions of 27±2°C, 75±5% relative humidity and 12h/12h day/night cycle. Larvae were reared at low densities in plastic trays in tap water and fed *ad libitum* with commercial alevin food (Tetramin® Baby). Pupae were collected in cups and placed

in 30×30×30 cm cages. Newly emerged adults were allowed to feed for three to five days on 5% glucose solution then starved for 16-18 hours before blood feeding on cattle.

Cattle hosts

Eight bull calves of the local Metis breed (obtained from cross breedings between Fulani zebus and Baoulé bulls) were used as hosts for *Anopheles* direct skin feeding assays. This study was carried out in accordance with the ethical guidelines for care of laboratory animals (Act n°00468, 24th January 1994) covering all West African French speaking countries.

Upon their arrival at the stable of the Centre International de Recherche Développement sur l'Elevage en zones Subhumides (CIRDES), the calves were treated with therapeutic doses of aceturate diminazene and albendazole to, respectively, cure potential trypanosomiasis (endemic in this area) and gastro-intestinal infestation with endoparasites. To our knowledge, no study reported an effect of these molecules on Anopheles survival or fecundity. The experiment started one month later. Calves were fed with a diet made of straw and cotton oil cake and provided with water and salt *ad libitum*. They were maintained in the stable, protected by a net to avoid any insect disturbance, and checked every other day by a veterinarian to ensure their wellness. They were weighted before the start of the experiment and after it was completed to determine their percentage of mass change over the course of the experiment, which was taken as a proxy of their well-being.

Manufacturing of slow-release formulations of Ivermectin

Injectable long-acting formulations of ivermectin were designed using the BEPO® technology [26] and prepared as described in [27]. Briefly, a triblock copolymer, PLA_{97}-PEG_{45}-PLA_{97}, and two diblock copolymers, $mPEG_{45}$-PLA_{130} and $mPEG_{7}$-PLA_{41}, were synthesized by ring-opening polymerization in bulk condition as already described (patent US 9,023,897 B2). Two long lasting formulations were synthesized: (i) Formulation A, composed of 5% (w/w) of ivermectin, 45% (w/w) of copolymer comprising PLA_{97}-PEG_{45}-PLA_{97} and $mPEG_{45}$-PLA_{130} and 50% (w/w) DMSO, and (ii) Formulation B, composed of 5% (w/w) ivermectin, 50% (w/w) of copolymer comprising PLA_{97}-PEG_{45}-PLA_{97} and $mPEG_{7}$-PLA_{41} and 45% (w/w) DMSO. To achieve their preparation, the tri- and di-block copolymers of each formulation were preliminary dissolved overnight in DMSO (Procipient, Gaylord Chemical), at room temperature and under continuous stirring. Ivermectin (Fagron, France) was then added to the polymer solution until its complete dissolution. The formulations were sterile filtered (using 0.2 µm filters (Minisart SRP 15, Sartorius)) and administered at 1.2 mg of ivermectin/kg of body mass (i.e. 24 mg of formulation/kg), to cattle using hypodermic syringe capped with 16-gauge needle.

Long lasting formulations of Ivermectin were imported in Burkina Faso under the clearance provided by the national “Direction of public and veterinary health and legislation” and the “General Direction of Veterinary Services” of Burkina Faso (visa N°14/107 on the 18th of November 2014).

Calves treatment

Calves were randomly assigned to receive either a placebo (*i.e.* the formulation without ivermectin (vehicle)) or a treatment formulation. The formulations were given by subcutaneous injection under the loose skin in front of the shoulder. Calf number 8 suddenly moved during the injection, provoking the withdrawal of the needle before the end of the injection. Hence, for this calf, the injection has been made in two distinct spots instead of only one. Two calves were assigned per arm (placebo A, treatment A, placebo B, treatment B).

Blood feeding

Three to five days old mosquitoes from the same batch were randomly introduced into 32 plastic cups covered with nets (n=50 to 70 mosquitoes per cup) 16-18 h before the direct skin blood-feeding and left with water only to increase their propensity to feed on the hosts. Four plastic cups were randomly assigned to each control and treated calf, disposed on the animal's sides, and held using a rubber strap arranged around the abdomen. Animals were carefully restrained using ropes to avoid rough movements or scratching. Mosquitoes were allowed to feed for 15 min, after which only fully engorged females were transferred in cardboard cups for survival follow-ups. Blood feeding of mosquitoes occurred in 14 instances during the experiment: before treatment and at different time-points after the administration of the formulations, i.e., at 1, 7,14, 21, 28, 49, 91, 105, 119, 155, 183, 195 and 210 days after the injection (DAI). The percentage of blood-fed mosquitoes was similar

between the 14 batches (*i.e.*90-95%), for each treatment and each calf (data not shown).

Ivermectin bioanalysis

After blood-feeding episodes (except for the times post-injection of 105 and 119 days) and for each calf, 5 ml of blood was withdrawn from the jugular vein in heparinized tubes (BD Vacutainer®PST™ tubes). Blood samples were centrifuged at 2500 g for 10 minutes at room temperature. 1.5 ml of the supernatant (*i.e.* the blood plasma) were transferred in plastic tubes and stored at -20°C until further processing. Samples were analyzed for their ivermectin content as described by Boussinesq *et al*.[27].

Survival of mosquitoes fed on treated and control cattle

Fully engorged females were randomly distributed and maintained in paper cups for the survival follow up. Four cups were used per calf with ten mosquitoes per cup and provided every day with cotton balls soaked in 2.5% glucose solution. Mortality was recorded every day from the day of blood feeding until the 30th day after. Mosquitoes seen alive the 30th day were registered as “censored”.

Statistics and modeling

All statistical analysis and the modeling were performed using the software RStudio Version 4.0.1 (2020-06-06). The data and the R codes are all available upon request.

Statistical analysis

Dynamics of IVM in cattle blood

Generalized additive model (GAM) of IVM concentration in function of the formulation, the time after injection, and for each cattle was computed to compare the formulations and to assess for potential cattle effect in the pharmacokinetics of IVM. A log-normal distribution was assumed together with a cubic regression spline smoother of time for each cattle.

Mosquito survival

Kaplan-Meier survival estimates were calculated to investigate whether females' longevities were affected by a blood meal taken on treated cattle at different DAI after injection of the different formulations. Censored data were used, as all mosquitoes weren't dead by the 30th day after blood feeding. The effects of the treatment, the formulations, the time after injection, and their interactions were further tested using Cox proportional hazards mixed models where cups and cattle were considered as nested random effects.

Analysis were performed following the three steps described above:

(a) To assess for potential confounding effects of cattle blood, which may differ in its nutritive values between animals and which may give different survival outputs for mosquitoes, cattle effect was characterized before the ivermectin formulations were injected (0 DAI) using Cox proportional hazards mixed models with the cup included as random effect, in order to ensure that any difference in mosquito survival was due, at least in part, to the treatments. Further analyses were performed before treatments as well to assess that there is no confounding effect of the group calves composition on the group mean mosquito survival. Cox proportional hazards mixed models were also used here, including the calf as a random effect.

(b) After treatment, mosquito survival time was examined in function of the treatments and the time after the injection. Kaplan Meier survival curves were plotted and comparisons between treatments and their vehicle have been performed using Cox proportional hazards models where the calf was considered randomly. Both formulations were further compared for their efficacy in relation to the released concentrations, with time and calves as random.

(c) We further explored the formulations efficacy by considering the probability that a mosquito having imbibed an IVM containing blood meal dies before it becomes infectious and able to vector *Plasmodium falciparum* sporozoites under 2 simplistic scenarii: (i) the mosquito takes the ivermectin blood meal after he ingested an infectious blood meal (ii) the mosquito takes the ivermectin blood meal before he ingested an infectious blood meal. Assuming a gonotrophic cycle of 3 days, and 10 days being the average number of days taken for the sporogony to be completed in other studies using Ivermectin (e.g. [29]), we examined the effect of the formulations for cumulative mortalities of n= 7 and 13 days after the ivermectin blood meal (see Figure 1 for a schematic representation of both scenarii). Data have been

considered as binomial (*i.e.* dead (1) or not dead (0) before t=n days post ivermectin blood feeding) and the probability of dying before the n^{th} day, analyzed in function of the concentration and the treatments using a generalized linear mixed model with binomial errors, logit link function and time and cattle considered as random effects.

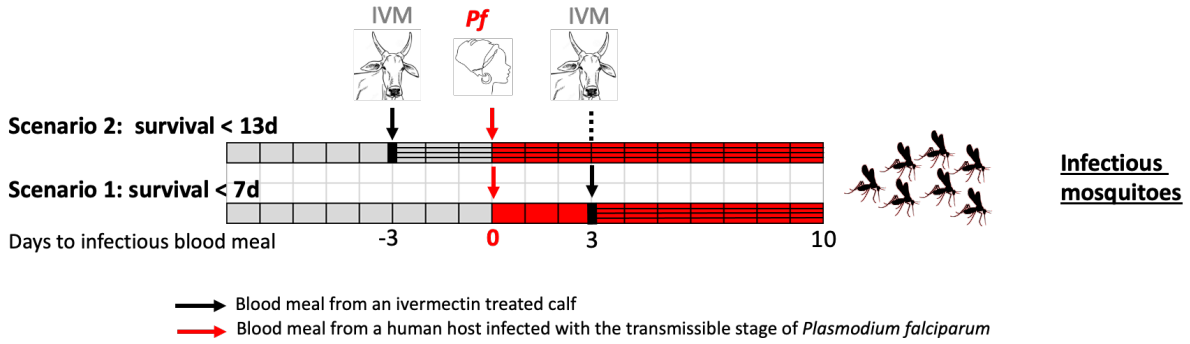

Figure 1: Schematic representation of the blood feeding simplistic scenarii that an anopheles could go through during its lifespan in an area where cattle is mass-treated with a long lasting formulation of Ivermectin FA or FB. IVM= Ivermectin, *Pf*: *Plasmodium falciparum*. In grey: the mosquito doesn't carry *Pf*. In red: the mosquito carries *Pf* and eventually becomes infectious. Hatched areas represent for each scenario, the time during which the mosquito should die after its Ivermectin blood meal so the transmission of sporozoites doesn't occur.

When needed, analyses were followed by *post-hoc* tests procedures to compare the levels of significant factors.

Modeling

Different parameters, taken from our data or from the literature were used to predict the impact of long lasting formulations' injection to cattle on malaria transmission (*i.e.*

on the mosquito population densities, either infectious or not) through modeling. These parameters and the model that we developed are detailed afterwards.

Dynamics of IVM in cattle blood:

The IVM concentration in the plasma of the four bovine treated with formulation B was fitted to the number of day post injection using a Generalized Additive Model (GAM) with automatic choice of smoothing parameters. We used the “gam” function of the “mgcv” package ([30]) in “R” (RCoreTeam, 2020). The GAM model was then used to predict the mean concentration of IVM in bovine plasma from 1 to 210 days post-injection.

Effect of IVM on Anopheles mortality

We assume that a vector biting on a given day after host IVM injection will experience an increased risk of death and, moreover, it will die at a new constant mortality rate governed by the amount of IVM in the cattle blood at the time the blood meal was taken. The GAM model was used to estimate this amount of IVM. A Cox proportional hazards survival model was used to describe how this concentration of IVM affects mortality. The relationship between the log of the concentration of IVM ingested by the mosquito and the induced mortality was modeled using a second-order polynomial function. The hazard from the survival model was converted into a mortality rate by multiplying the baseline mortality rate (0.1, from Slater et al., 2014) by the relevant hazard for mosquitoes biting on each day post host IVM injection. We used the “coxph” function of the “survival” package [31] in “R” for this task.

Malaria transmission model:

Susceptible-Exposed-Infectious (SEI) model of *P. falciparum* transmission

The model described by [32] was modified to account for two types of host (cattle and human) and for the effect on vector mortality of two interventions (IVM injected to cattle and LLINs to protect humans from mosquito bites).

State variables and parameters of the model are further described in the Supplementary material S1, table S1 therein.

Each day, a proportion of mosquitoes takes a blood meal on calves' population (*1-HBI*), in which a proportion (based on the IVM coverage rate C_{ivm}) contains IVM. These vectors then move to a new compartment where they will have a higher mortality rate based on the amount of time after IVM injection where the blood meal was taken. Once vectors move to the new compartment on a specified day, they will have the corresponding mortality rate for the rest of their lifespan. The mortality rate for vector biting on day *i* post IVM injection is denoted μ(di)_v and is given by the vector mortality model (see below). The mortality rate of all "non-IVM" mosquitoes that didn't imbibe blood meals containing IVM equals the baseline mortality rate μ_v0.

Each day a proportion of susceptible mosquitoes takes a blood meal on humans (*HBI*). A proportion of these vectors will move to the infected compartment based on the *P. falciparum* prevalence (P_{pf}) in the human population and on a probability to be infectious (*k*). Infected vectors will move to the infectious compartment after *n* days (duration of the extrinsic incubation period of *P. falciparum*).

Among the vectors that take a blood meal on humans, a proportion μ_h will die due to the presence of LLINs.

<u>Vector behavior and mortality model</u>:

A vector behavior and mortality model was developed to feed the SEI model of *P. falciparum* transmission with credible values of (i) probability that a vector will feed on cattle (1 - *HBI*) and (ii) probability of death due to the LLINs μ_h (for vectors encountering a human protected under a LLIN) under various environmental and entomological scenario:

- varied cattle:human ratio in the host population (*i.e.* more cattle than humans, equal number of humans and cattle, or more humans),

- varied levels of LLIN coverage in the human host population (0, 50 or 100%) and,

- varied host preference phenotype (human *vs.* cattle) in the *Anopheles* population (zoophilic, opportunistic or anthropophilic).

We assume that the probability for a vector to choose a type of host (human or cattle) is independent of the origin of a previous blood meal and that LLINs have no remote effect (*i.e.* no deterrence). Parameters and equations of the vector behavior and mortality model are further described in Supplementary material S1, Table S2 therein.

RESULTS

Formulations, ivermectin concentration, and steady release

The generalized additive modeling of the ivermectin plasmatic concentrations in our experiment (assuming a log-normal distribution of the values) revealed that, when comparing both treatment arms, the ivermectin controlled release formulations *(i.e.* the polymers mixture that traps the ivermectin) did not yield different plasma concentrations of the molecule (formulation effect, F = 0.062, p=0.8). In each treatment arm, the ivermectin plasma-concentration time profile varies over time with

more or less fluctuations depending on the calve (Figure 1). Significant fluctuations in the concentration-time profile are observed for B3(FA) and B6(FB) (*i.e.* a significant time effect, B3:$F=16.58$, $p<0.001$; B6: $F=22.867$, $p<0.001$), while for the other calves, the concentration remains steady (time effect, B1(FA): $F=1.551$, $p=0.278$; B8(FB): $F=1.612$, $p=0.211$). For both B3 and B6, ivermectin concentrations show an initial burst reaching highest values of more than 40ng/ml 7 days post-injection, and remain most of the time above 10ng/ml. It is worth noting that for these calves, plasmatic concentrations dramatically decrease between days 165 and 185, which corresponds to the targeted duration of release. After day d=185, ivermectin concentrations continue to decrease until reaching values close to the limit of quantification (0.1 ng/ml). For B1 and B8, the plasma-concentration time profile unexpectedly looks different with an absence of an initial burst release and relatively steady concentrations of ivermectin in the range of 2-10 ng/ml from day 0 to 210.

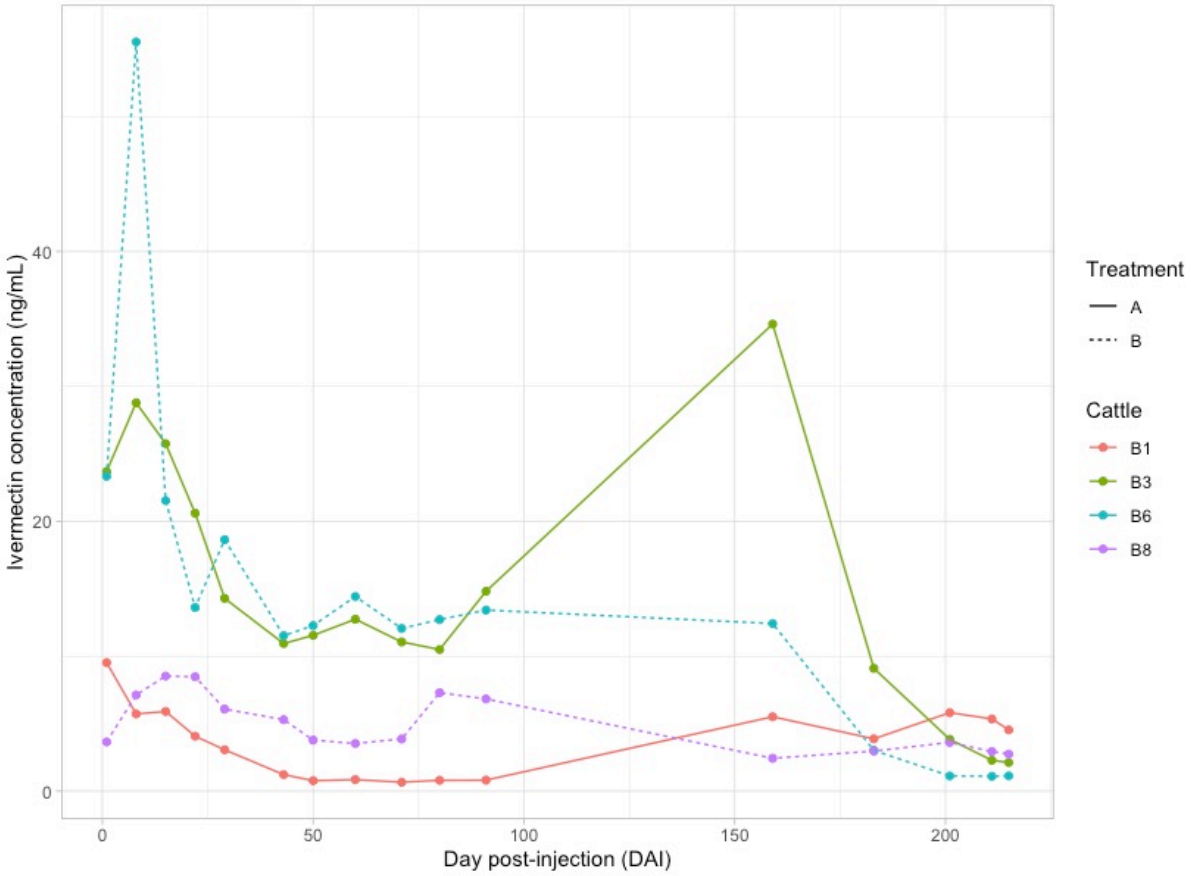

Figure 2. Ivermectin plasmatic concentrations (ng/ml) over time in experimental calves after injection of the BEPO-IVM formulations. B1 and B3 received the FA formulation while B6 and B8 received FB.

Efficacy study: overtime survival of mosquitoes after skin-feeding assays at different time points post injection of the formulations

Preliminary skin-feeding experiment has been performed after the calves' acclimation to the stable and one month before the treatments to evaluate potential confounding host effects on mosquito survival. The modeling of survival using the coxph function (where the cage numbers have been considered as random effect) revealed a strong host effect (LRT X^2_7=41.69, p<0.001). However, when survival has been compared between treatment groups that have been composed randomly, no difference has been revealed (host identity and cage number have been both considered as random effect, LRT X^2_3 = 0.68, p=0.88), which confers reliability on

the further efficacy study (corresponding Kaplan-Meier survival curves are given in the supplementary figure SF1, A and B).

For each treatment arm (PA, PB, TA, TB) and at each delay post-injection of the formulations, at least 100 mosquitoes were exposed on the flank of the restrained animals and allowed feeding for 30 minutes. Only fully engorged mosquitoes were further considered for survival studies. In total, 3378 mosquitoes were observed daily for 30 days. Kaplan Meyer survival curves for each group after treatment have been drawn (Figure 3). The effects of the Delay after injection (DAI), the treatments, and their interaction on mosquito survival have been further modeled. We found a significant effect of the DAI, the treatment and their interaction on mosquito survival (DAI effect: $X^2_{10}=192.05$, $p<0.001$; treatment effect: $X^2_3=27.42$, $p<0.001$; DAI x treatment effect: $X^2_{30}=289.67$, $p<0.001$), which means that the effect of the DAI is not the same for each treatment. For each delay post-injection, hazard ratios were calculated for all treatment combinations (supplementary table S3). During 6 months post-injection, the formulation A was significantly better than its vehicle at killing mosquitoes on all instances except at 49 and 91 days after injection, whether the formulation B was efficient until DAI=155, where its effect was only marginally significant. Both formulations were no more inducing significant mosquito mortality at day 183 post-injection and at the subsequent timepoints (*i.e.* DAI= 197 and 210, data not shown).

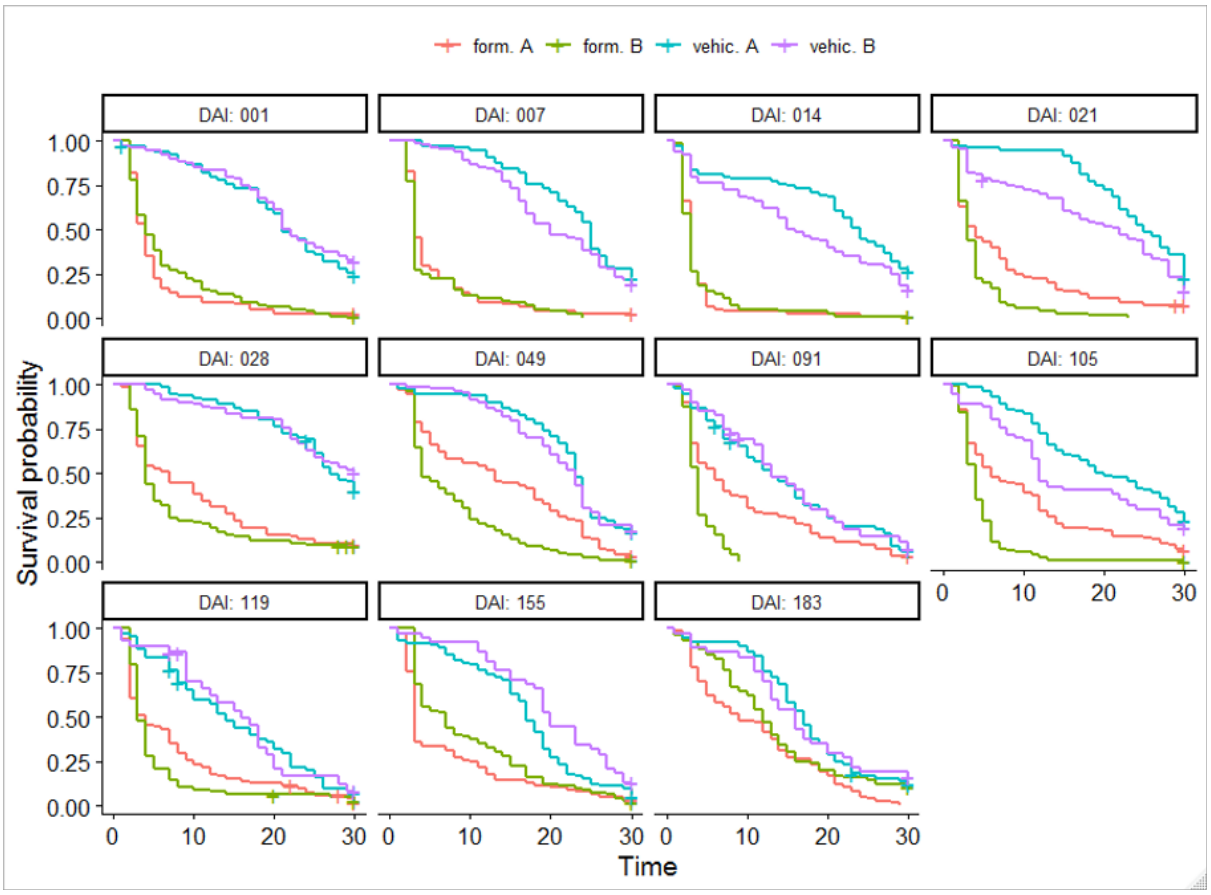

Figure 3. Kaplan Meier survival curves for mosquitoes fed on the calves of each experimental arm. Curves are drawn for each delay after injection at which mosquitoes were fed during direct skin feeding experiments. DAI: days after injection.

When compared together, both treatment formulations showed identical efficacy at any considered time point (See Figure 3 and supplementary table 3). These results are further illustrated by plotting the ratios of the probability to die between mosquitoes fed on calves treated with FA *vs* FB and corresponding confidence intervals (Figure 4, FA/FB). As previously described, the formulations A and B are different from their vehicle, except for specific timepoints (49 and 91 days post injection) for formulation A and, as expected by the formulations design, at t = 180 days for both A and B (Figure 4, A/T_A, B/T_B).

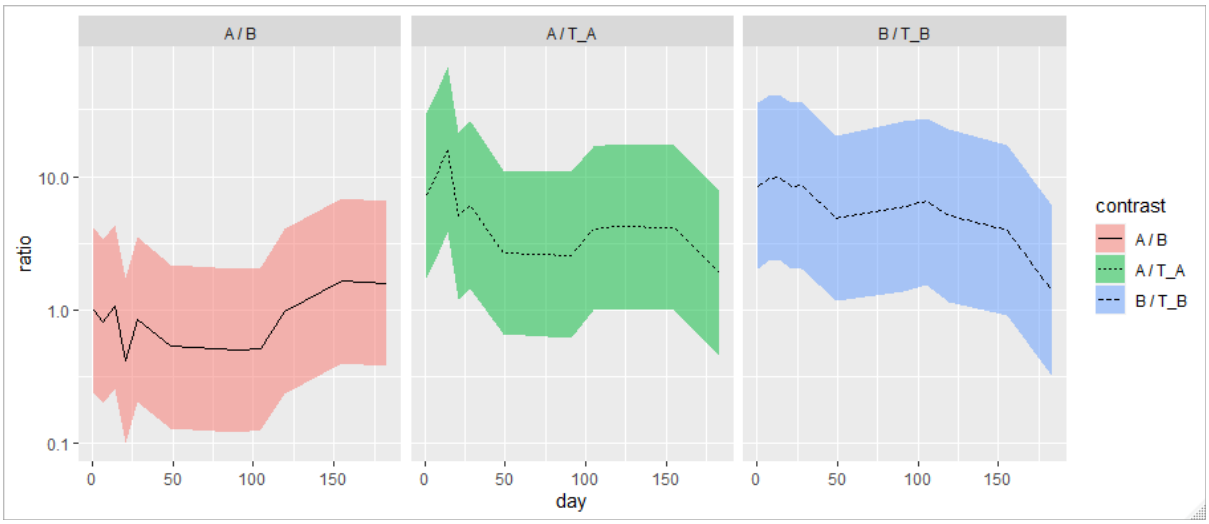

Figure 4. Hazard Ratios and corresponding confidence intervals for comparisons of induced mortality between FA and FB (Panel A/B), and FA and FB and their corresponding vehicle (Panel A/T_A and B/T_B, respectively).

Efficacy study was further elaborated by taking into account the plasmatic concentrations of IVM that were found in treated calves only, at different survival observations (same as in Figure 3, except for DAI=105 and 119 where IVM concentration was not available). The mixed cox survival modeling approach has been used to characterize the impacts of the concentration, the formulation and their interaction on mosquitoes survival, while considering the day after injection and the cattle effects as random. The model shows that there is a significant effect of the concentration ($LRT\chi^2_1=184.57$, $p < 0.001$) , the formulation ($LRT\chi^2_1=6.57$, $p = 0.01$), and their interaction ($LRT\chi^2_1=22.86$, $p < 0.001$), which means that the effect of the formulations differs in function of the plasmatic ivermectin concentration.

All data taken together, the model predicts an increase of the probability to die when the concentration of ivermectin was increased in the range of 1 to 50 ng/ml ($p<0.001$). Per additional 1ng/ml, daily mortality rate is multiplied by 1.08 (*i.e.* HR=exp(0.07) with the formulation A, and by 1.05 (*i.e.* HR=exp(0.074-0.027) with

form B. The interaction between the concentration and the formulation is significant, which means that both formulations are not acting the same for equivalent concentrations. For smallest ivermectin concentrations in the plasma (<4ng/mL), the hazard of death is predicted to be better if the formulation B is used, while the formulation A seems better for killing mosquitoes at the highest concentrations (>26ng/mL) (Figure 5).

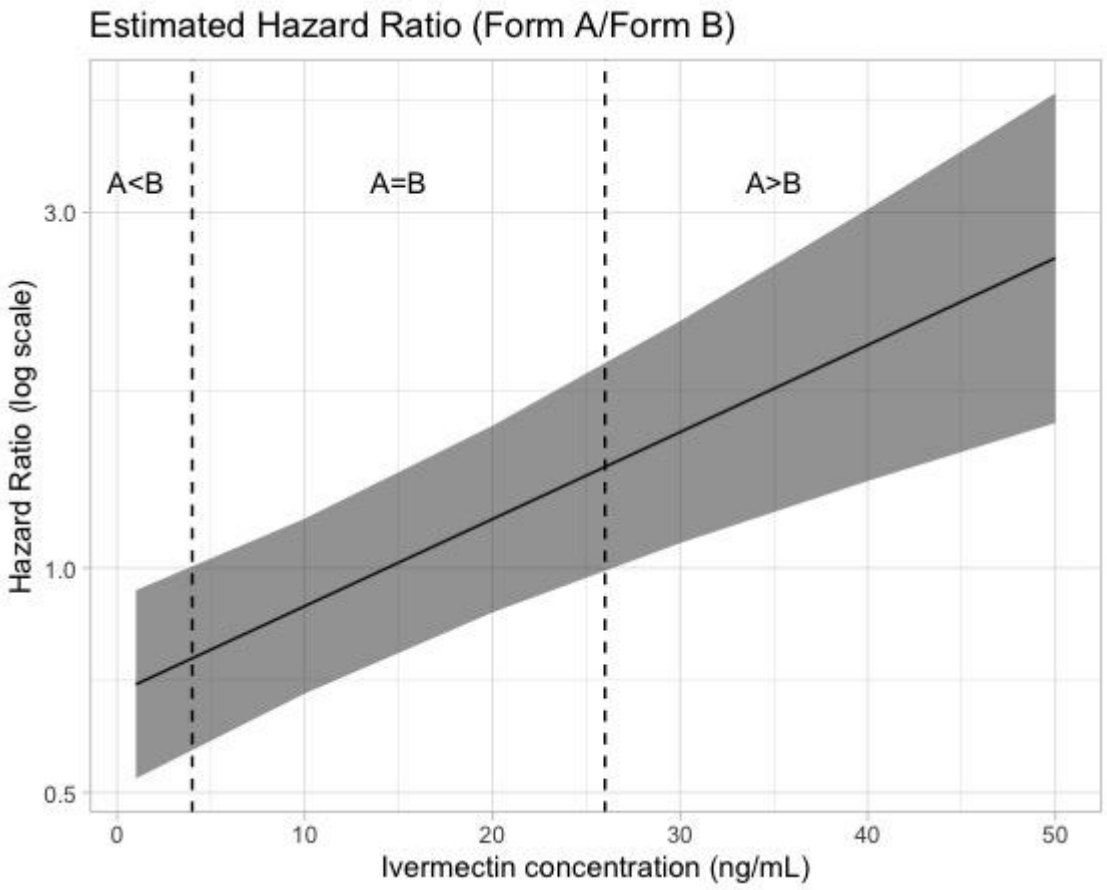

Figure 5. Linear relationship of ivermectin plasmatic concentration and the ratio between the probability to die for a mosquito after it ingested a blood meal containing ivermectin from FA and FB. For each ivermectin concentration and each formulation, the HRs (hazard ratios) induced by the formulations FA and FB are estimated by modeling (see text), and their ratios further computed.

Under the two scenarii defined in the Material and method section (*i.e.* the IVM blood meal taken after or before the Infectious blood meal, Figure 1), the cumulative mortality model predicts that the formulation B would induce a 90% mosquito mortality before sporogony is achieved for ivermectin plasmatic concentrations of 13 and 9 ng/mL, for scenario 1 and 2, respectively, while formulation A reaches this mortality level for higher concentrations of 15 (range:12-16 ng/ml) and 11 ng/mL. Furthermore, the LC50 associated with the probability of dying before 7 days (scenario 1) are 5 ng/mL (range:4-7) and 6 ng/mL (range: 5-8) for formulation B and A, respectively. For scenario 2, the initial probability of dying is above 50%, then there are no LC50 values (figure 6). The probability to die before the 7th day post-Ivermectin blood meal was significantly different between the A and B formulations (OR A/B_13=0.52, p=0.019; OR A/B_15=0.46, p=0.025) but not the probability to die before 13 days post meal (Figure 6).

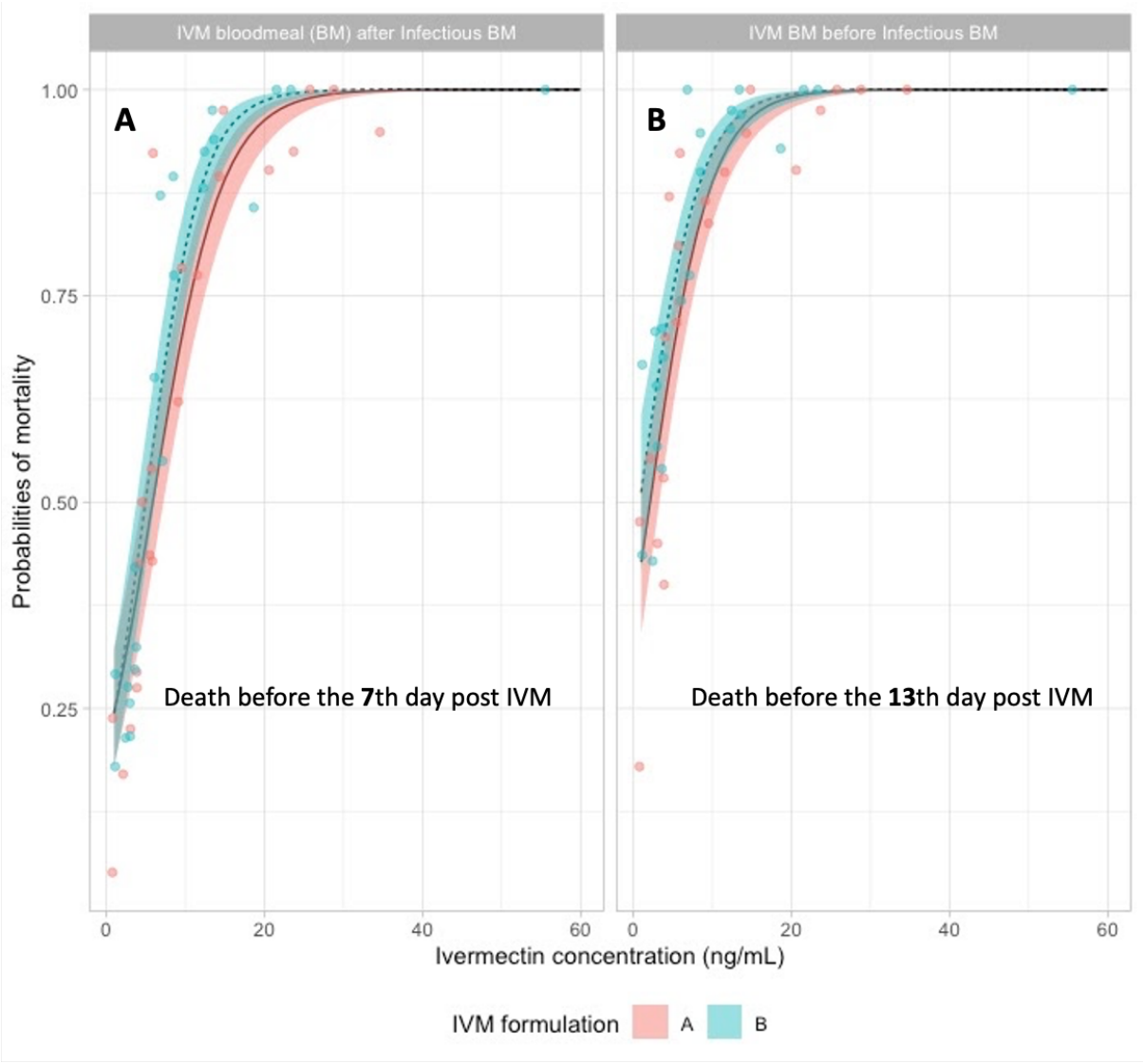

Figure 6. Models prediction of mosquitoes cumulative mortalities when they take a blood meal containing Ivermectin IVM on hosts treated using formulations FA (solid black line) and FB (dotted black line). Red and green shaded areas are confidence intervals for FA and FB, respectively, while red and green dots are experimental data points. Probability to die is explored under 2 scenarii (see Figure 1): (i) on the right panel, IVM is taken after the mosquitoes had fed on an infected host (the mosquito should die in 7 days following this last blood meal), (ii) on the left panel, IVM is taken while feeding on a treated host before feeding on an infected host (the mosquito should die in the 13 days following the IVM blood meal).

Effect of the formulation B on mosquito population in function of different field scenario

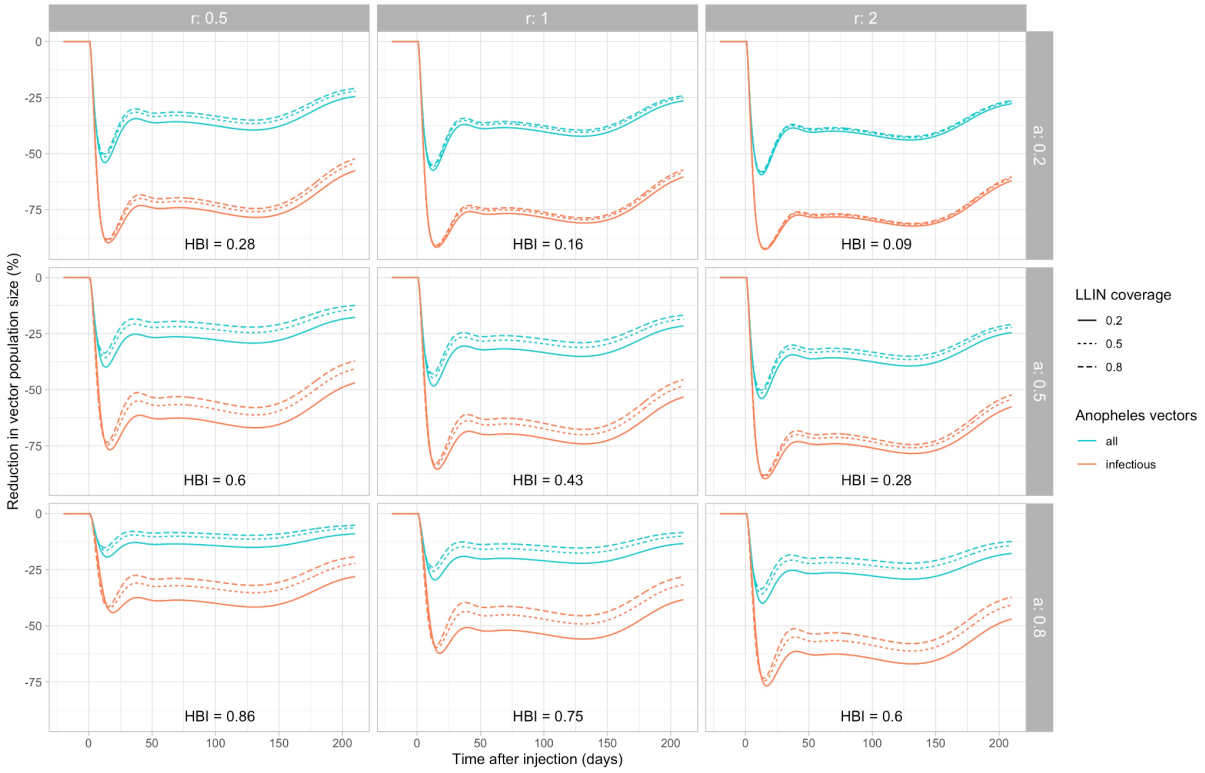

Figure 7. Model's prediction of malaria vector's population size reduction over time following a mass treatment of calves using the 6-months lasting formulation of Ivermectin FB. Different scenarii were tested where the ratio between calves and humans (r), the mosquitoes preference for human hosts (a), the LLIN coverage ratio and the infectious status of the vectors in the population were allowed to change to account for the great variability of field situations. r values of 0.5, 1 and 2 represent the scenarii where humans are present in twice, equal or half the number of the calves, respectively. The HBI (Human Blood Index) represents the realized proportion of blood meals taken on humans when parameterizing the model under the different scenarii. Here the values given are for a LLIN usage of 50%. The reduction of vector population size represents the added efficacy value of treating calves using Ivermectin BEPO® formulations and is given relatively to the basic LLIN intervention (coverage of 0.2, 0.5 or 0.8).

The added percentage of reduction in malaria vector's population obtained by treating calves using the 6-months lasting formulation of Ivermectin FB is given relatively to the use of bednets only and varies in function of the different tested scenarii (Figure 7). As it could be presumed, this One-Health approach would be the most efficient when the calves *vs* humans ratio is the highest and the vector's proclivity to feed on humans the lowest (*i.e.* lowest *a* value). For this scenario, the percentage of LLIN coverage has no effect on the treatment impact, and a supplementary 30-60% reduction of the whole mosquito population could be reached during the 6 months. On the contrary, the approach is the least efficient for contexts where few calves are present and the vector the most anthropophagic, in other words, when the probability that it takes at least one blood meal on a treated animal host during its lifespan is the lowest (around 10% more reduction than bed nets only).

The added value of treating animals using Ivermectin is influenced by the bednet usage when the vectors' population is the most anthropophagic and humans in greatest numbers: Ivermectin relative efficacy decreases when the LLIN use increases, in other words, when the vectors are already killed in great proportion by the bednets' insecticides. In all scenarios, a greater impact is expected for epidemiologically relevant mosquitoes, *i.e.* those potentially carrying *Plasmodium*. This stands because an infectious vector is at least 12-15 days old (which comprises the number of days before it takes its first (infectious) blood meal (2-3 days) and the extrinsic incubation period of the Plasmodium (8-12 days), and probably resumed 3 to 4 gonotrophic cycles starting by as many blood meals. Hence, the probability to ingest blood from a treated host is naturally increased for older infectious mosquitoes. The percentage of infectious population reduction, by comparison to the

use of bednets only, is therefore always greater than when the whole population of mosquitoes is considered, and could reach 75 - 95% for the first scenario (r=0.5, a=0.2) for instance.

For classical scenarios of west-african areas where main malaria vectors are predominant (which are highly anthropophagic), the LLIN coverage is high and humans are the most abundant host species, treating calves using a long lasting formulation of ivermectin lasting 6 months would enhance the reduction of the infectious vectors population by about 40% (but see the discussion section).

DISCUSSION

Our results establish the proof of concept that long lasting ivermectin prototype formulations using BEPO® technology could allow the release, from a single injection, of mosquitocidal plasmatic concentrations of Ivermectin that kill malarial vectors during at least 6 months. This sustained efficacy and the associated logistical ease for mass administration opens great perspectives to the One-Health approach in combating malaria [33].

For the two tested formulations, intra- and inter-animals variability in the pharmacokinetic profiles has been noted. Ivermectin plasmatic concentration fluctuation is not unexpected, and among the casualties, the release rate of the drug from the depot is likely not steady over the time course, and physiological factors, like body mass index, inherent to each individual animal may carry some degree of variability. The body fat, where Ivermectin is accumulated, could act as a reservoir from which Ivermectin is released, notably in function of the individual's metabolism

([34], [29]) as it is reported as well for interspecies variations of IVM metabolism, and related drug efficacy overtime ([35]). Recently, [36] showed that Ivermectin metabolization produces compounds that may extend the action of the core molecule in humans. Different IVM metabolizations occur among different species ([35]), and quantitative differences are likely to be found from an individual to the other. We showed that for specific ranges of ivermectin concentrations, identical plasmatic amounts seem to induce different mosquito mortality depending on which formulation is used.

As formulations' compositions are not yet entirely disclosable, their inherent quantitative or qualitative differences or their potential different induced ivermectin metabolites production cannot be assessed at this stage. Moreover, even with the random effect integrated in the model, the outcomes might be influenced by the limited number of calves per experimental arm. Further studies with the long-acting Ivermectin formulations, incorporating more animals per group, are definitely needed to understand release PK and efficacy variability as a function of formulation composition and dose, so the formulations' impacts on vectors' populations could be predicted as accurately as possible.

Previous attempts using the same technology allowed a year round release of microfilaricidal ivermectin concentrations active against natural infections of zebus by *O. ochengi* ([27]). These concentrations are reached as well by our present formulation, and collateral benefits of long lasting technologies could therefore comprise several other endo or exo-parasitic diseases of animals health concerns, including zoonoses that are transmitted to humans ([37]).

On a large-scale implementation perspective of this new tool for malaria control, technical and logistical gaps that were recently identified by the WHO

instances could then be circumvented (WHO, 2021, preferred product characteristics: endectocide for malaria transmission control, https://cdn.who.int/media/docs/default-source/malaria/vector-control/who-ucn-gmp-2021.11-eng.pdf?sfvrsn=7454d2c7_10), with both an Ivermectin concentration greater than the LC50 for Anopheles species and an efficiency duration over the rainy season or more. Of course, the added value of Ivermectin administered to animals as a complementary tool is not expected to be great in all field contexts. Working on suitability scores combining malaria prevalence, the densities of cattle and the presence of *Anopheles arabiensis* wich displays both zoophagic and anthropophagic feeding proclivities, [38] identified the sahelian zone in sub-saharan Africa as being the region which would benefit the most from this approach. However, the proportion of the *Plasmodium* transmission that is driven by *Anopheles arabiensis* is quite variable, depending in majority on its relative density over time and among other Anopheline species in the area, which is not taken in account in the former study.

Hence, to evaluate the potential impact of our formulation in the western african context where humans and livestock are often in close vicinity, we developed an efficacy model that is a population model, exploring our formulation efficiency at killing epidemiologically relevant mosquitoes in complement to bed nets, if calves of a village are all treated in a One-Health approach. We tested different reality-based field scenarios, where the product efficacy appears to be, logically, mainly a function of the proportion of vectors that actually fed on calves *vs* humans (*i.e.* the effective, realized blood meal, given the field constraints). Our model is not dynamic and mosquitoes that fall in a feeding compartment (human or calf) will remain on it whatever their success at feeding. This is simplistic and in the fields, among the

most anthropophagic vectors that prefer humans for their blood meal, a proportion is killed by the bed net that protects the host, some are successful at blood feeding despite the bed net (physiologically and behaviourally resistant mosquitoes), but some others behave following an uncharacterized pattern, among which a proportion of mosquitoes may be diverted from humans to alternative hosts like calves ([39]). This proportion of mosquitoes could be assimilated in our model as expressing a lower HBI than expected from their innate feeding preference. Hence, our current model probably underestimates the efficacy of our formulations because mosquitoes that present an anthropophagic preference are in fact plastic : their realized choice of hosts includes peridomestic animals. Such mosquitoes are actually represented in western sub-saharan Africa by the main vectors of *Plasmodium* like for instance *An. coluzzii* and *An. gambiae s.s*. In some villages, more than 50% of *An. coluzzii* and *An. gambiae s.s.* mosquitoes could take their blood meals from peridomestic animals ([40], in the vast majority from calves, despite an innate marked preference for humans reaching 0.8-0.9 in olfactometer based lab experiments ([41]). This has been associated with a high ratio of bednet use and the unavailability of humans while peridomestic animals were accessible to the bite in the vicinity. Underestimation of the IVM effect of our approach by the model should then be the greatest for the scenarios where the LLIN coverage is the highest, and vectors physiologically resistant to currently used insecticides, which is the scenario that is overrepresented in western Africa during the rainy season. Hence, the efficacy of treating peridomestic animals to control residual malaria transmission will capitalize on the zoophagic behavior of malaria vectors, whether it is innate or induced, and would be best assessed by characterizing their realized blood meals in the targeted area instead of considering innate feeding preferences. Assessing beforehands the

realized blood choice of the mosquitoes could orient the IVM treatments on any peridomestic animals, depending on the proportion of blood meals actually taken on the different available species.

Another slow releasing subcutaneously implantable ivermectin formulation has been designed ([42]) and is equivalent to ours in the release duration and the plasmatic concentrations that were reached in different animal species, which were mosquitocidal for at least 6 months ([43] [42]). However, the implanted solid units in Chaccour et al. studies required surgical incisions to be placed in the animals subcutis, whereas our long-acting formulation only required a standard injection device. Ivermectin approach to combat malaria is promising, and would be of course the most efficient if humans were to be treated together with animals or instead of them.

Among all formulations that have been tested so far, ours offers the unique advantage of being injected on a single act and of resorbing progressively while releasing active, mosquitocidal concentrations, equivalent to the therapeutic ones reported in the litterature for humans and animals treatments of parasitic diseases, for a duration that could largely encompass the *Plasmodium* transmission season. For these reasons, our formulation seems one of the best Ivermectin formulation candidates to be amenable until clinical phases in the near future. Increased dosages, multiple administration schemes, and a persistent implant, are indeed hardly compatible with field realistic scenarios.

Implementation of the “Ivermectin approach” to animals and/or humans, whatever the treatment scheme, will necessitate taking into account, ahead of the implementation, IVM resistance appearance risks, in the helminths parasites

populations that are classically treated using Ivermectin ([44] [37], but also in Anopheline populations. The selection pressure exerted by Ivermectin will dramatically increase if the approach was to be deployed, and Ivermectin resistance appearance would be only a matter of time, like it was the case for all other insecticidal compounds widely used to date. Ideally, mitigation strategies based on a careful resistance appearance follow up, using existing or to be found markers, should be proposed and implemented together with the approach, in all targeted field contexts.

Mitigation strategies should be explored as well concerning environmental toxicity that the IVM excreted in the treated hosts feces will undoubtedly provoke, with potential dramatic consequences on non targeted fauna, whether terrestrial or aquatic, among which are numerous species of dung degrading insects that are crucial for soil fertilizations ([45]. Ivermectin has been reported to be phytotoxic as well [46]). Today more than ever, we shall be concerned with these issues, even if ivermectin seems to be the panacee, potentially increasing human and animal's health at once in a theoretically virtuous loop, killing malaria mosquitoes and parasites responsible for humans and animals diseases that impair local development. Studies on ivermectin amounts released in the feces when calves are treated with already marketed and BEPO® ivermectin formulations are ongoing, with attempts to measure the contaminated dung toxicity on reference non targeted coprophagic species, which should give a first hint on associated risks in the fields (ANIVERMATE project https://anr.fr/Project-ANR-17-CE35-0013). Treatments and mitigation measures should be defined and developed with the help and assessment of local herders and peasants, as a community based integrated development.

Other vectors than malaria mosquitoes, biting humans and/or animals, are sensitive to Ivermectin as well ([37]), making this novel intervention a potential broader control measure targeting Malaria and other Neglected Tropical Diseases of zoonotic origin. Conceptually, there is, definitely, an opportunity for integrating this innovative tool within a One-Health context. In an approach where both humans and animals are treated, other endectocides or alternative class of molecules with ectoparasiticidal efficacy against the vectors of interest are possible mitigation strategies to address the risk of ivermectin resistance in both helminths and anopheles.

CONCLUSION:

Our study illustrates, by combining experimental and modeling approaches, that a formulation allowing the release of mosquitocidal concentrations of Ivermectin during 6 months could provide a great complementary tool to current approaches in malaria endemic areas where peridomestic animals are daily associated with human beings in urban like in rural environments. More studies are needed to better control inter-individual variability and initial bursts of concentrations, using new biopolymer combinations and *ad hoc* experimental design. By being able to adjust the released IVM concentration and its duration, the BEPO technology offers virtually all possible time *vs* concentrations possibilities to be efficient in numerous epidemiological contexts. Ultimately, a technological transfer of such formulation to humans and a concomitant treatment of humans and animals would definitely impact malaria transmission with the strength needed to have substantial effect on incidence, in ranges expected by the WHO.

Conflict of interest

The patent related to the formulation used during this study belongs to MedinCell S.A. There is no conflict of interest between the co-authors and present or past affiliation with MedinCell and the co-authors affiliated at the IRD, the CIRDES and the IRSS. Co-authors affiliated with IRD, CIRDES, or IRSS have no specific interest (i.e., shares) or commercial relationship (i.e., consulting) with MedinCell. In the event of a commercial development of the long-acting formulation of IVM described in the present publication, MedinCell would benefit from the scientific outcomes of the present study. However, the co-authors with present or past affiliation at MedinCell did not contribute to the data analysis, interpretation of results, and related conclusions and opinions, which reflects the views of scientists from IRD, CIRDES, IRSS and University of Dédougou only.

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