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TESI DI DOTTORATO DI RICERCA

A new strategy for mosquito biocontrol based on the improvement of *Bacillus thuringiensis* effectiveness

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Summary

Abstract

Mosquitoes are insects with a worldwide geographical distribution. Several species are known to spread animal and human illnesses, such as the Zika fever, Dengue fever, and malaria. This is due to the vectorial competence of adult females that is the ability to acquire a pathogen from an infected host during a blood meal and transmit it to an uninfected host during the following meals.

Several anthropic activities have favoured the diffusion of invasive mosquito species of sanitary importance to non-endemic areas raising concern due to the increasing number of outbreaks of neglected diseases in these regions. Due to the lack of vaccines and prophylaxes strategies, mosquito management is a pivotal aspect to control the spread of these diseases. Beside adult stage management, larval management is also extremely important to decrease the density of adult populations and to prevent the spread of neglected diseases. However, biorational strategies for their control are still scarce. To date, the most effective bioinsecticides targeting mosquito larvae are based on the entomopathogens *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *Lysinibacillus sphaericus* (*Lsph*). These products, active by ingestion, include *Bti* alone or in combination with *Lsph* and are safe for the environment while displaying a high and specific toxicity against the larvae of Culicidae. The key issues related to their use is due to *Bti* short residual activity after the application and to the possibility of the onset of resistance phenomena in target population. To preserve the efficacy of these formulations it is necessary to develop proper delivery systems able to protect the active ingredients maintaining their effective concentration. With these purposes we developed MosChito raft, a biodegradable floating hydrogel constituted of molecules of natural origin, namely chitosan and genipin. This product has been designed to include a *Bti*-based formulation to induce larval toxicity, *Saccharomyces cerevisiae* cells to induce phagostimulation and air bubbles to allow its flotation in water. Our results have shown the efficacy of MosChito rafts in laboratory conditions and in semi-field conditions against laboratory colonies and colonies with a genetic background comparable to that of natural populations of the Asian tiger mosquito, *Aedes albopictus*, and the common house mosquito, *Culex pipiens*. Differently from what stated for the *Bti*-based product how it is, its inclusion in MosChito raft prolongated its efficacy of at least 15 times (i.e., from two days to one month) solving one of the main issues related to its application. Beside what we expected, yeast cells inclusion did not improve the attractiveness of the hydrogel, anyhow, their role can be rethought as biofactory to produce and administrate immune-modulating double-stranded RNA molecules with the aim of weaken larval immune system while boosting the toxicity of *Bti*. Further studies must be carried out assessing the ecotoxicological effects of our product against

off-target organisms and in field conditions. Looking at the results obtained so far, MosChito rafts could represent a valid alternative for the larval control of two highly anthropophilic mosquito species of sanitary importance at the same time in the urban context.

1. Introduction

1.1 Biology and ecology of mosquitoes (Culicidae Meigen, 1818)

Culicidae (Meigen, 1818) is a family belonging to the suborder Nematocera (Diptera Linnaeus, 1758) that encloses those insects commonly referred to as mosquitoes (figure 1). These animals are characterized by having a single pair of wings and a couple of thin and filamentous antennae [Scortecci 1960, Foster and Walker 2019, Mullen and Durden 2019]. Culicidae comprehend in turn two subfamilies, Culicinae and Anophelinae. To date, there are a total of 113 genera and 3618 formally recognized mosquito species in the Valid Species List of the Mosquito Taxonomic Inventory (updated 06 January 2024) [Harbach 2024]: 110 of the 113 genera belong to the Culicinae subfamilies.

Mosquitoes are holometabolous insects where commonly immature stages and adults do not share the same environmental niche (figure 2) [Howard 1901].

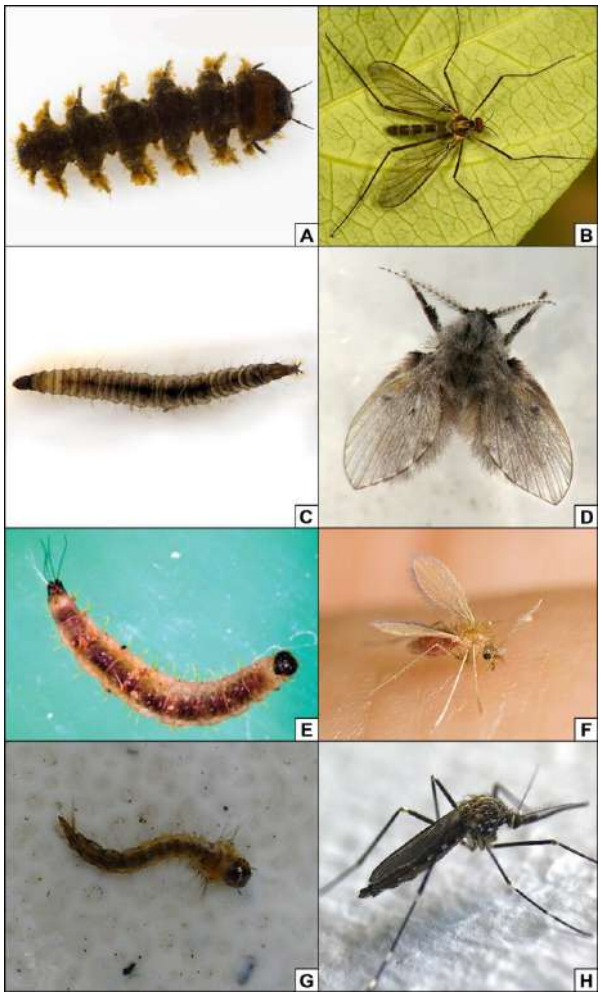

Figure 1: larvae and adults of *Agathon comstocki* (Diptera: Blephariceridae) (A, B), *Clogmia albipunctata* (Diptera: Psychodidae) (C, D), *Phlebotomus perniciosus* (Diptera: Psychodidae) (E, F), *Aedes japonicus* (Diptera: Culicidae) (G, H).

Holometabolism, also called complete metamorphosis, is a developmental strategy in which the immature stages (larvae) do not morphologically resemble the imago (adult), and metamorphosis from larva to adult takes place during a pupal stage (figure 2). Depending on the species, mosquito eggs can be laid individually or in clusters and their shape varies from ovoid to spherical or spindle-like (figure 3 A-C).

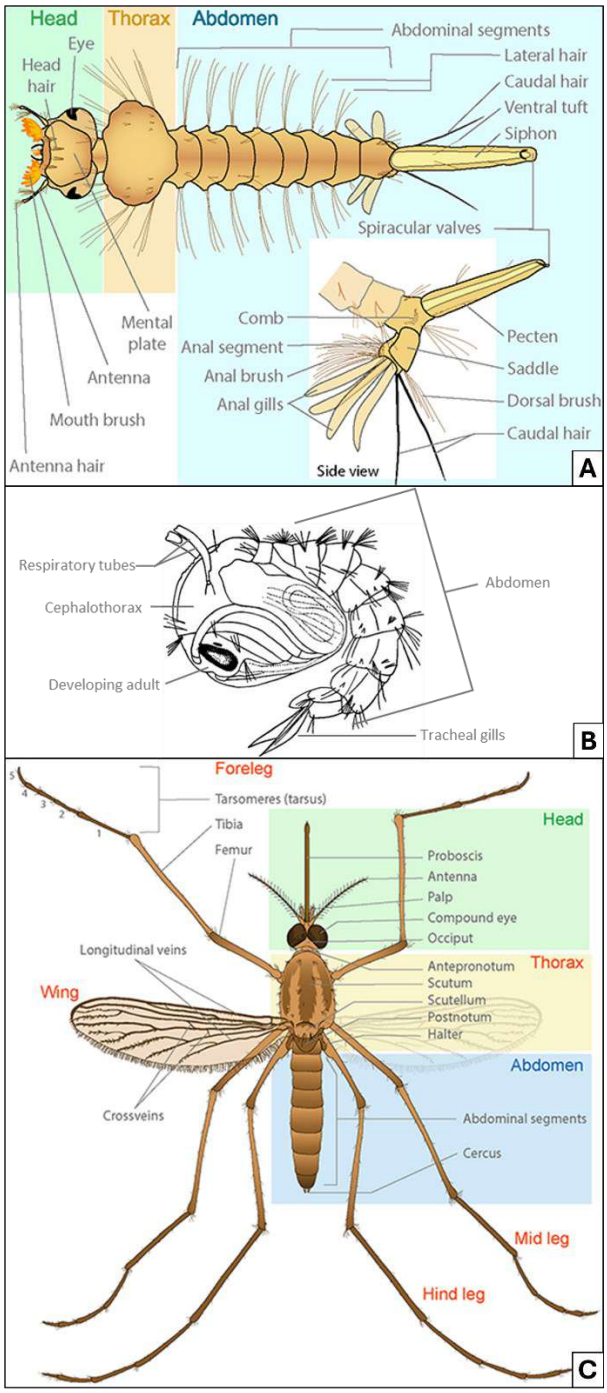

Figure 2: general representation of the different developmental stages of mosquitoes. Some of the main morphological structures are pointed out by arrows. A) larva, B) pupa, C) adult female. A) and C) credits: LadyofHat (http://ladyofhats.com/)

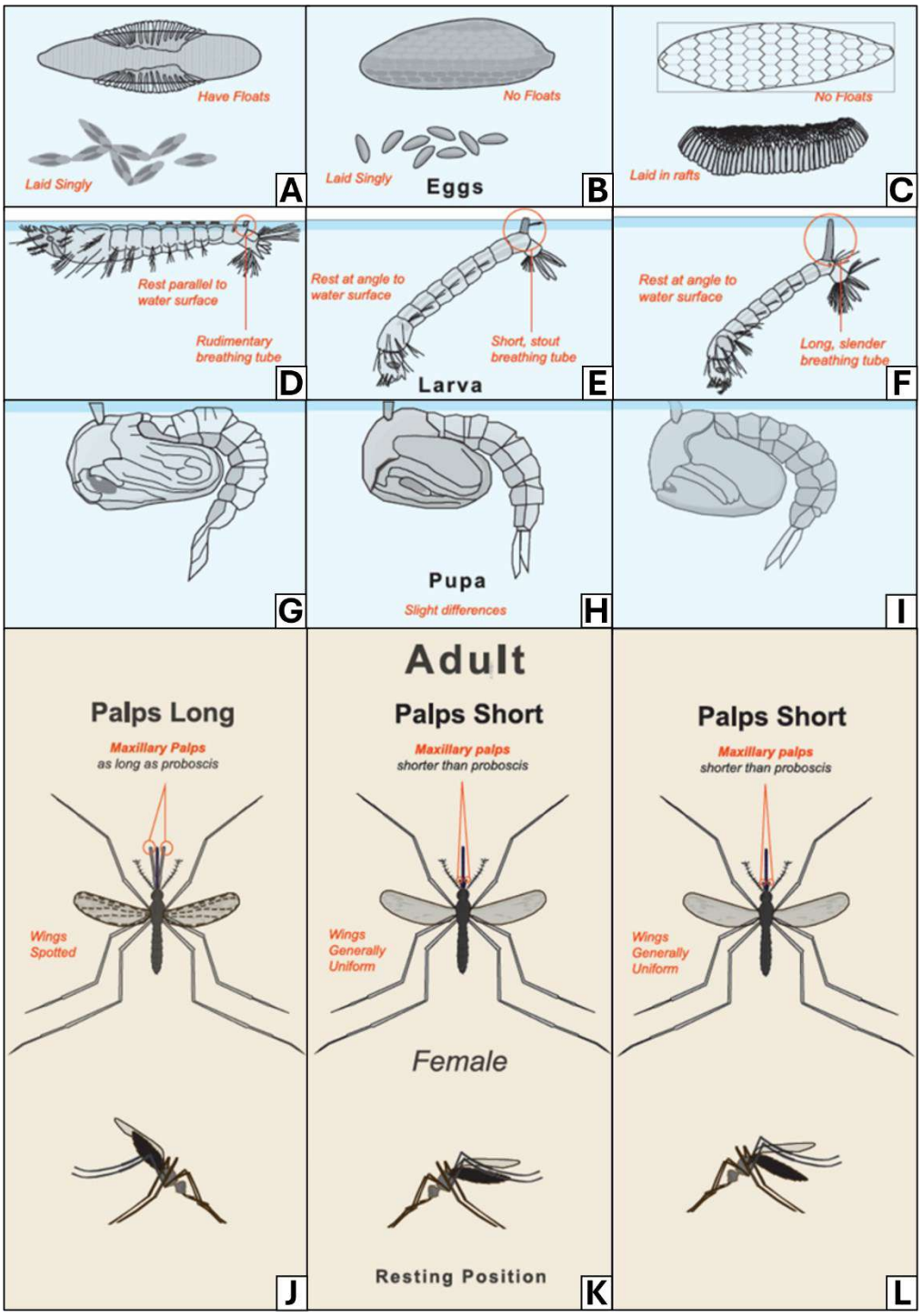

Figure 3: morphological differences among the developmental stages of three different genera of mosquito. A, D, G, J: eggs, larva, pupa, and adults of *Anopheles* mosquitoes; B, E, H, K eggs, larva, pupa, and adults of *Aedes* mosquitoes; C, F, I, L eggs, larva, pupa, and adults of *Culex* mosquitoes. Modified from Global Learning and Observations to Benefit the Environment (GLOBE) 2018.

Eggs are oviposited individually on the water surface by the females of the subfamily Anophelinae and in many species of the subfamily Culicinae, while several other species belonging to the Culicinae oviposit the eggs aggregated to form a floating raft (figure 3 A-C). The eggs of the genus *Mansonia* are layed clustered and anchored to the aquatic vegetation [Day 2016]. The time necessary

to complete the embryonic development and hatch can vary from two days to more than a week. Aedini tribe represents an exception since their eggs are layed above the water surface on a substrate that will probably be submerged in future [Day 2016, Bova et al. 2019, Foster and Walker 2019]. These eggs can resist months or even years to cold and desiccation into a quiescent status until they are submerged by water, which is the main stimulus to hatch [Denlinger and Armbruster 2014].

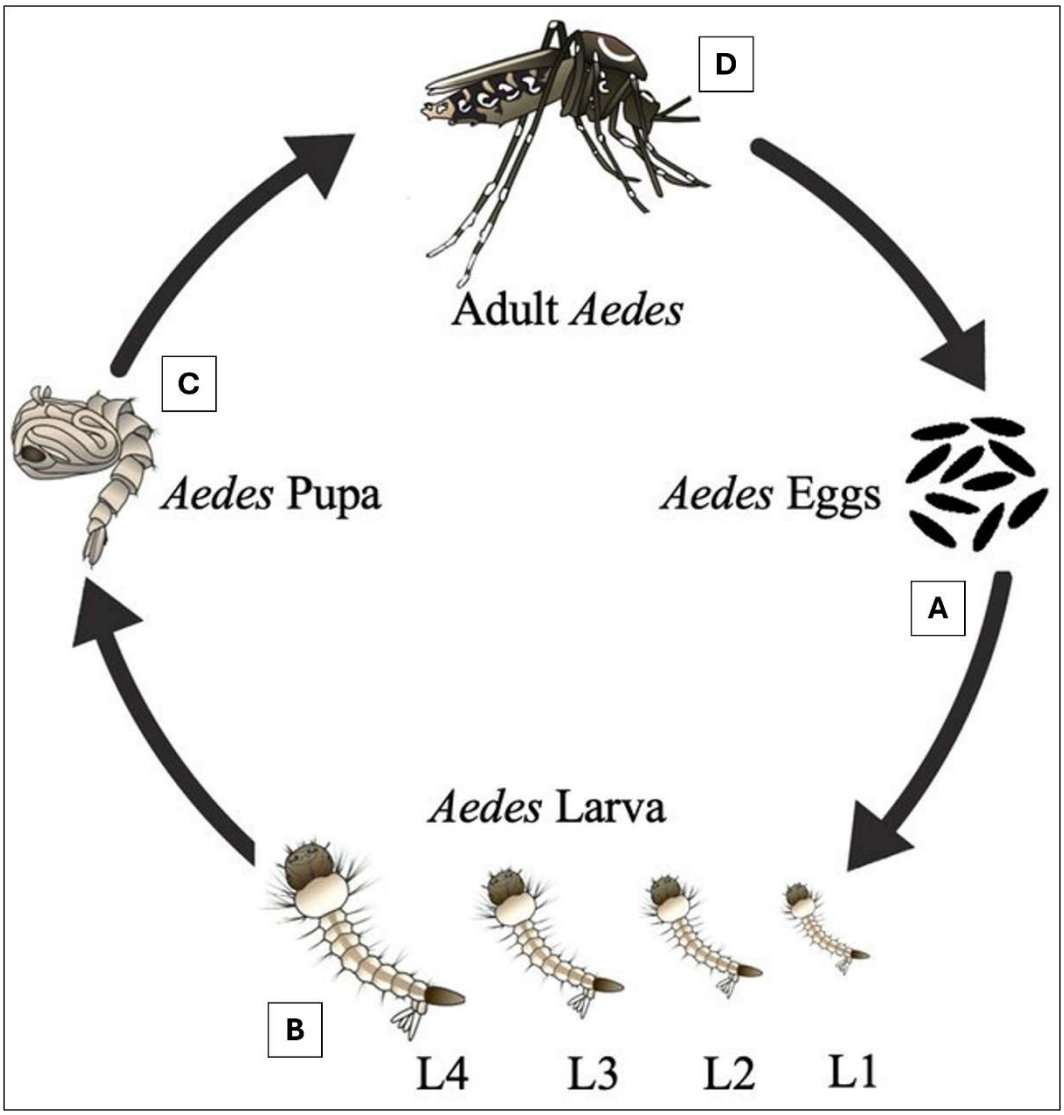

Figure 4: postembryonic development of *Aedes* mosquitoes. After egg (A) eclosure, the mosquito develops through 4 larval stages (B), followed by a pupal stage (C). From the pupae emerge the adults (D) that mate and reproduce. Adult females (D) need to take multiple blood meals on mammal or bird hosts to trigger eggs development. Then, they lay the eggs near stagnant water since larval and pupal stages are aquatics. Modified from Hossain et al. 2022.

The larva hatches from the egg and passes through 4 instars morphologically similar aside for the increase in size (figures 3 D-F, 4) [Christophers 1960, Carvajal-Lago et al. 2021]. The head bears a pair of visual organs, chewing mouthparts, and a couple of antennae. The body is segmented with a bulbous thorax and a

cylindrical abdomen that ends with an anal segment where 4 anal papillae (or anal gills) are present (figures 2, 3 D-F). These structures contain the chloride cells that enable the larvae to acquire salt and water from the aquatic environment and thus contribute to insect homeostasis maintenance. A pair of spiracles or an elongated siphon is located on the dorsal side of the terminal part of the body (figures 2, 3 D-F). The 4th instar larva moults into the pupa which is still aquatic and able to swim but does not feed. The shape of the pupa resembles that of a comma (figures 2, 3 G-I, 4). The head and the thorax are fused together into a cephalothorax, and a pair of respiratory tubes extends from the mesothorax [Scortecci 1960] (figures 2, 3 G-I). When the adult is fully developed, it emerges from the pupal exuvia. Adults have a slim body with long and thin legs [Foster and Walker 2019]. On the head are present a pair of compound eyes and a pair of long and filamentous antennae that usually display sexual dimorphism (figure 3 J-L). The mouthparts of the adult are modified into a long proboscis where are located the stylets used by the female to sting the host skin in blood-feeding species. The pattern formed by scales, setae, and fine pile that cover the body surface is different in each species. The abdomen is elastic to accommodate large volumes of blood or sugar solution and terminates with the opening of the reproductive organs. Mosquito species can be found everywhere on the globe except for the Antarctica continent, with a higher density found in tropical and subtropical regions [Rossati 2017, Laporta et al. 2023] (figure 5).

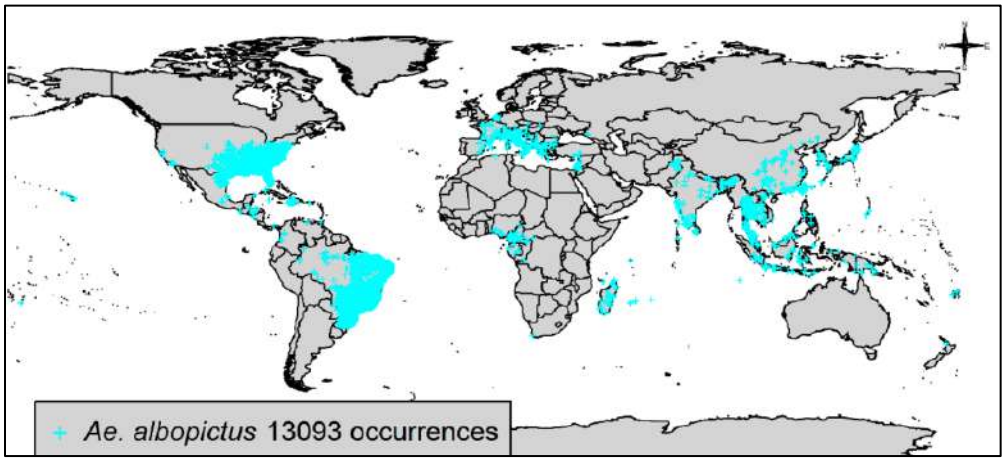

Figure 5: *Aedes albopictus* occurrence map updated to 2023 based on adults and larvae detection data. From Laporta et al. 2023.

Culicidae larvae are adapted to aquatic environments that can range from ephemeral to permanent water bodies in natural environments or artificial containers. Their dietary habits can also range from detritivorous to predatory behaviours. In the former and most common case, the larvae feed on microorganisms and particulate organic matter that they filter from the surrounding environment, while in the latter case they prey on larvae with also cannibalism phenomena where intraspecific competition is high [Harbach 2007,

Chandrasegaran et al. 2020]. Pupae do not feed but rely only on the nutrients stored during the larval stage. They float thanks to the presence of an air pocket, allowing them to stay just below the surface of the water body and acquire oxygen from the troposphere [Mullen e Durden 2019]. They are also able to actively dive deeper as an anti-predatory behavioural response or in response to other environmental stimuli [Brackenbury 1999, Rodríguez-Prieto et al. 2006, Awasthi et al. 2012]. Adults eventually emerge from pupae. They are glycyphagus and forage the sugar they need mainly from nectar and honeydew. Adult females of most species are also obligate hematophagous, as they require a blood meal on a vertebrate host to complete oogenesis [Foster 1995].

The duration of each developmental stage varies significantly according to environmental conditions, even intraspecifically [Jepson et al. 1947, Crans 2004, Wilkerson et al. 2021]. Once the larva hatches, it takes a minimum of 5 days to pupate in species that inhabit warmer habitats and up to three weeks in species confined to cooler regions. In extreme cases the larval stage can last for months. Pupal stage lasts generally from two to more than 6 days. Adult mosquito males usually emerge from the pupae before the females. At the time of emergence, the individuals of some species are already sexually mature and start to mate [Kliewer et al. 1966, O'Meara and Lounibos 1981], while in other species sexual maturation can takes hours as well as up to 4 days after emergence to be completed [Smith and Gadawski 1994]. To copulate, males usually form flight swarms where females are attracted by the release of a volatile pheromone [Downes 1969]. Nevertheless, this is not the only reproductive strategy of Culicidae. Adults' lifespan varies depending on the species, environmental factors, but it is also strongly affected by the energy acquired from the food sources by the larvae as well as the energy spent during the immature stages to complete the life cycle (figure 4) [Nayar and Sauerman 1971, Wilkerson et al. 2021]. It is also common a sexual dimorphism on this trait: in fact, adult females tend to live longer than males [Tsuda et al. 2001]. Furthermore, in many species, adults enter diapause, a state of halted or retarded development to overcome the cold period of the year [Denlinger and Armbruster 2014].

1.2 Impact of mosquitoes on human and animal health worldwide and in Italy

Culicidae represent a high public health concern because of their high vectorial competence. Specifically, pathogens can be vectored to humans and animals through the bite of an infected adult female during the blood meal. Indeed, the mosquito is considered one of the deadliest animals on the planet with over 700,000 indirect deaths caused per year (with peaks in tropical and subtropical regions, especially among poorer populations) [WHO 2020]. There has been a

strong vector-pathogen coevolution so that mosquitoes of the genus *Aedes* are responsible for the transmission of arthropod-borne viruses (arboviruses) that cause Zika fever, yellow fever, Rift Valley fever, dengue, and chikungunya that are respectively Zika virus (ZIKV), Yellow fever virus, Rift Valley fever virus, Dengue virus (DENV), and Chikungunya virus (CHIKV) [Jánová 2019]. The etiological agents of Japanese encephalitis and West Nile fever are vectored by mosquito of the genus *Culex*, while malaria, the most known and studied neglected disease, is caused by different species of protozoan belonging to the genus *Plasmodium* that can be transmitted by *Anopheles* mosquitoes. These three genera of mosquito and *Mansonia* include species able to vector different nematodes that cause lymphatic filariasis, i.e., *Brugia* spp. and the most known *Wuchereria bancrofti* [WHO 2020, WHO 2023, Mathison and Sapp 2021]. The latter can also be vectored by *Coquillettidia juxtamansonia*. The nematodes *Setaria* spp. and *Breinlia annulipapillata* can also be vectored by some mosquito species and are the etiological agents of ocular filariasis [Nabie et al. 2017, Koehler et al. 2021].

These parasites exhibit a huge variability in their life cycles. Typically, juveniles are acquired from an infected host by the vector, which is called intermediate host. Once they reach the infective stage they can be transmitted to the definitive host and complete the development, and the sexual reproduction can take place: this is the time that clinical symptoms can manifest (diagnostic stage). Once new juveniles are produced, they migrate into the host's blood stream to reach the peripheral vascular system where they are ready to be acquired by an uninfected mosquito female during a blood meal. The disease can also affect dead-end hosts species, accidental hosts in which the parasite is not able to complete its life cycle living as juvenile until its dead (or the death of the parasitized host) [Knauer 1998]. Human beings, cattle, and pet animals (but not only them) can represent the definitive host or a dead-end host in the lifecycle of the pathogen depending on the parasite. Moreover, many vertebrate species can represent a reservoir for the parasite, usually remaining asymptomatic [Baum 2008, Mandl et al. 2015].

Arboviruses have a different lifecycle: they are maintained in a horizontal transmission cycle between mosquito female and the vertebrate host [Jánová 2019], although some cases of vertical transmission have been also reported [Lequime and Lambrechts 2014]. Arboviruses are acquired by the female during the blood meal on an infected host. The virus colonizes the intestinal epithelial cells and starts to multiply. After that, the virus spreads systemically and reach the salivary glands where its copies grow exponentially. At this point, when the mosquito female takes the blood meal, virus transmission to the new host with the salivary secretion occurs. So, it replicates and spreads in the new host and the cycle starts again [Wu et al. 2019].

Since pathogens reproduce at the expenses of the host, they trigger its immune system to vanquish them: in mosquito, the immune pathways involved in response to the abovementioned viruses and parasites are Toll, immune deficiency (IMD), Janus kinase/signal transducers and activators of transcription (JAK-STAT), and RNA interference (RNAi) pathway [Mussabekova et al. 2017]. Nevertheless, if the pathogens suppress the immune response of their host, they could expose it to infection by other organisms and viruses being counterproductive. Accordingly, evolution rewarded those arboviruses and parasites that managed to reproduce inside the mosquito by evading its immune system, even though the mechanisms at the base of this phenomenon are still not completely clear. Moreover, mosquito susceptibility to infection is an unstable equilibrium that vary greatly in each population since it depends probably by environmental factors as well as by a genotype-by-genotype interaction that occurs among vector and pathogen [Bartholomay et al. 2010, Sim et al. 2014].

Finally, many mosquito species can occasionally act as phoront for at least one dipteran insect. Phoresy is the mechanism by which a larger individual transports an organism of another species to find a new host or a food source. The adult mosquito can vector mechanically the eggs of *Dermatobia hominis* (Diptera: Oestridae). This insect causes myasis in many cattle species as well as in humans and other warm-blood species as birds [Goldman 1945, Marchi et al. 2012, Alencar et al. 2017].

Development of vaccines or other prophylaxis strategies for mosquito-borne diseases is very challenging due to several aspects: the great number of pathogens vectored by Culicidae and their fast biological cycles, the different serotypes of arboviruses, the different organisms involved in their life cycles and their respective immune systems, globalization of tropical areas and the removal or loosening of commercial borders that cause the enlargement of mosquito areal, the impossibility to avoid contacts with mosquitoes and so on. To date, effective vaccines against mosquito-borne diseases are available only for Yellow Fever Virus and Japanese encephalitis virus [Wiwanitkit 2007, Ferguson 2018, Huang et al. 2019]. Nowadays, the best way to reduce the socioeconomic burden of these neglected illnesses remains the control of mosquito populations.

1.2.1 Invasive mosquito species

During its 4.5 billion years of history, Earth has passed through climate changes. However, nowadays almost the entire scientific community agrees that we are facing a human-caused climate change [Lynas et al. 2021, Myers et al. 2021]: the raise of temperature registered during the last decades naturally would need centuries to take place. We are moving toward a hotter future. Global warming

together with globalization and the removal of trade borders allowed many species to expand their areals [Hulme 2009, Seebens et al. 2017, Bertelsmeier 2021]. Outside of their natural areal these species are considered alien species. If they manage to invade and reproduce in a new region, they are referred to as invasive alien species. When they successfully colonize a new areal, they occupy free ecological niches or undermine an endemic species by competitive exclusion [Gioria and Osborne 2014]. Usually, the success of invasive alien species is determined by a combination of characteristics as the high water and nutrient acquisition ability, high phenotypic plasticity, dietary generalism, fast life cycle and the lack of local predators as well as a great dispersal capacity [Davidson et al. 2011, Gioria and Osborne 2014, Mathakutha et al. 2019]. However, the outcome of whether an alien species will become invasive or not depends on the place and the time of invasion [Mathakutha et al. 2019].

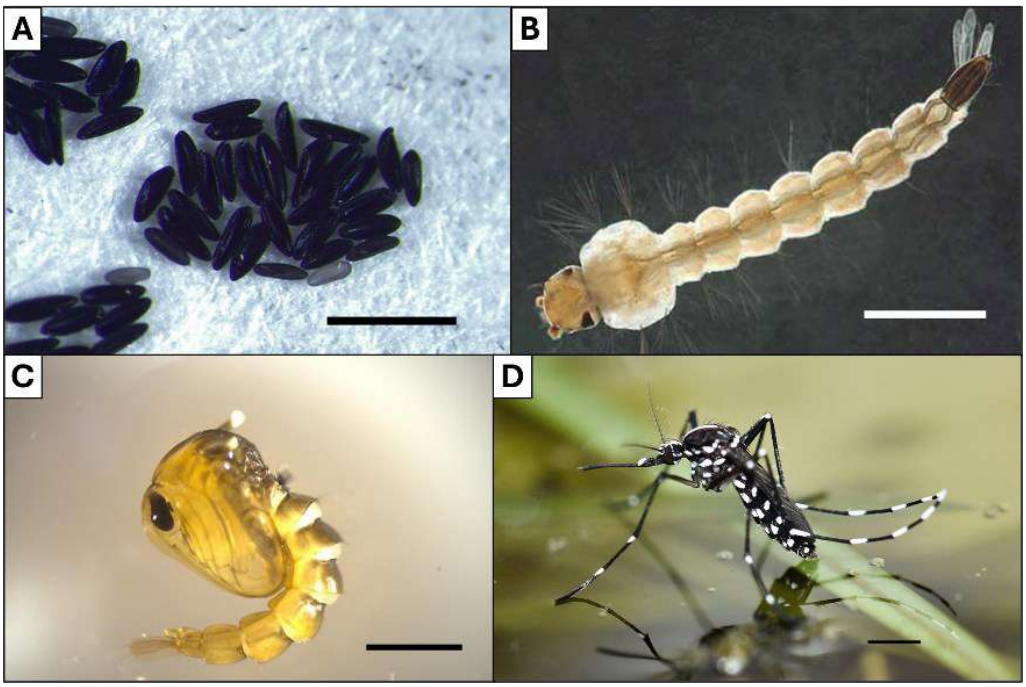

Figure 6: pictures of the different developmental stages of *Ae. albopictus*: A) eggs clutch, B) larva, C) pupa, D) adult mosquito. In D is clearly visible the white stripes pattern that allows the morphological identification of this species. Bars: 1 mm.

The impact of climate change on pathogens depends on the effect it has on their hosts and vectors [Finch et al. 2021]. Extreme climatic events are increasing in frequency and create the conditions for invasive alien species to settle down [Masters and Norgrove 2010]. By consequence, pathogens can enlarge their area of distribution causing diseases in populations that have never faced these organisms [Campbell et al. 2015]. Indeed, the ecological and socioeconomic impact of invasive species could be heavy. In addition, invasive alien species are one of the main causes of biodiversity loss together with climate change [Masters and Norgrove 2010]. Their cumulative effects are even worse. The damages can be

direct or indirect (i.e., caused by the pathogens they vector) and range from the already mentioned biodiversity loss to the destruction of entire crops or livestock and new epidemics in non-endemic areas.

Aedes albopictus, also called Asian tiger mosquito, is an extremely annoying and well-known invasive alien species. This Culicidae is native to the tropical Asian forest, but it spread to the point that is now cosmopolite [Watson 1967]. The spread of this species, its development, and survival are boosted by the higher temperatures (figure 6) [Jánová 2019].

1.2.2 Mosquitoes present in Italy

Italy is considered one of the major biodiversity hotspots globally since it falls almost completely within the Mediterranean Basin hotspot. This is confirmed by the presence of 25'000 plant species of which 13'000 are endemic to this area, representing the 4.3% of the known plant species globally [Myers et al. 2000]. As for the flora, Italian topography and climate offer a wide variety of habitat for many faunal species, with the Culicidae finding ideal conditions in marshy areas, ephemeral pools of brackish water, and glacial lakes scattered throughout the country.

Severini et al. in 2009 published a massive work describing the Italian mosquito fauna, with a focus on those of medical importance, taxonomical keys for the identification of various of these Culicidae and so on [Severini et al. 2009]. They described 64 species of mosquitoes belonging to two subfamily and 8 genera whose presence was reported in Italy: subfamily Culicinae is the most represented and includes the genera *Aedes*, *Ochlerotatus*, *Coquillettidia*, *Culex*, *Culiseta*, *Orthopodomyia*, and *Uranotaenia;* however, *Ae. albopictus* was not included in this list even if we know it arrived most probably in the early '90s.

In 2022, Severini et al. published what can be considered an updated version of the paper from 2009 [Severini et al. 2022]. The list of Italian mosquitoes increased from 64 to 65 species (with the addition of *Ae. albopictus*), included 8 species that are reported as not observed from at least 25 years in Italy. *Ae. aegypti* is one of these 8 species: its last sighting record dates back to the year 1971 and the previous were most likely due to accidental imports. It is also worth mentioning that even if the two species can be found in sympatry, *Ae. albopictus* is able to outcompete and replace *Ae. aegypti* under certain environmental conditions [Juliano 2010].

In 1970, Italy has been added to the "WHO official register of areas where malaria eradication has been achieved" and it is nowadays considered a malaria-free country. However, among the 65 Italian mosquito species, the presence of *An. labranchiae*, *An. superpictus*, and *An. atroparvus* is still reported. These species played a crucial role in the past as vector of the malarian *Plasmodium vivax* and *P.*

falciparum in Italy [Romi et al. 2001, Severini et al. 2022]. The first two Anophelinae species were the main vectors together with *An. sacharovi* (that is no longer present in the country), while *An. atroparvus* has played a role as secondary vector for the spread of malaria in the country.

Within the Culicinae subfamily, *Ae. albopictus* is not the only alien species that became invasive in Italy: *Aedes koreikus* is a species that originated in North-Eastern Asia from where it expanded its areal and since 2011 is present in Italy and *Aedes japonicus* whose presence was limited almost only to Japan and Republic of Korea (South Korea) - no data are available for the Northern region of Korean peninsula since the mid XX century -, while since 2015 it is stabilized also in the Italian area [Montarsi et al. 2019, Gradoni et al. 2021, Arnoldi et al. 2022]. Differently from these two species, *Ae. albopictus*' distribution is not limited to the Northern Italian regions: its range expansion has accelerated rapidly during the post-war period with the advent of global trades. It started from East and Southeast Asia to invade other Asian countries, then it was imported from Asia in the '70s-'80s to the United States travelling as eggs in used tires [Enserink 2008], and from the US it migrates to Latin America. Moreover, Albania has been the first European country to record the presence of *Ae. albopictus* in 1979 which was probably imported from China. In Italy, the first record dates back to 1990 in Genoa. Nowadays, the Asian tiger mosquito can be considered endemic of the Italian peninsula (figures 5, 6) [Enserink 2008].

One of the major concerns related to the stabilization of alien hematophagous mosquito species is the possible importation of neglected diseases: given the expansion of the vector's areal, even the arboviruses or the parasites could expand or shift their distribution [Campbell et al. 2015]. West Nile virus - the etiological agent of West Nile fever - and Usutu virus - the etiological agent of Usutu virus disease that infect mainly birds, but with reported cases of human infections - are mosquito-vectored tropical arboviruses that can be considered endemic to Italy by this time. The first record of West Nile fever diagnosed in Italy is from 2008 and after that we had even up to more than 100 cases per year. Instead, CHIKV, DENV, and ZIKV are under surveillance in Italy due to the several imported cases and/or the outbreaks registered in the country over the past few decades. Autochthonous CHIKV infection outbreaks occurred in 2007 and in 2017 vectored by *Ae. albopictus* adult females [Bonilauri et al. 2008, Rezza 2018]. For what concern DENV, we had 11 autochthonous registered infections in 2020 [Del Manso et al. 2020], while in 2023 the cases of autochthonous infection rose up to 82 [Del Manso et al. 2023]. ZIKV presence in Italy, differently from the previous arboviruses, is due to imported cases only and no autochthonous outbreaks have happened in Italy to date. In the last decade, we had just two cases of autochthonous transmissions: the first in 2014 probably due to a sexual transmission and the second in 2019 has been

hypothesized to be due to a vertical transmission from the mother to the child [Del Manso et al. 2019]. These events are still sporadic, but it exists the possibility that in the near future their frequency and magnitude in non-endemic areas will increase facilitated by travels, trading, and climate change. In Italy, this risk is heightened because of the stable presence of *Ae. albopictus* and *Cx. Pipiens* in the territory [Di Luca et al. 2016]: local mosquitoes could transmit imported infections causing local outbreaks from which endemic transmission could be triggered.

1.3 Integrated management of insect pests (IPM) and vectors (IVM)

The necessity to improve pest control arrived between 19th and the 20th century with the advent of globalization and urbanization and the demand for agricultural product shifted from subsistence to commercial purpose. Pest control became pivotal to improve agricultural yield. Until the 1940s, mosquito population control was performed only using environmental and mechanical methods [WHO 2012]. During those years the insecticidal properties of dichlorodiphenyltrichloroethane (DDT), a neurotoxic organochlorine compound, were observed. The high toxic efficacy observed against insects and the erroneous belief related to its safety on off-target organisms induced people to a massive use of DDT-based insecticides (figure 7) [Cameron and Burgess 1945, Ware 1974].

Figure 7: picture showing a boy being dust with DDT in Naples, Italy, about 1944 [Stapleton 2005].

Over time the negative effects of the insecticide and its degradation products became known: off-target effects [Turusov et al. 2002], persistence in environment that can last decades [Jürgens et al. 2016, Quadroni and Bettinetti 2019] and several resistance phenomena in target mosquito populations [Moyes et al. 2017]. The ban of DDT started in 1970 in Sweden, while in the following years many other countries took the same decision [Council of the European Community 1979].

Nowadays we are facing the same problematics due to the massive use of chemical pesticides during the 1900's. Towards the mid-1900s the necessity to find alternatives to synthetic pesticides began to be felt and in 1966 Smith and Reynolds firstly defined the concept of Integrated Pest Management (IPM) as "a pest population management system that utilizes all suitable techniques in a compatible manner to reduce pest populations and maintain them at levels below those causing economic injury" [Deguine et al. 2021]. IPM is in use since 1970s with the aim of keeping pest populations density below a level that would cause excessive economic loss. European Union legislated redefined IPM in 2009 making its adoption in agriculture mandatory since 2014 [European Parliament and Council of the European Union 2009]. IPM can be schematized as a pyramid (figure 8) in which prevention strategies are prioritized over intervention strategies. In turn, interventions must be performed relying on environmental-friendly strategies first while resorting to more environmental-impacting strategies just in extreme situations [Oerke 2005, WHO 2012, European Parliament and Council of the European Union 2009]. So, IPM is a methodical approach that uses different levels of analysis to maximize the application of various tools and techniques to make insect control successful and sustainable.

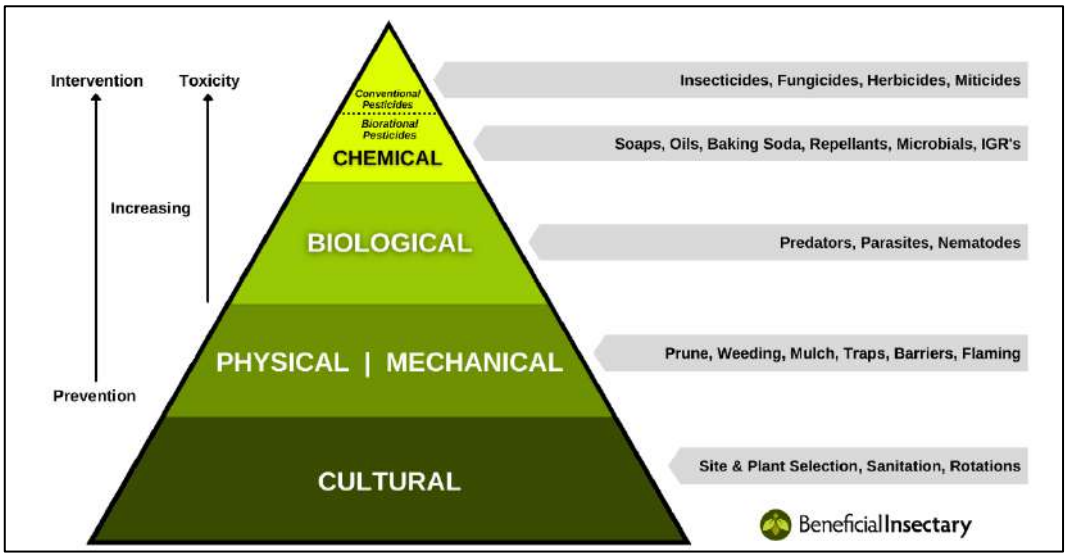

Figure 8: schematic representation of IPM and IVM strategies. The types of interventions vary from prevention at the base to intervention methods always more invasive as you move up. Toxicity as well as environmental risk rose while moving to the tip of the pyramid. From BeneficialInsectary (https://insectary.com/ipm/)

Integrated Vector Management (IVM) approaches, introduced soon after IPM, is based on the same pillars of IPM and focus on the control of disease vectors (figure 8).

A vector is a species that can carry and spread the causing agents of the so-called vector-borne diseases such as viruses, bacteria, or parasites that can be harmful to plants, animals, or humans [WHO 2014]. These organisms that can vector pathogens are usually arthropods and can cause severe diseases in humans, livestock, and pets [WHO 2014]. Arthropod vectors of relevance on human health comprehend, for example, triatomine bugs, also known as kissing bugs, that are the main vectors of *Trypanosoma cruzi*, the etiological agent of Chagas disease [Schaub 2021] and *Phlebotomus* spp. that are the main vectors of several species of the protozoans belonging to the genus *Leishmania* [Svobodová et al. 2009, Karmaoui 2020]. Several mosquito species can be at the same time noxious, because of the puncture of adult females and the subsequent hitch, as well as vectors because of viruses, bacteria, and eukaryotic parasites that can be vectored by mean of the puncture [Nabie et al. 2017, Jánová 2019, Koehler et al. 2021, Mathison and Sapp 2021]. IVM campaigns use as a basis the involvement of communities through citizen science projects, vector surveillance, and the management of larval stages beside the adulticide strategies [WHO 2012, WHO 2013]. As IPM, IVM implementation should have a pyramidal structure and thus the use of synthetic chemicals should be used when unavoidable, e.g. outbreaks of neglected disease [Oerke 2005, WHO 2012].

The use of vector control strategies is ruled by the "Biocidal Products Regulation (BPR), Regulation (EU) no 528/2012" that is a document produced with the aim to protect the environment and the health of people and animals of the European Union providing a list of active ingredients that can be used as biocidal products in the EU together with the conditions for the approvement of new ones [European Parliament, & Council of the European Union 2012, ECHA 2024].

1.4 Surveillance and control strategies of mosquitoes

The introduction of new allochthonous mosquito species may increase the frequency of new outbreaks of neglected diseases absent in Italy [Venturi et al. 2017, Rezza 2018, Lazzarini et al. 2020]. Surveillance programs are fundamental components of IPM and IVM strategies to prevent and eventually control mosquito-borne diseases to reduce their incidence and transmission [ECDC 2012, Ferguson 2018, Caputo and Manica 2020] as well as to detect as early as possible the presence of alien species [Osório et al. 2014, Yemshanov et al. 2019]. In the case of mosquito-borne diseases, surveillance is comprised of two separate yet interdependent functions: a first surveillance program monitor arbodiseases within animal and human populations to check the presence of pathogens and prevent

outbreaks, and to retrieve important data about them, for example, seasonality and geographic distribution. The second fundamental aspect is the surveillance performed on local mosquito populations for the acquisition of data related to virus activity in vectors and non-human vertebrate hosts, and other pertinent environmental data [Caputo and Manica 2020, CDC 2022].

In Europe, the adopted mosquito surveillance guidelines have been defined by the European Centre for Disease Prevention and Control (ECDC) and by the World Health Organization [ECDC 2012, WHO 2017a, WHO 2017b]. Sampling methods for mosquito surveillance can be categorized according to the stage being sampled, which includes eggs, larvae and pupae, and adults [WHO 2016].

Eggs are sampled by mean of the use of oviposition traps (or ovitraps) that are highly effective, low cost, and easy to use. Placing the ovitraps in the exact same spot every time is pivotal to observe the fluctuation in mosquito populations and their oviposition habits [Bellini et al. 2020]. Larvae and pupae are actively searched in possible breeding sites or passively sampled using funnel traps to identify the species composition of a given area and are essential for the individuation of larval hotspots. Indeed, adult samplings are important to obtain data related to species composition, their density and seasonality, and for the presence of pathogens. Sampling methods for adult mosquitoes include the use of mosquito bug nets, mouth or battery-powered aspirators, or the use of falling or sticky traps: attractant molecules, visual cues, or a combination of both are sometimes used to attract mosquitoes to these traps [WHO 2016].

1.4.1 Adult and larval mosquito control

At present, in Europe, mosquito control is performed both on larval and adult stages [Baldacchino et al. 2015, Roiz et al. 2018]. If on the one hand adult populations management is carried out to decrease mosquito-transmitted disease burden and to reduce the nuisance derived by their puncture, on the other hand the management of larval stages has the advantage to be acted in advance, preventing their development to the vectorial stage [Roiz et al. 2018]. Moreover, larval control is more targeted than adult management because of the confined nature of larval breeding sites. Anyway, they are both fundamental for IVM strategies and the available management methods that can be used in Europe can be subdivided as follow:

- Environmental: these are the easier and cheaper methods to perform larval control. The aim is to reduce oviposition sites and immature stages breeding sites such as containers with ephemeral accumulation of water (for example flowerpot dishes and bucket) or small permanent water basins that can become hotspot for mosquito proliferation (figure 9) [Richards et

al. 2008]. The operations consist in the constant emptying or, when possible, the overturning of the containers and the cover of the basins [Randell et al. 2010]. Since it is not uncommon to find these elements in private properties, the inclusion of the citizens plays a pivotal role to obtain a positive outcome with environmental control as well as monitoring and repetitiveness. This is particularly true for what concern the control of highly anthropophilic species (e.g., *Ae. albopictus*) whose larval stages live in unpredictable and ephemeral habitats such as containers in domestic and peridomestic areas [Abramides et al. 2011, Healy et al. 2014, Faraji and Unlu 2016, Baldacchino et al. 2016]. Indeed, WHO prompted an increase in citizen involvement and education concluding that community engagement is essential for the effective accomplishment of IVM strategies [WHO 2012, WHO 2004].

Figure 9: examples of possible *Ae. albopictus* mosquito larval breeding sites. A) tree hole, B) leaf axil, C) decommissioned tyres, D) flowerpot saucer.

- Mechanical: these methods which rely on the use of traps are mostly cheap and target adult mosquitoes. Traps can target gravid females looking for an oviposition site or adult females in seek of a partner to mate with or a host to take a blood meal [Lühken et al. 2014]. For the first case can be used both sticky traps, on which the individuals stick and die, or gravid traps which offer insecticide-treated oviposition sites, while for partner/host seeking females are used BG-sentinel traps (Biogents) that however necessitate an electrical power

supply to function [Li et al. 2016]. These traps can also be implemented by the addition of attractant molecules [Dormont et al. 2021].

- Chemical: to date, the chemical methods for mosquito control in Europe rely on the use of Insect Growth Regulators (IGR) as larvicidal and ovicidal [Mulla 1995, Suman et al. 2013, ECHA 2024] or the use of pyrethroids as adulticides [Faraji and Unlu 2016, Bellini et al. 2020]. IGRs are insecticidal molecules that delay or inhibit insect development [Mulla 1995, Suman et al. 2013]. Two widely used IGRs are chitin synthesis inhibitors (e.g., Diflubenzuron), and analogues of the Juvenile Hormone (e.g., Methoprene) causing morphological aberrations [Lawler 2017, Sankar and Kumar 2023]. These molecules are safe for non-target organisms and are implemented in IVM targeting larval sites, but they can also be used for the so called autodissemination where adults are exploited as carriers for IGRs to oviposition sites [Bellini et al. 2009, Devine et al. 2009, Seixas et al. 2019]. Even though some IGRs are implemented in national and regional mosquito management plans in Italy and can be directly applied to larval breeding sites [Ministero della Salute 2019, Regione Emilia-Romagna 2023, Regione Veneto 2023a, Regione Veneto 2023b], the observation of resistance phenomena in several mosquito populations have brought to the cease of their use in certain areas or the application mandatorily in combination with microbial bioinsecticides in other areas [Porretta et al. 2019, Fotakis et al. 2020, Mastrantonio et al. 2021, ECDC 2023].

  Pyrethroids are neurotoxic synthetic chemicals derived from pyrethrins, natural insecticide molecules produced by certain chrysanthemum plants [Dorta et al. 1993, Bradberry et al. 2005, Gajendiran and Abraham 2018]. Their toxicity is high already at low dosage but poorly specific as it also affects off-target organisms such as non-pest insects, other arthropods, and aquatic organisms [Gajendiran and Abraham 2018, Corcos et al. 2019]. In addition, resistance phenomena in target insect populations have been also recorded [Pichler et al. 2017, Amelia-Yap et al. 2018]. While pyrethroid-based products are sprayed for ground treatments in IVM programs, Europe allows aerial treatments with any kind of pesticides only during outbreaks [European Parliament and Council of the European Union 2009]. Beside pyrethroids, picaridin (also known as icaridin or KBR 3023), N,N-Diethyl-metatoluamide (DEET), and ethylbutylacetylaminopropionate (or IR3535) are chemical molecules that can be used as repellent for individual protection or to produce insecticide-treated materials [Ministero della Salute 2019, ECHA 2024].
- Biological: these methods rely on the use of mosquito natural enemies. The organisms used include for example entomopathogenic bacteria and fungi or their products, essential oils, and predators such as fishes and copepods. Bacterium-based mosquito management rely on the use of *Saccharopolyspora*

spinosa, *Bacillus thuringiensis* (*Bt*) and *Lysinibacillus sphaericus* (*Lsph*, formerly known as *Bacillus sphaericus*) [Valtierra-de-Luis et al. 2020, Silva-Filha et al. 2021, Hernández-Santana et al. 2022]. Spinosad, an insecticide obtained through the fermentation processes of *S. spinosa*, contains spinosyn A and spinosyn D as active components, which are known for their efficacy against mosquito larvae [Hertlein et al. 2010, Bacci et al. 2016]. These neurotoxic molecules are applied into water containers. Spinosad shows a high toxicity and lack of cross-resistance in mosquito populations resistant to other neurotoxic pesticides. Nevertheless, its photosensitivity and its adsorption by particulate matter are high and can lead to a fast degradation of the product when released in the environment [Santos and Pereira 2019, European Commission 2022]. Different varieties of *Bt* which target different organisms are known and the one specific against mosquito larvae is *Bt*var. *israelensis* (*Bti*). Both *Bti* and *Lsph* are Gram-positive spore-forming entomopathogenic bacteria. During sporulation they produce toxins with larvicidal activity, in fact insecticides based on *Bti* or *Lsph* or both are used to treat larval breeding containers. These products are highly specific against target species and environmental-friendly, although *Bti* degradation by biotic and abiotic factors is fast and damages to chironomid and Odonata fauna have been associated to its use [Gerstle et al. 2022], while *Lsph* has a longer environmental persistence due to its ability to proliferate in carcasses [Berry 2012]. *Beauveria bassiana* and *Metarhizium robertsii* (formerly known as *Metarhizium anisopliae*) are entomopathogenic fungi which are used as biological weapons for mosquito management. In *Ae. aegypti*, adult survival and reproduction are negatively affected by *B. bassiana*, while *M. robertsii* has ovicidal, larvicidal, and adulticidal activities against *Aedes* mosquitoes [Blanford et al. 2005, Vivekanandhan et al. 2020]. Entomopathogenic fungi could be applied in breeding sites in coformulation with oils, but they could also be used to treat clothes [Sousa et al. 2013]. Anyway, they still are not in use in Europe due to problematics such as the need of high doses to achieve an effective management, their low persistence in environment and the high cost related to their production [Sharma and Sharma 2021].The exploitation of mosquito natural predators can be performed by protecting target species or by their introduction in mosquito larvae-inhabited artificial containers. In the EU, these strategies can rely on the use of indigenous copepods belonging to the order Cyclopoida, especially *Mesocyclops* spp. [Pauly et al. 2022, Lühken et al. 2023, Russell 2023]. In Italy positive results have been achieved using *Macrocyclops albidus* (Copepoda: Cyclopidae) [Veronesi et al. 2015, Baldacchino et al. 2017]. Their breeding is cheap, but these organisms are usually more effective for the management of the genus *Culex*, they prey only first instar larvae and they necessitate water and food sources -

in addition to mosquito larvae - to survive. Other indigenous species that play a crucial role in controlling mosquito larvae populations must be protected, including Odonata, whose larvae serve as natural predators of aquatic invertebrates. [Saha et al. 2012].

In the end, essential oils extracted by a variety of plant species could represent an alternative in the next future for the biological management of mosquito populations. Essential oils are composed by different classes of chemical compounds with different mechanisms of action from neurotoxicity to effects on insect development [Satyan et al. 2009, Prophiro et al. 2011, Pereira Filho et al. 2021]. For this reason, they can have adulticidal, larvicidal, or ovicidal action. The effect of different essential oils can be combined to achieve a synergic effect and the risk of resistance phenomena is low due to their complex composition and mode of action [Dias and Moraes 2014; Baldacchino et al. 2015]. BPR, Regulation (EU) no 528/2012 allows the use of lavender oil and peppermint oil as biocide active ingredients [European Parliament, & Council of the European Union 2012]. Nevertheless, in depth and comprehensive information about their precise mechanisms of action and ecotoxicological data are scarce. Moreover, they show low selectivity, high volatility, and the yield of their production is variable. These issues affect their commercial availability, indeed a few products based on essential oils as active ingredient are available in the EU market, and none of them recommended for mosquito control [Aljameeli 2023, Assadpour et al. 2023, Luker et al. 2023, ECHA 2024].

The complete lists of biocidal products and repellent products available on the market for European countries are available at https://echa.europa.eu/it/information-on-chemicals/biocidal-products which also report other essential information such as the active substance and if and how they can be applied in the field [ECHA 2024]. Anyway, it is worth mentioning that in Italy and in the rest of Europe, mosquito control is mostly implemented using formulates based on *Bti*, *Bti* and *Lsph*, or IGRs [Brühl et al. 2020, ECHA 2024].

1.4.2 Mechanism of action of microbial biopesticides used for larval mosquito IVM and resistance management

The discovery of *Bt* dates to 1902 when Shigetane Ishiwatari isolated it from dead silkworms *Bombyx mori*. Ishiwatari initially named it *Bacillus sotto* [Milner 1994]. Following its discovery, many other strains of *Bt* have been found living ubiquitously all over the world [Ohba and Aizawa 1986, Mizuki et al. 1999]. Some *Bt* varieties displayed toxicity against distinct insect groups including *Bti*, the variety of *Bt* active against Culicidae larvae, that have been discovered in 1976 by L. J. Golberg and J. Margalit in a stagnant pond in the dry riverbed of Nahal Besor, a

region located southeastern of the actual Gaza Strip [Golberg and Margalit 1977, Margalit 1990]. The specificity of toxic *Bt* strains depend on the number of different crystal proteins (δ-endotoxins) they produce and on their toxicological and functional differences [Bravo et al. 2012]. Crystal toxins (Cry) are a major component of the parasporal crystals produced by the bacterium during sporulation [Lacey 2007, Ben-Dov 2014]. *Bti* parasporal crystals contain the Cry protoxins Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, and Mpp60A/Mpp60B (previously known as Cry60A/Cry60B), and the cytolytic (Cyt) protoxin Cyt1Aa. Some *Bti* strains can produce also little amounts of Cyt2Ba and Cyt1Ca [Valtierra-de-Luis et al. 2020, Silva-Filha et al. 2021] and can lack *mpp60* gene [Berry et al. 2002, Valtierra-de-Luis et al. 2020].

After ingestion, the parasporal crystals reach the midgut lumen where they are solubilized releasing the Cry and Cyt protoxins. The latter are proteolytically activated into toxins: at this point, Cry proteins bind different gut membrane receptors while Cyt toxins directly interact with membrane lipids, thus acting as additional receptors for Cry toxins. After binding, the Cry proteins oligomerise and insert into the membrane causing the formation of pores (figure 10). The larva finally dies showing several histopathologic damages such as columnar cells fragmentation, microvilli disruption, vacuolization of the cytoplasm and others (figure 10) [Melo et al. 2014, Zhang et al. 2017, Valtierra-de-Luis et al. 2020, Silva-Filha et al. 2021]. Importantly, each Cry toxin (i.e., Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa) has a unique mechanism of action, as each Cry toxin bind to unique domains and/or isoforms of the receptors on brush border membranes [Valtierra-de-Luis et al. 2020].

Differently from *Bt* which has been in used as insecticides for more than 40 years, the use of *Lsph* has begun more recently and the knowledge about its mechanism of action is still scarce [Park et al. 2010, Riaz et al. 2020, Silva-Filha et al. 2021]. During sporulation *Lsph* produces crystals that contain the Bin protoxins BinA and BinB [Park et al. 2010, Sharma and Kumar 2022]. As it happens for *Bt* toxins, once these crystals are ingested by the target organism, the alkaline pH of the midgut solubilize them releasing the protoxins. Once the protoxins are activated into toxins by the action of midgut proteases, they combine forming the heterodimeric toxin Bin. BinB subunit specifically binds to a single class of midgut membrane receptors, while BinA subunit is most likely involved in the formation of pores in the epithelium [Silva-Filha et al. 2021, Sharma and Kumar 2022]. As already stated, the cellular events leading to the death of the organism after the ingestion of *Lsph* spores is unclear, but it could be induced by a cascade apoptotic mechanism mediated by mitochondrial stress [Berry 2012, Sharma and Kumar 2022].

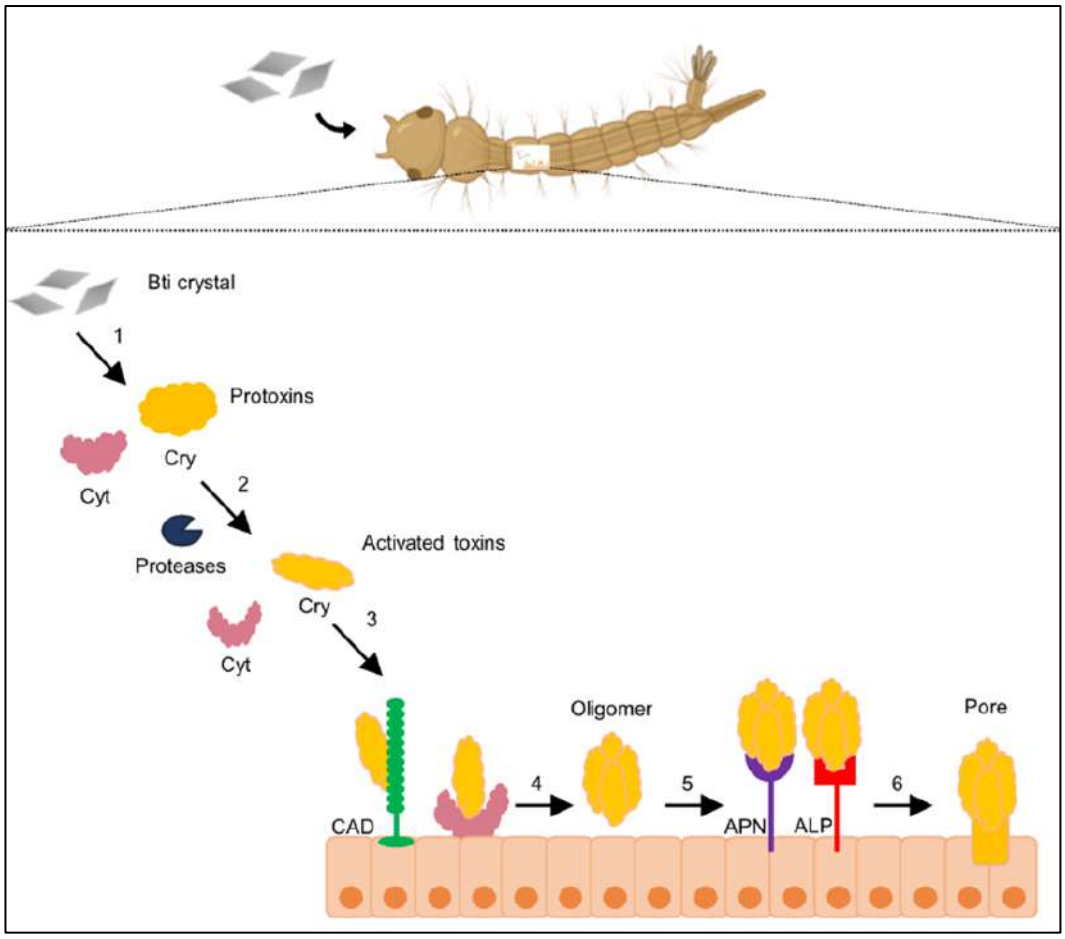

Figure 10: schematic representation of the mechanism of action of *Bti* toxins. The parasporal crystals contains Cry and Cyt proteins that are produced as protoxins: only after ingestion by mosquito larvae, the alkaline pH of gut lumen induces the solubilization of the crystals causing the release of the proteins. The protoxins are activated into toxins by a proteolytic cut acted mainly by serine proteases present in the intestinal lumen. Activated Cry proteins bind to cadherins, which are membrane receptors, to form oligomers while activated Cyt toxins interact with membrane lipids altering membrane permeability and subsequentially act as additional receptors for Cry proteins. Therefore, these two different toxins act synergically. The binding of Cry with cadherins or Cyt promote the formation of Cry oligomers that bind preferentially to different secondary receptors of the midgut epithelium with high affinity - isoforms of aminopeptidases (APN) and alkaline phosphatases (ALP) enzymes - that allow the insertion of oligomers into the membrane forming cation channels called pores. From Silva-Filha et al. 2021.

The insecticidal activity of *Lsph* is functionally monocomponent, as it is based on the action of the dimer Bin formed by the two toxins present in the crystal. This feature led to the development of *Lsph* resistant populations [Darboux et al. 2007, Wirth et al. 2010]. However, the use of *Lsph* in combination with *Bti* has proven to be effective due to the synergistic effects of their toxins. Moreover, Cyt1Aa has been observed to help the internalization of BinA reducing the selective pressure exerted by the Bin toxin on the genome of target species, while *Lsph* replication in larval corpses compensate the low persistence of *Bti* toxins in environment [Silva-Filha et al. 2021]. In contrast to *Lsph*, the toxicity of *Bti* is due to a combination of several toxins that make resistance onset extremely unlikely: the different Cry toxins

bind to different receptors and the presence of Cyt toxins, which act with a different mechanism, can synergize with Cry toxins (figure 10) [Valtierra-de-Luis et al. 2020, Silva-Filha et al. 2021]. Indeed, although tolerance phenomena to single *Bti* toxins have been observed under laboratory [Lee et al. 2014, Stalinski et al. 2016] and field conditions [Tetreau et al. 2012, Carvalho et al. 2018], lack of *Bti* efficacy in the field and control failure has never been detected [Tetreau et al. 2012, Becker et al. 2018, Carvalho et al. 2018]. Despite the lack of *Bti* resistance in the field to date, monitoring the resistance to each *Bti* toxin in mosquito field populations while boosting its efficacy over time is pivotal to overcome resistance [Tetreau et al. 2013, Stalinski et al. 2014].

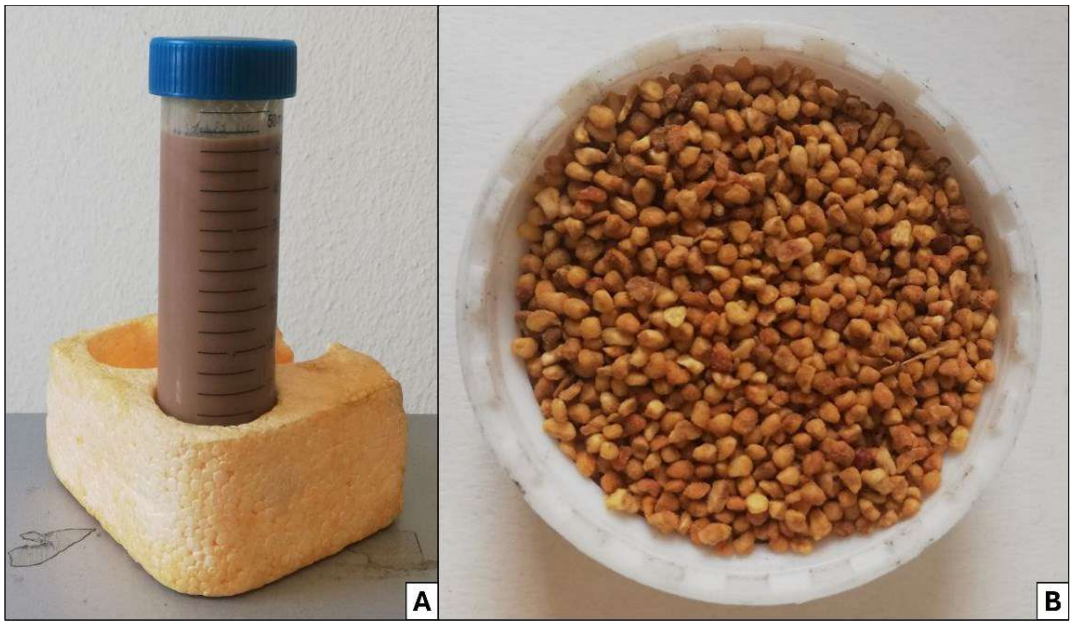

Figure 11: appearance of two *Bti*-based bioinsecticides: A) the liquid insecticide VectoBac® 12AS [Valent BioSciences 2019] and B) the granular product named VectoMax® FG which contains also *Lsph* toxins [Valent BioSciences 2017]

Several commercial *Bti*-based bioinsecticides are available on the market. Due to their importance for the research performed for this PhD thesis, it is worth mentioning VectoBac® 12AS (Valent BioSciences) that is a liquid formulation which toxicity is due to the fermentation product of *Bti* strain AM65-52 [Valent BioSciences 2019] and VectoMax® FG that is a granular formulation and its toxicity is due to the fermentation products of *Bti* strain AM65-52 and *Lsph* ABTS-1743 (figure 11) [Valent BioSciences 2017, Hernández-Santana et al. 2022].

1.5 Current research efforts and development of novel strategies for mosquito control

Beside the management approaches mentioned above, in recent years, alternative control methods are under study and evaluation. They include, for example, RNA interference (RNAi)-mediated control, Sterile Insect Technique (SIT), and symbiotic control.

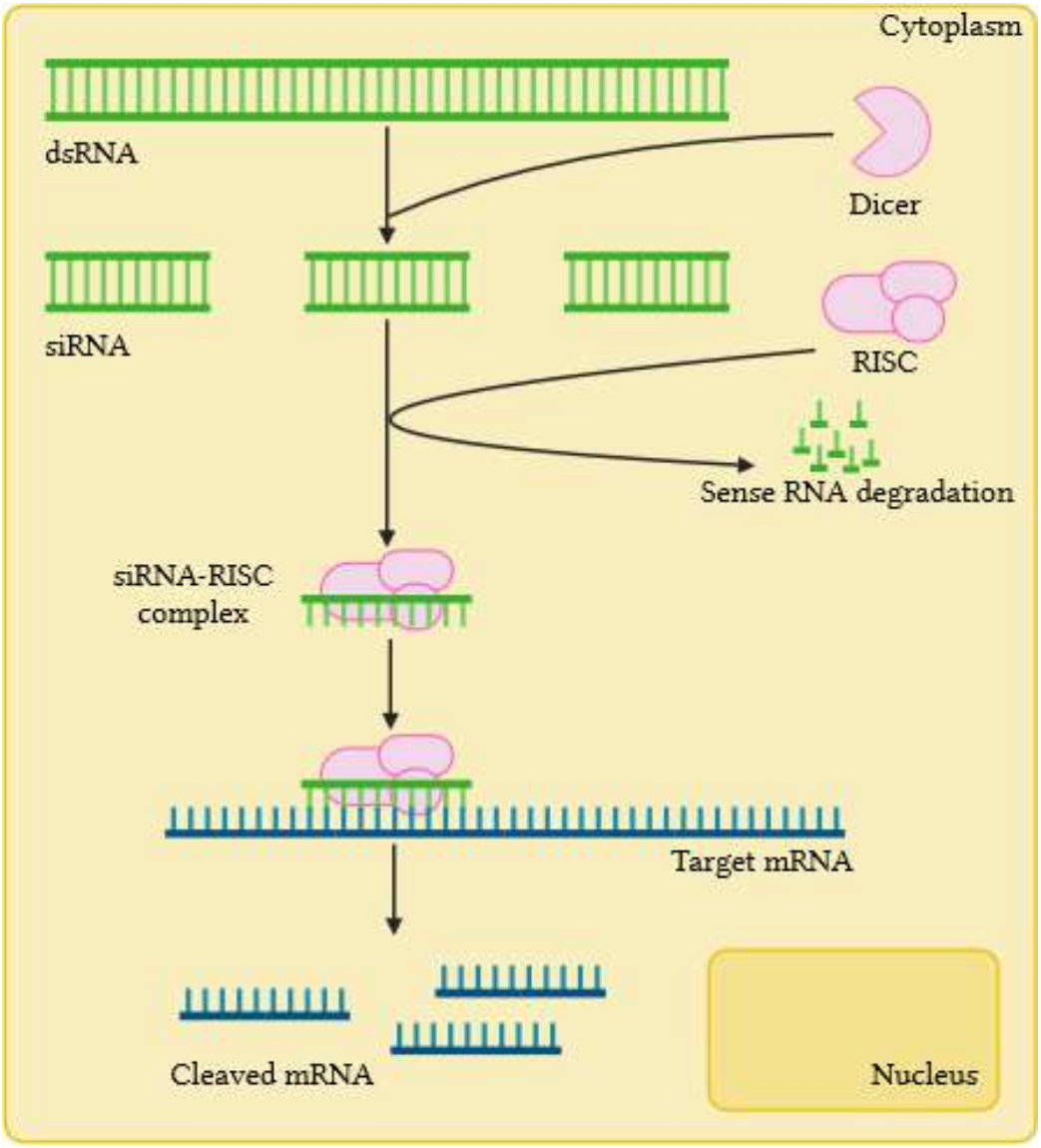

Figure 12: schematic representation of intracellular RNA interference pathway triggered by exogenous double stranded RNA (dsRNA). Dicer enzyme recognizes and cut the dsRNA into short interfering-RNAs (siRNAs). At this point, these siRNAs bind to the RNA-induced silencing complex (RISC) with the release and consequent degradation of the sense strand (the so-called passenger strand). The anti-sense strand (guide strand) together with RISC form the siRNA-RISC complex that target messenger RNA (mRNA): the target mRNA is recognized and cleaved causing the silencing of the translation. Created with BioRender.com

RNAi-based bioinsecticides are eco-friendly pest control methods. They exploit the RNA interference pathway, which is shared among most eukaryotic organisms, including insects (figure 12) [Timmons and Fire 1998, Mussabekova et al. 2017]. RNAi can be induced by the oral administration of double-stranded RNA (dsRNA), molecules specifically designed to target genes fundamental for the survival or the development of the animal. Once inside the body, the components of the RNAi pathway convert these molecules into small single-stranded RNA molecules that can silence the expression of a target gene [Bona et al. 2016, Airs and Bartholomay 2017, Balakrishna Pillai et al. 2017]. The great advantages of this method are the

possibility to design highly species-specific dsRNAs and their eco-compatibility; anyway, these molecules, if released in environment as such, would be degraded rapidly and the massive *in vitro* production of specific dsRNAs would be extremely expensive. For these reasons, the future of RNAi-based management depends on both the development of biofactories to synthesized dsRNA molecules, that would make RNAi-based control cost-effective, and on the development of delivery systems capable of protecting them by environmental biotic and abiotic degradation factors [Hapairai et al. 2017, Mysore et al. 2019]. In mosquito, RNAi induction by oral feeding has proven to be effective and many candidate genes have been identified. Among the different delivery systems that have been explored so far for larval management, microbial-based ones seem to be most effective and promising, while attractive toxic sugar baits can be exploited for the delivery of interfering RNAs to the adult stages [Airs and Bartholomay 2017, Wiltshire and Duman-Scheel 2020]. Recently, the first proof of efficacy in semi-field experiment of a delivery system based on a yeast-producing interfering RNA to control vector mosquito species have been published [Stewart et al. 2023].

SIT has been in use since many years to successfully control different pest species [Klassen et al. 2021]. To date, field trials started using SIT with the purpose of controlling different mosquito species [Bellini et al. 2013, Kumar and Shagal 2022]. This technique is based on the release in nature of massive quantities of sterile adult mosquito males that would cause the reduction of the progeny inducing the development of non-vital embryos in wild females or by reducing the probability that fertile mating take place by competition with wild males. Sterilization of mosquito males is performed by exposing them to γ-irradiation or by using sterilizing chemical compounds that, however, can impact the fitness of these mosquitoes, for example, by reducing their mating competitivity [Helinski et al. 2009, López-Martínez and Hahn 2014]. Another problem related to this technique is represented by the need to breed and select an enormous number of male mosquitoes in a narrow time period. Nevertheless, the major obstacle for the actuation of SIT strategies is represented by the combination of conditions that must coexist to achieve a long-term efficacy, such as, for example, the ethology of the target species and the orography of the treated area [Dufourd and Dumont 2013]. Despite this, SIT application in pilot field-trials have shown its efficacy, with positive results achieved even in Italy [Bellini et al. 2013]. To improve the effectiveness of SIT in mosquito control operations, conventional control strategies that seek to lower wild *Ae. albopictus* populations must be put into place in parallel [Becker et al. 2022].

Symbiotic control of mosquitoes relies on the use of symbiont microorganisms. It can be implemented, for example, by the Incompatible Insect Technique (IIT) that exploits the obligate intracellular parasite *Wolbachia* to achieve a reduction in the

number or the local extinction of target vector mosquito species [Crawford et al. 2020, Wang et al. 2022, Weng et al. 2023]. This is a Gram-negative Alphaproteobacterium which has been found to naturally infect several invertebrate species including Culicidae. *Wolbachia* localizes inside the cells of different tissues including the germline from where it is vertically transmitted to the progeny of its host. Certain strains induce Cytoplasmic Incompatibility (CI) which is the pillar mechanism of IIT, while other strains can cause male killing, feminization, or parthenogenesis [Fialho and Stevens 2000, Kremer et al. 2009, Kern et al. 2015, Xiao et al. 2021]. It has been proposed that CI-inducing *Wolbachia*-infected males produce a modification factor that is transferred to the females with sperm during copulation: if the females are also infected with a CI-inducing Wolbachia they produce a rescue factor that blocks the action of the modification factor, while uninfected females are sterilized (figure 13) [Wang et al. 2022].

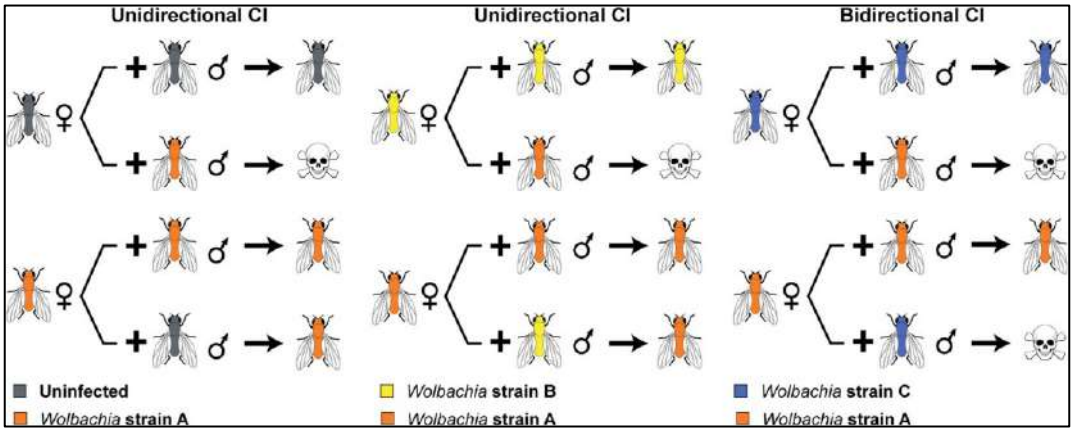

Figure 13: two different CI mechanisms are known: in unidirectional CI, sterile mating occurs among an infected male and an uninfected female, while every other mating is fertile even if the couple is infected by different, but compatible, *Wolbachia* strains, while in bidirectional CI also mating among individuals infected by incompatible *Wolbachia* strains will end with sterilization. It is not rare that different *Wolbachia* strains infect the same population as it happens for *Ae. albopictus* which can be infected by the strains wAlbA, wAlbB, or both. From Wang et al. 2022.

Additionally, even if it is not a mosquito management technique, it worth mentioning the use of *Wolbachia* to act population replacement strategies. Their aim is to reduce mosquito-borne diseases incidence and transmission by substituting natural populations with lab grown *Wolbachia*-infected populations. In fact, it has been observed that mosquitoes infected with certain *Wolbachia* strains are refractory to viruses and/or other parasites they can spread [Walker et al. 2011, Weng et al. 2023]. The efficacy of this technique has already been proven in field trials [Hoffman et al. 2011, Ross et al. 2022]. However, the implementation of these strategies requests continuous releases of massive numbers of infected individuals and constant monitoring of the target populations [Crawford et al. 2020, Alphey 2023]. Moreover, there are evidence that *Wolbachia* infection can

even boost mosquito infection by other pathogens, so further research is needed [Hughes et al. 2014, Dodson et al. 2023].

These techniques are just a few of those that could strengthen mosquito management in the future, but there are many others that will struggle to be accepted in the EU because of the use of genetically modified organisms such as the release of insect carrying a dominant lethal gene or the release of insect made refractory to viruses and/or other parasites by RNAi or CRISPR/Cas9 [Baldacchino et al. 2015, Viktorov 2021, Weng et al. 2023].

2. Aims of the PhD project

The overall objective of my PhD project is the development of biorational tools to control mosquito populations by killing larval stages. Reduce populations of mosquito competent vectors can mitigate the incidence of mosquito-borne diseases or prevent their establishment in non-endemic areas. The idea is to enhance the effectiveness of the *Bacillus thuringiensis* var. *israelensis* (*Bti*)-based bioinsecticides which represent to date one of the main biological weapons to control mosquito larvae. Indeed, these products display a high toxicity together with a high specificity, while their environmental impact is incredibly low. Even if some off-target effects have been observed in natural populations of Chironomidae, a Family that is phylogenetically close to Culicidae, *Bti*-based products still represent the best compromise between effective management and environmental safety. In addition, although no resistance phenomena have been registered to date to *Bti* formulates, on the base of reported decrease susceptibility of single formulate components, the future onset and the spread of resistant populations cannot be excluded. Nevertheless, another big issue of these products is represented by their low persistence in environment because of the degradation acted by biotic and abiotic agents.

The aim of this work is practical and consist in the development of a product prototype that will increase and boost the effectiveness of *Bti*-based formulates. Maintaining public health, containing environmental contamination, and safeguarding non-target wildlife all depend on the effectiveness of available biorational alternatives to control insect pests and vectors. In particular, the objective of the research will be to develop a delivery tool to control mosquito larvae in water that:

(i) will include a *Bti*-based formulate as active ingredient;
(ii) will be effective in protecting *Bti* from degradation by biotic and abiotic factors that usually reduce the persistence of the formulation to just a couple of days after its release [Valent BioSciences 2019]: this aspect is pivotal to guarantee a continuous and constant toxic effect without the need of frequent reapplication that can be costly [Becker et al. 2022];
(iii) will consist of molecules of natural origin;
(iv) will be able to float and attract mosquito larvae that typically rest at the water-air interface to be able to breath.

3. Papers

3.1 First paper: “Biodegradable floating hydrogel baits as larvicide delivery systems against mosquitoes”

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Biodegradable floating hydrogel baits as larvicide delivery systems against mosquitoes†

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Biological methods for mosquito larvae control are completely biodegradable and have null or limited effects on nontarget organisms. However, commercially available products have a low residual activity, with the consequent need for multiple applications that inevitably increase costs and the risk of resistance phenomena insurgence. Smart delivery systems made of hydrogels proved their efficacy in increasing the action duration of biolarvicides up to several months, but the lack of an efficient baiting mechanism to strongly attract the target pest remains a problem in practical applications. In this work, we investigated two novel hydrogel-based formulations of completely natural composition for baiting and killing larvae of *Aedes albopictus* mosquitos. The proposed materials consist of charged crosslinked polysaccharides (chitosan and cellulose) and are specifically manufactured to float in water, simulating organic matter usually present at breeding sites. Within the hydrogels' matrix, yeast colonies of *Saccharomyces cerevisiae* were embedded as phagostimulants alongside a biolarvicide (*Bacillus thuringiensis* var. *israelensis* (*Bti*)). Despite the similar chemical nature and structure, chitosan-based hydrogels exhibited a markedly superior baiting potential compared to those made of cellulose and also succeeded in efficiently killing mosquito larvae just after a few hours from administration. We are confident that the proposed smart delivery hydrogel made of chitosan can be an enabling tool to attract mosquito larvae towards biopesticides of different nature without delocalizing active ingredients away from the breeding site and to simultaneously increase their residual activity, thus holding the potential of minimizing environmental pollution related to pest control and vector-borne disease prevention.

1. Introduction

Mosquitoes are one of the most widespread public health problems, in Europe and worldwide, because of their vector capacity for viruses, bacteria and protozoa which causes them to be among the deadliest animals for humans.[1] Recent environmental changes accentuated the strong dynamism of such animal species and modulated, on a global scale, the spatio-temporal distribution of vectors, hosts and pathogens.[2,3] It is indeed not uncommon to register epidemic outbreaks of mosquito-related diseases (*e.g.* dengue, yellow fever, West-Nile, chikungunya, zika, filariasis and malaria) in non-endemic areas.[4]

Since mosquito adult forms are more difficult to control, due to the non-confined area where they live, it is essential to implement eradication strategies targeting immature stages (*i.e.* eggs, larvae, and pupae). Chemical larvicides have been dismissed in recent years because of water pollution, bioaccumulation and resistance insurgence related problems and are being substituted by those of biological origins.[5–8] Biolarvicides can be derived from plants, bacteria, algae, lichens and fungi;[9] some of the most extensively studied are oomycetes species (*Leptolegnia chapmanii*, *Pythium* sp. and *Lagenidium giganteum*),[10] herbal species (*Azadirachta indica*)[11] and bacterial species (*Bacillus thuringiensis* var. *israelensis* (*Bti*) and *Bacillus sphaericus* (Bs)).[12,13] Their increasing use in integrated control programmes, where environmental management, personal protection and careful use of insecticides are crucial, is owed to specificity towards mosquito larvae with no side effects on mammals.[14–16]

Even if biolarvicides have the advantage of limited ecological impact, they have the drawback of a short duration of action

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due to high susceptibility to external atmospheric agents (*e.g.* sunlight and high temperature).[17,18] The consequent need for multiple applications makes biolarvicides an expensive solution and increases the possibility of the emergence of resistance in mosquitoes.[19] Therefore, to achieve an environmentally friendly formulation with a long shelf-life and efficacy, it is necessary to find an effective method to protect the active principle while simultaneously preventing its dispersion into the environment.

To overcome the above-listed challenges, a great deal of research is being focused on the development of new biolarvicide formulations and, in particular, smart delivery systems (*i.e.* engineered technologies for the targeted delivery and/or controlled release of substances) made of hydrogels showed very promising results.[20]

Hydrogels are crosslinked polymers capable of absorbing and retaining a large amount of water within their three-dimensional network. This is due to the presence of hydrophilic side groups on the polymer chains that compose the molecular backbone.[21] A unique characteristic of hydrogel-based materials is the possibility of finely tuning their swelling and release properties by adjusting intrinsic parameters like crosslinking density, monomer types and network's charge.[22] Therefore, if active ingredients (*e.g.* chemicals, cells, and enzymes) are encapsulated in the bulk architecture of the hydrogel, they will be released with a desired kinetic profile depending on their chemical nature, the hydrogel's matrix features and also the ionic composition of the external aqueous environment.[23] Such technologies have been developed mainly for pharmaceutical applications but, in the last decades, they have also been engineered for biopesticide release.[24–26]

Only a few examples of smart delivery hydrogels employed specifically against mosquito larvae can be found in the literature. For instance, *Borge* and colleagues[27] improved the release of the hydrophobic larvicidal extract of the plant *Dendranthema grandiflora*, by dispersing it in a block co-polymer of polybutylene succinate (PBS) and polyethylene glycol (PEG), causing mortality in larvae of *Aedes aegypti*. In another recent study, the residual activity of *Bti* against *Aedes albopictus* larvae was shown to increase up to several months when loaded into hydrogel capsules of PEG and hexadecanol.[28]

The fact that the hydrogels' matrix can act as a shield for those active principles that can be inactivated by environmental agents is a potential enabling solution to diminish the number of biolarvicide administrations, therefore reducing both production costs and, most importantly, minimizing the risk of resistance insurgence in the target species.[19,20] However, despite the undeniable efficiency upon increasing the residual activity of biolarvicides, the translation from laboratory to field conditions might not be that straightforward. As a matter of fact, it is not uncommon to attest a significant decrease in larvicide performance once placed in a real application environment (*e.g.* fountains, manholes, flowerpot dishes, and small ponds).[29,30] Such incongruency can be attributed to the incapacity of the formulation to strongly attract towards itself the target species.[31] A baiting mechanism is therefore paramount to ensure the specific insect to come in direct contact with the pesticide. Mosquito larvae are known to be attracted towards microorganisms that represent a feeding source and floating objects where they can find shelter from predators.[32,33] To our knowledge, no hydrogel-based biolarvicide formulation has yet been reported of being both capable of floating and attracting like a phagostimulant.

Due to increased attention to environmental issues, many research interests are now moving towards the use of green and biodegradable substances. For this reason, in the context of control of pests and vectors, hydrogels made of natural polymers have become preferred over those of synthetic origins.[31,34] Polysaccharides such as chitosan, alginate and cellulose have an attested renewability, biocompatibility, and nontoxicity and have already been successfully engineered to be used as smart delivery capsules against mosquito larvae.[35–39]

In this work, the potential of two novel smart delivery hydrogel-based formulations of completely natural origin is investigated in baiting and killing mosquito larvae. In more detail, the first one is a cationic hydrogel composed of crosslinked chitosan (ChitH) whereas the second one is an anionic hydrogel made of crosslinked cellulose (CellH). They were manufactured using an *ad hoc* liquid foam templating technique that allows generation of closed macro-pores entrapped in the matrix, so as to enable buoyancy in water. Within these hydrogels, colonies of the yeast *Saccharomyces cerevisiae* were embedded as phagostimulants and a commercially available *Bti* formulation as a biolarvicide. Physico-chemical analysis suggested that active ingredients (*Bti* and yeasts) were administered to the target species mainly by material erosion, framing the proposed hydrogels in the broad category of erodible smart delivery systems. However, only the ChitH formulation was proved to possess efficient biological activity towards *Ae. albopictus* larvae, both as a phagostimulant and as a larvicide. We strongly believe that the proposed smart delivery hydrogel made of chitosan can be a suitable ecofriendly tool against many different targets representing a public health concern or a challenge for the agriculture sector.

2. Experimental section

Chitosan (medium M_W, 75–85% degree of deacetylation), sodium dodecyl sulphate (SDS) and citric acid were purchased from Sigma-Aldrich. Medium-viscosity hydroxyethylcellulose (HEC) was purchased from Farmlabor while carboxymethylcellulose sodium salt (CMCNa) and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Iris Biotech. Genipin was purchased from Wako Chemicals. All chemicals were used without further purification. *Bti* (Vecto-Bac® 12 AS-Bti) was purchased from I.N.D.I.A (INDUPHARMA Srl). All solvents were of ACS grade or higher and were obtained from Sigma-Aldrich. Ultrapure water (resistivity 18.2 M cm) was obtained using a water purification system (MilliQ® Direct, EMD Millipore, Germany).

Chitosan hydrogel formulation

One chitosan hydrogel (ChitH) tablet was prepared by slowly dissolving 10 mg of chitosan in 1 mL of a 1% v/v acetic acid solution and by adding 10 μL of 20 mM SDS solution. The mixture was stirred for 1 h at 300 rpm at room temperature (RT). Then, 100 μL of 44 mM genipin solution in EtOH 10% v/v were added, and the mixture was stirred at 600 rpm at RT for 30 min to blend everything together. For ChitH with *Bti* and yeasts (ChitH@Bti-Y), the active ingredients were added in the polymer mixture right after the genipin solution. The hydrogel preparation was performed from the microgram to multigram scale.

Cellulose hydrogel formulation

One cellulose hydrogel (CellH) tablet was prepared according to a modified procedure described by *Sannino et al.*[40] First, a mixture of HEC (8 mg) and CMCNa (24 mg) was dissolved in 1 mL of water by magnetic stirring at RT, until a clear and homogeneous solution was obtained. The final polymer content was 3 wt%, with a CMCNa/HEC weight ratio of 3 : 1. After the addition and mixing of 32 mg of EDC as a crosslinking agent, 50 μL aqueous solution containing 1 wt% of citric acid was added as a gelation catalyst. For CellH with *Bti* and yeasts (CellH@Bti-Y), the active ingredients were added in the polymer mixture before the citric acid solution. The hydrogel preparation was performed from a microgram to a multigram scale.

Liquid foam templating technique

Before starting the liquid foam templating technique ChitH pre-polymer solution was cured at 42 °C for 35 min, whereas CellH pre-polymer solution was cured for 20 min at RT. Bubbles were generated inside the partially cured pre-polymer mixtures by injecting air at 10 μL min^{-1} with a syringe pump (KD Scientific, Thermo Fisher Scientific) in combination with vigorous stirring (1000 rpm). The procedure lasted 2 min, and then, the liquid foams were pipetted in the designated aluminium mould and were left to crosslink overnight. ChitH was cured at 37 °C whereas CellH was cured at RT. All hydrogels were moulded as small cylinders (∅ = 16 mm).

Gel fraction

Gel samples were dried in an oven at 37 °C until a constant weight was reached, and then they were left to swell in MilliQ water for 4 days at room temperature. Swollen samples were again put in the oven at 37 °C until a constant weight was reached. The gel fraction degree (GF%) was obtained using formula (1):

$$GF\% = \frac{W_f}{W_0} \times 100 \quad (1)$$

where W_0 and W_f are the weight of the dried gel before and after swelling treatment, respectively. GF% values were averaged from three repeated measurements.

Swelling degree

Cured hydrogels were weighed before swelling in a large excess (150 mL) of NaCl aqueous solutions of increasing ionic strength (IS) (0 mM, 5 mM, 50 mM, and 500 mM). When the constant weight was reached, samples were removed from the aqueous solutions, gently tapped onto filter paper and weighed again. The swelling degree at equilibrium (Q%) is described using formula (2):

$$Q\% = \frac{(W_f - W_0)}{W_0} \times 100 \quad (2)$$

where W_f is the weight of the gel after swelling and W_0 is the weight of the gel before swelling. Q% values were averaged from three repeated measurements.

Weight loss

After reaching the maximum swelling degree in the NaCl swelling solutions (0 mM, 5 mM, 50 mM, and 500 mM), the weight loss of the gels was monitored over a period of 28 days. The weight loss (W%) was calculated using formula (3):

$$W\% = 100 - \left[\frac{W_0 - W_i}{W_0} \times 100\right] \quad (3)$$

where W_0 is the sample weight at the maximum swelling and W_i represents the sample weight after a designated time. W% values were averaged from three repeated measurements.

Uniaxial compression mechanical tests

Uniaxial compression tests were performed on hydrogel tablets with cylindrical shape (∅ = 16 mm) with a deformation rate of 5 mm min^{-1}, using a traction machine (TVM-N, Sauter) coupled with a dynamometer FH-10 (Sauter) and the software AFH-FAST/FD (Sauter). Before testing, the samples were swelled in water for 24 h at RT. Three independent specimens were tested for every condition analysed. The stress (σ) was evaluated as the ratio between the forces measured by the load cell and the undeformed specimen cross-section. Strain (ε) was determined as the ratio between the crosshead displacement and the specimen height. Elastic moduli were obtained considering the elastic region of the stress–strain curve up to 10% of deformation. For CellH samples, the Young's modulus (E) was calculated whereas for ChitH samples the secant modulus (E_s) was calculated.

Mosquito breeding

The mosquitoes used in the present work are derived from the Rimini strain of the Asian tiger mosquito *Aedes* (*Stegomyia*) *albopictus*, established in 2004 from mosquito eggs collected in Rimini.[41] The colony was maintained under standard conditions of humidity (70–80%) and temperature (28–30 °C) in the Insectary of the University of Milan. Adult mosquitoes were fed with animal blood to complete the reproductive cycle, while the larvae were reared with fish food (Tetra-fish, Melle).

Fluorescent yeast culture for matrix inclusion

Saccharomyces cerevisiae cells that were GFP-labelled (SY2080 with integrative plasmid pRS306) were used to perform matrix inclusion and feeding experiments on *Ae. albopictus* mosquito larvae. Cells were grown in generic Yeast Extract–Peptone–Dextrose (YPD) medium enriched with 2% raffinose as a nutrient source and induced for 5 h with 30% galactose. Cells post-incubation were counted to reach a concentration of 10^7 cells mL^{-1} to be included in each hydrogel tablet.

Ingestion test and fluorescence analysis

Two pools of five *Ae. albopictus* larvae were starved for 12 h in glass Petri dishes containing 50 mL of deionised water. One hydrogel tablet with yeast-GFP included (ChitH@Y or CellH@Y) was then added to the water. An empty matrix served as a control. After 24 h or 72 h larvae were dissected, separating the intestine from the carcass, and the midguts were observed using a SIM A1 confocal microscope (Nikon).

Transmittance studies

Hydrogel samples filled with *Bti* and yeasts (CellH@Bti-Y and ChitH@Bti-Y) were immersed in 150 mL of a 50 mM NaCl solution for 24 h and the transmittance (*T*%) spectra of the gels were recorded over a period of 15 days, using a UV-vis spectrophotometer (Cary 100 UV-vis, Agilent Technologies, Santa Clara, CA, USA). The wavelength range with steps of 1 nm was set between 550 and 800 nm for CellH@Bti-Y and between 700 and 800 nm for ChitH@Bti-Y. The *T*% values were averaged from three repeated measurements. Each measurement was performed on an independent gel tablet.

Mortality bioassays

Pools of ten *Ae. albopictus* larvae were placed in Petri dishes containing deionised water. Larvae were exposed to a hydrogel tablet of different formulations (CellH@Bti-Y or ChitH@Bti-Y) placed onto the Petri dish; the empty matrices were used as a control for the test. The monitoring of live and dead larvae was carried out for a week.

3. Results and discussion

Hydrogels' design and manufacture

The rational design behind the proposed hydrogel formulations considered the need for a biodegradable floating system with baiting capability towards mosquito larvae that could be used as a delivery protective tool for the administration of biolarvicide molecules.

For this purpose, the molecular backbone of both engineered hydrogels was completely made of natural polymers, in particular of polysaccharides, respectively, chitosan and cellulose. Polysaccharide-based hydrogels have indeed recently been attested

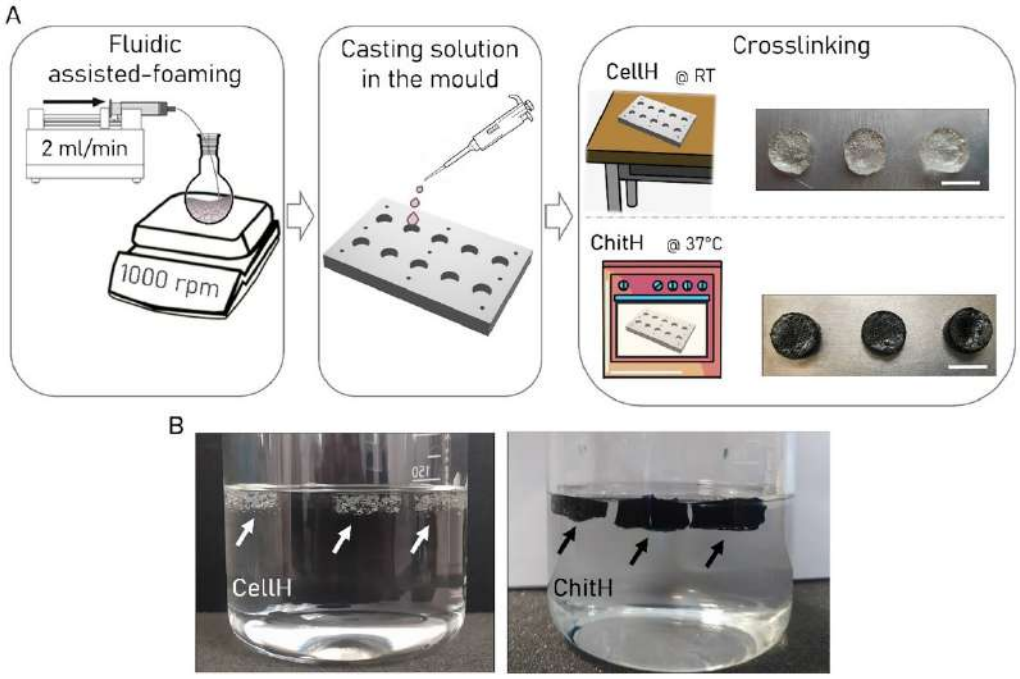

Fig. 1 (A) Illustration of the manufacturing procedure used to mould floating hydrogels. ChitH was crosslinked in an oven at 37 °C, whereas CellH was crosslinked at RT. The scale bar is 16 mm. (B) Pictures of ChitH (left) and CellH (right) samples floating on water.

to increase *Bti* residual activity up to several months.[39] ChitH samples were crosslinked using genipin which is far known to have markedly lower cytotoxicity as compared with alternative crosslinkers such as glutaraldehyde.[42] After crosslinking, they appeared as a dark-blue coloured tablet, a trend mark of oxygen radical-induced polymerization of genipin as well as its reaction with chitosan amino groups.[43,44] This feature can both provide better *Bti* covering from UV radiation and more efficient larva baiting.[33,45] CellH samples were instead obtained by crosslinking an aqueous mixture of HEC and CMCNa through the water-soluble carbodiimide EDC, under acidic conditions. This well-known chemical reaction induces the formation of ester bonds between the carboxyl groups provided by CMCNa and the hydroxyl groups provided by HEC. The crosslinking agent EDC is not incorporated into the gel structure, but it is changed into a urea derivative (EDU), which displays a very low degree of cytotoxicity and can be easily washed out from the polymeric network, increasing the biocompatibility of the final product.[40]

In both cases ionic hydrogels were obtained. While ChitH displayed positive charges, thanks to the presence of $-NH_3^+$ groups in the polymeric backbone, the negative fixed charges ($-COO^-$) of the polyelectrolyte CMCNa induced the formation of anionic CellH.

The crosslinking efficacy was measured by gel fraction (GF%) experiments. The GF% of ChitH reached 79 ± 3% indicating a good degree of crosslinking formed in the polymer hydrogel network. On the other hand, the GF% value of CellH was found to be around 54 ± 1%, demonstrating and confirming that EDU molecules did not participate in the gel network, and they were free to spread out after swelling.

Afterwards, buoyancy was studied. In fact, floating objects have a natural baiting potential for mosquito larvae, since these insects normally exploit leaves or other organic materials to hide beneath and protect themself from natural surface- and bottom-predators or simply as an available microbial food resource while ensuring safer filter feeding.[33] For such a reason small bubbles of air were entrapped in the hydrogel matrixes in order to allow them to float on water. This was achieved by developing an *ad hoc* liquid foam templating manufacturing procedure (Fig. 1A). A syringe pump was set to inflate air through a needle inside the pre-polymer solution, while a magnetic stirrer was used to break big bubbles into smaller ones (diameter in the millimetre range). When the liquid foam was generated, the solution was pipetted in the designated mould for crosslinking. A key aspect to ensure liquid foam stabilization before the onset of gelation is viscosity. Pre-polymer solutions possessing high viscosity can stabilize bubbles more efficiently and will prevent aging mechanisms (*i.e.* coalescence, coarsening and drainage) that inevitably disrupt the liquid foam.[46,47] Both the ChitH and the CellH solutions proved to be not viscous enough to stabilise a foam. For this reason, we decide, on an empirical basis, to partially cure the ChitH pre-polymeric solution for 35 min and the CellH one for 20 min before starting the fluid-assisted foaming. Using this approach, we manage to successfully develop two floating *Bti* formulations (Fig. 1B). The versatility of liquid foam templating in converting virtually all kinds of pre-polymeric solutions into porous hydrogel materials makes this technique very appealing also in the context of preparing larvicide formulations. Moreover, the entire manufacturing procedure required mild conditions throughout the whole process, which not only ensure safety but also maintained low production costs, thus facilitating industrial scalability.

Hydrogels' behaviour in aqueous environments

In hydrogels, the driving force for swelling arises from a difference between the water–polymer thermodynamic mixing force and the retractive force of the polymer chains.[22] If charged species are present on the molecular backbone of the matrix's network and as free ions in the imbibing solution then the swelling process will be controlled by the previous two contributions as well as the ionic interactions between charges (*i.e.* Donnan's contribution).[21]

Here, the swelling experiments were performed on both the investigated hydrogels in a solvent having a simple and well-defined composition. In particular, NaCl solutions with different concentrations (0 mM, 5 mM, 50 mM and 500 mM) were prepared in order to mimic the ionic strength of aqueous

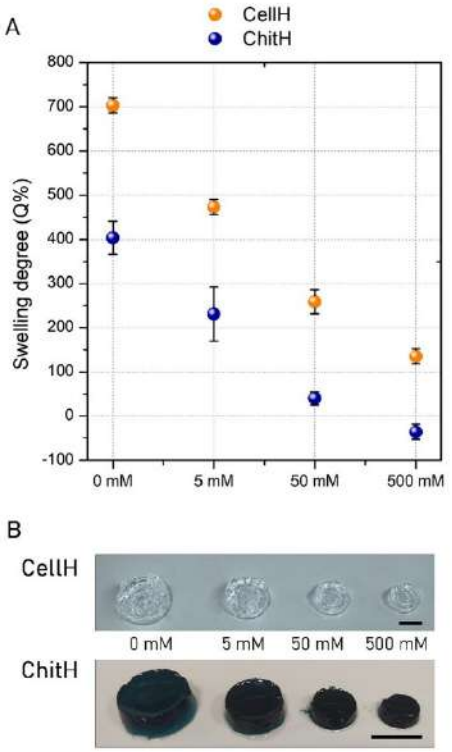

Fig. 2 (A) Swelling degree measurements of CellH and ChitH at increasing ionic strength (0 mM, 50 mM, 50 mM and 500 mM). (B) Picture of CellH (up) and ChitH (down) after 24 h of being imbibed in water solutions. Scale bar is 10 mm.

environments of practical interest (*e.g.* fountains, manholes, flowerpot dishes and small ponds). The hydrogels were immersed in a large reservoir of solvent and the variation in weight was recorded once the swelling equilibrium point is reached. The 0 mM NaCl solution (MilliQ water) represents the simplest solvent model tested, since the ion swelling pressure depends only on the ionic charges within the hydrogel. As expected, both the gels were able to absorb a large amount of water (Fig. 2A). However, the swelling capability is much higher in CellH ($Q\%_0 = 703 \pm 17\%$) than in ChitH ($Q\%_0 = 404 \pm 37\%$), suggesting that the negative charge of CellH imparted higher ionic strength with respect to the positive charge of ChitH. This result was further confirmed by varying the NaCl concentration. CellH samples showed a gradual decrease in swelling capability with the increase of salt concentration, due to reduction of the osmotic pressure between the hydrogel and the outer solution. ChitH samples resulted in a similar trend but with smaller swelling degree values. Also in this case, the maximum swelling degree was observed in ultrapure water and a gradual decrease and shrinkage were observed as the ionic strength of the outer solution increased up to 500 mM. When the salt concentration reached 50 mM, ChitH was near to be at osmotic equilibrium with the outer solution since the swelling degree value was close to 0% ($Q\%_{50} = 40 \pm 15\%$). By further increasing the salt concentration, the ionic strength of the reservoir solution exceeded the ionic strength in the hydrogel's network, and this resulted in material shrinkage ($Q\%_{500} = -36 \pm 17\%$).

When *Bti* and yeasts were included in the matrices (CellH@Bti-Y and ChitH@Bti-Y) the swelling degree trend of both hydrogels remained the same (Fig. S1, ESI†), indicating that active ingredients did not significantly impact water absorption dynamics. However, the overall values were slightly lower with respect to the empty matrices, suggesting a possible interaction between the charged amino acids of *Bti* and the charged side groups displayed in the hydrogels' polymer backbones that decreased the osmotic pressure inside the gel networks.

The hydrogels' weight variation was also monitored in the same range of ionic strength over a period of 28 days at RT. From the graph shown in Fig. 3A it can be observed that CellH samples maintained a steady weight in the 50 mM to 500 mM ionic strength range, whereas it underwent full degradation in 0 and 5 mM NaCl solutions after nearly a month. A hydrogel

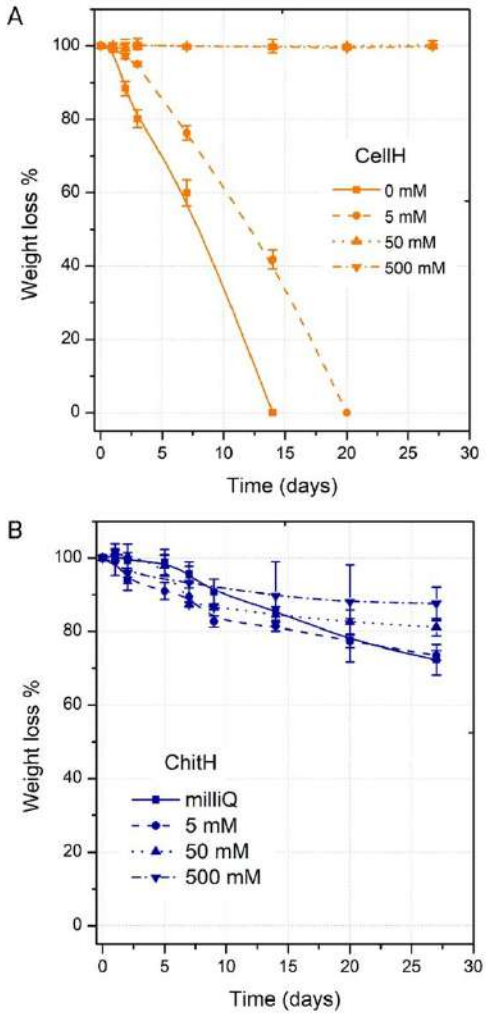

Fig. 3 Weight loss (*W*%) of CellH (up) and ChitH (bottom) samples after 7, 14, 21, and 28 days in solutions of increasing ionic strength (0 mM, 50 mM, 50 mM and 500 mM).

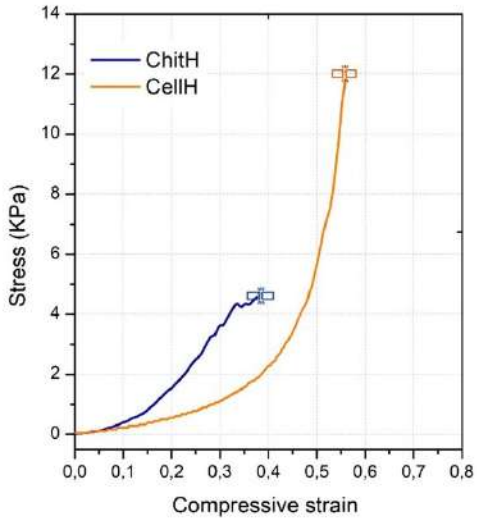

Fig. 4 Representative stress–strain curve of a CellH and ChitH sample.

Table 1 CellH and ChitH elastic modulus, compressive strength and ultimate compression

	Modulus ($N\ m^{-2}$)	Compressive strength (kPa)	Ultimate compression (mm)
CellH	3.4 ± 0.4	12.3 ± 2.9	0.6 ± 0.3
ChitH	2.1 ± 1.8	4.9 ± 0.4	0.4 ± 0.1

dimensional stability in a specific water solution mainly depends on its capacity of absorbing and retaining the solvent within the three-dimensional network. CellH massive swelling under 0 ($Q\%_0$ = 703 ± 17%) and 5 mM ($Q\%_5$ = 473 ± 17%) conditions exceeded the extensibility of crosslinked polymer segments to withhold together and eventually tore the material apart into many fragments.[21] On the other hand, ChitH samples always maintained their weight above 70% of the initial value because the reduced amount of imbibed water did not compromise irreparably matrix integrity over the tested time window (Fig. 3B).

Hydrogels' mechanical properties

The hydrogels' mechanical behaviour was assessed through uniaxial compressive mechanical tests. Fig. 4 shows the stress–strain relationship of the materials when compressed at a constant rate until fracture. CellH specimens showed a clear rubber-like elasticity behaviour. In fact, stress remained proportional to strain up until 30% of compression with respect to the initial height. After this value, yielding started to occur and deformation became plastic. On the other hand, ChitH specimens never quite manifested clear elastic deformation even at low strain values. Because of such differences in mechanical curves, we calculated the Young's modulus for the CellH samples (3.44 ± 0.4) and the secant modulus for ChitH (2.1 ± 1.8) (Table 1). Due to shorter crosslinks among polymer chains, ChitH also reported lower values of compressive strength (CS = 4.9 ± 0.4 kPa) and ultimate compression (UC = 0.4 ± 0.1) when compared to CellH (CS = 12.3 ± 2.9 kPa; UC = 0.6 ± 0.3). As a matter of fact, genipin is quite a short molecule and made the crosslinked network of the ChitH material extremely brittle.[48] In contrast, the longer bridges holding together the cellulose polymer chains allowed the CellH material to experience sustained plastic deformation after the yield point.[49] When matrices were filled with *Bti* and yeasts, mechanical properties did not change significantly (Fig. S2, ESI†).

Hydrogels' biological activity

To address whether ChitH and CellH could represent an attractive feeding source for *Ae. albopictus* 3rd instar larvae, matrices

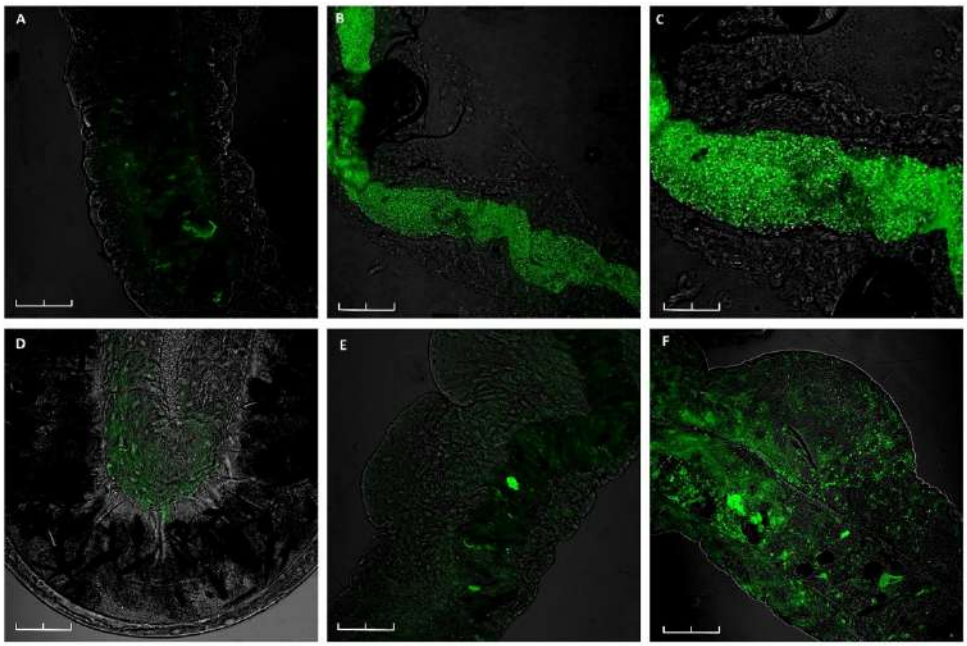

Fig. 5 Fluorescence emission observed in the dissected digestive tracts of larvae exposed to hydrogel tablets containing GFP-yeast. (A) No evident fluorescence was detected in larvae exposed for 24 h to empty ChitH tablets, only a weak fluorescence halo due to autofluorescence of larval tissue and/or undigested organic debris. (B and C) Fluorescent yeast cells were detected in the digestive tract of larvae treated with ChitH@Y tablets, after 24 h. (D) Only a widespread autofluorescence emitted by larval tissue was detected in larvae treated for 24 h with empty CellH tablets. (E) A weak fluorescent signal was detected in larvae exposed to CellH@Y matrices after 24 h. (F) The fluorescence signal associated with the presence of yeast cells, slightly increased after 72 h in larvae exposed to CellH@Y tablets. The scale bar is 200 μm in A and B and 100 μm in C–F.

only filled with fluorescent *S. cerevisiae* cells (yeast-GFP) were placed in water. Confocal microscopy images illustrate a clear green signal along the entire gastro-intestinal tract of the larvae when exposed to ChitH@Y already after 24 h (Fig. 5B and C). In contrast, from Fig. 5E and F it is visible that the intestine of larvae fed with CellH@Y did not present many cells inside the digestive tract within the same period of time and that a weak fluorescence signal could be observed only after 72 h of treatment.

Chitosan-based hydrogels (ChitH@Bti-Y) proved also to be extremely efficient in killing *Ae. albopictus* larvae; in particular, the results obtained after mortality bioassays showed that, in the presence of these tablets, all the exposed larvae died after 12 h. In contrast, when the larvae were exposed to the CellH@Bti-Y hydrogels, they were still alive even after 72 h. Considering that *Bti* has an immediate effect once ingested, such a late mortality might suggest that larvae died upon starvation rather than the direct activity of the biolarvicide.

In order to elucidate the mechanisms of action of the hydrogel formulations, and in particular to what extent *Bti* and yeasts were retained/released from the matrices, transmittance analysis over a period of 15 days was performed. Hydrogel tablets filled with both *Bti* and yeasts were left to fully swell in a 50 mM NaCl solution and matrix optical transmittance ($T\%$) was monitored over time. Such ionic strength was chosen because the hydrogel swelling ratio in larvae culture medium (*i.e.* mineral water) was similar to that registered in the 50 mM NaCl solution (Fig. S3, ESI†). A clear change in optical properties in the presence of active ingredients was particularly evident with cellulose-based hydrogels, since the empty matrix was completely transparent ($T\% = 100$), while in the presence of *Bti* and yeasts, a significant quantity of light was absorbed

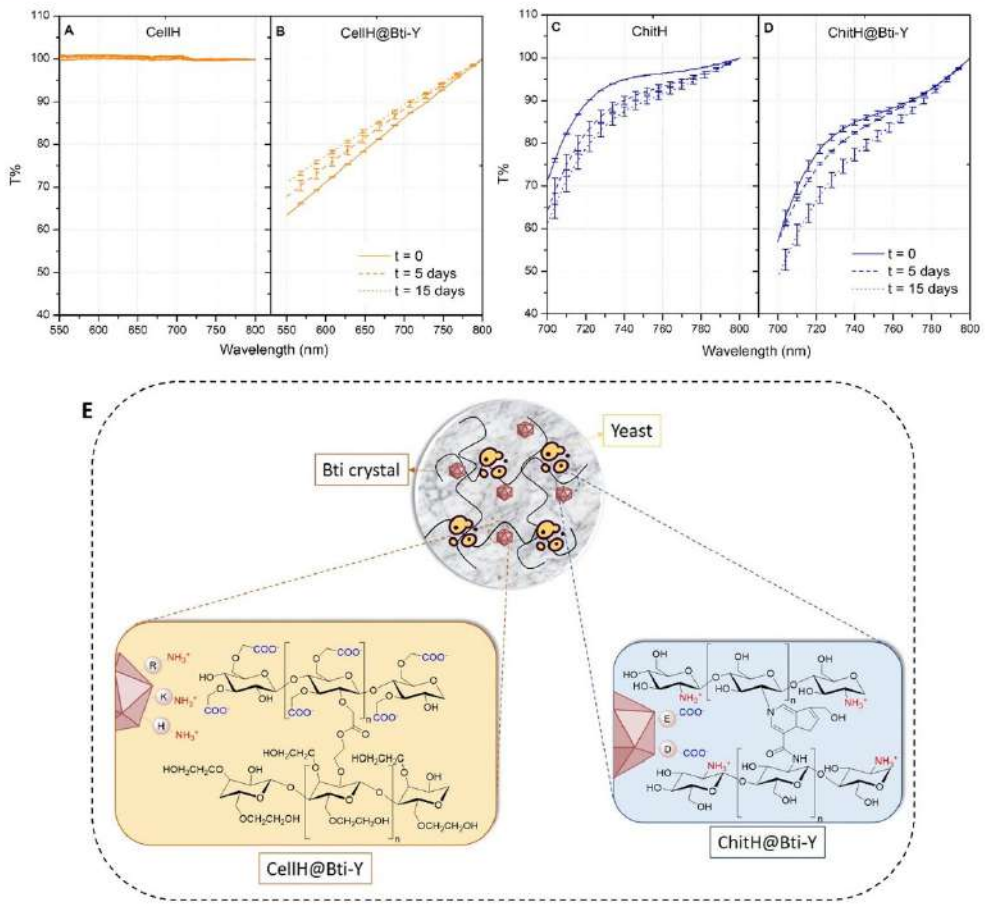

Fig. 6 Transmittance spectra of CellH (A), CellH@Bti-Y (B), ChitH (C) and ChitH@Bti-Y (D) at maximum swelling ($t = 0$), after 5 days and after 15 days of being immersed in a 50 mM NaCl solution. (E) Illustration of the ionic interactions between *Bti* charged amino acids and the hydrogel molecular backbone.

and/or scattered by the system (Fig. 6A). *T*% remained quite stable over the period tested with a small decrease of the transmittance value in the range of 550–800 nm (Fig. 6B), suggesting that only a small portion of active ingredients were released from the matrix. On the other hand, the transmittance spectrum of empty chitosan-based hydrogels already showed significant optical absorbance, in particular below 700 nm, which is why a narrow wavelength range (700–800 nm) was considered for comparative analysis. Despite all, a difference between empty matrices and those with *Bti* and yeasts was still evident, since at time zero ($t = 0$) the *T*% function was shifted downwards (Fig. 6C). Most importantly, even for this formulation, *T*% remained quite unvaried after 15 days of immersion in the salty solution (Fig. 6D).

We hypothesized that active ingredient retention within the hydrogel matrices (Fig. S4, ESI†) can be influenced by two phenomena: electrostatic interactions and size exclusion. The *Bti* active principle consists of the sporulation products of *B. thuringiensis* which contains two types of toxins, crystal (Cry) and cytolytic (Cyt). These toxins are crystal proteins with a similar C-terminal hydrophilic region rich in basic and acidic amino acids such as lysine (K), arginine (R), histidine (H), aspartic acid (D) and glutamic acid (E).[50] Therefore positively (R, K, and H) and negatively (E and D) charged amino acids can electrostatically interact with the anionic CellH network ($-COO^-$) or with the cationic ChitH network ($-NH_3^+$) respectively, thus preventing *Bti* crystal diffusion in the outer solution (Fig. 6E). For what concern yeast cells instead, we believe that their diameter ($\varnothing \approx 30$ μm) was way too large compared to the theoretical mesh size (ξ) of hydrogels ($\xi \approx 1$–100 nm), giving a possible explanation of why they could not be released by diffusion.[51,52]

All together, these results point in the direction that active ingredients were mainly administered to larvae upon hydrogel matrix erosion and/or natural degradation and not because of diffusion through the meshes of crosslinked polymeric chains. The substantial superiority of the ChitH biological effect in terms of bating and killing the target species is therefore a reflection of its brittle nature and lower stability in aqueous environments. As a matter of fact, the immediate availability of yeast cells and *Bti* crystals contained in the chitosan-based matrix suggested that this material can be easily eroded by the filtering buccal apparatus of the animal right after immersion in the culture solution, enabling delivery of many yeast cells and of a lethal amount of *Bti*. In contrast, the marked rubber-like mechanics of the cellulose-based matrix most probably hindered its erosion, with the consequence of just a small number of yeast cells detected in the larvae intestinal tract as well as the complete absence of *Bti* residual activity.

Besides the larvae's inability to erode a substance with such a gelatinous consistency, attractiveness was further reduced due to CellH's transparent appearance. During biological assays it was observed that larvae preferred the blue-coloured ChitH and remained in its proximity for a longer time. Dark coloured objects can be indeed more easily confused with organic feeding sources as well as hiding places from predators, making a chitosan-based tablet a perfect bait for larvae.[33]

4. Conclusions

In this article, we reported a novel strategy for encapsulating microorganisms and biolarvicides in biocompatible floating materials acting as a shield against environmental agents and smart delivery systems. To validate our system, mosquito larvae of *As. albopictus* were proven to be drawn towards hydrogel matrices made of polysaccharides embedded with a commercially available *Bti* product and *S. cerevisiae* cells. The ChitH formulation proved to have more efficient phagostimulant activity compared to the CellH one and also to efficiently cause larvae mortality within just a few hours. These results are ascribable to the intrinsic brittleness of the crosslinked chitosan matrix in aqueous environments that made the material easily crumble by the larva brushes during feeding. Physicochemical analysis suggested indeed that active ingredients (*Bti* and yeasts) were almost fully retained within the hydrogel network and could be delivered only by matrix erosion and/or degradation. We believe that such features, along with the natural composition of ChitH matrices that ensure environmental biocompatibility and nontoxicity, make the proposed engineered smart delivery hydrogel an appealing option for many applications. This might be considered as a proof-of-concept product to be possibly conjugated with other biolarvicides and/or microorganisms effective on different insect species that represent a problem for agriculture or public health.

Author contributions

Conceptualization: C. Bandi, S. Caccia, S. Epis, C. Lenardi. Investigation and validation: E. Brambilla, L. Giussani, S. Locarno, A. Negri, M. Piazzoni, S. Pitton. Data curation: S. Locarno, M. Piazzoni. Writing – original draft: M. Piazzoni. Writing – review & editing: C. Bandi, S. Locarno, A. Negri, C. Lenardi, M. Piazzoni.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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3.2 Second paper: "MosChito rafts as effective and eco-friendly tool for the delivery of a *Bacillus thuringiensis*-based insecticide to *Aedes albopictus* larvae"

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MosChito rafts as effective and eco-friendly tool for the delivery of a *Bacillus thuringiensis*-based insecticide to *Aedes albopictus* larvae

Simone Pitton[1,9], Agata Negri[1,2,3,9], Giulia Pezzali[1], Marco Piazzoni[4,5,6], Silvia Locarno[5,7], Paolo Gabrieli[1,2,3], Roberto Quadri[1], Valentina Mastrantonio[8], Sandra Urbanelli[8], Daniele Porretta[8], Claudio Bandi[1,2,3], Sara Epis[1,2,3]✉ & Silvia Caccia[1]✉

Adult mosquito females, through their bites, are responsible for the transmission of different zoonotic pathogens. Although adult control represents a pillar for the prevention of disease spread, larval control is also crucial. Herein we characterized the effectiveness of a suitable tool, named "MosChito raft", for the aquatic delivery of a *Bacillus thuringiensis* var. *israelensis* (*Bti*) formulate, a bioinsecticide active by ingestion against mosquito larvae. MosChito raft is a floating tool composed by chitosan cross-linked with genipin in which a *Bti*-based formulate and an attractant have been included. MosChito rafts (i) resulted attractive for the larvae of the Asian tiger mosquito *Aedes albopictus*, (ii) induced larval mortality within a few hours of exposure and, more importantly, (iii) protected the *Bti*-based formulate, whose insecticidal activity was maintained for more than one month in comparison to the few days residual activity of the commercial product. The delivery method was effective in both laboratory and semi-field conditions, demonstrating that MosChito rafts may represent an original, eco-based and user-friendly solution for larval control in domestic and peri-domestic aquatic habitats such as saucers and artificial containers in residential or urban environments.

Mosquitoes (Diptera: Culicidae) are a major threat in public health since adult females are able to transmit parasites and pathogens to humans and animals during the blood meal[1–3]. In addition, globalization and climate change have loosened biogeographic barriers and species with high invasive potential have spread worldwide creating concerns about exotic vector-borne zoonoses outbreaks[2,4–6]. In this scenario, the Asian tiger mosquito *Aedes albopictus* (Skuse, 1894) (Diptera: Culicidae) represents a case point because records of appearance in novel habitats have increased exponentially in the last decades[7,8]. Indeed, indigenous to South-East Asia, islands of the Western Pacific and Indian Ocean, *Ae. albopictus* is now present worldwide[7,8]. *Ae. albopictus* females are aggressive biters throughout the day, and they are competent vector for at least 22 arboviruses[2,7–9]. Since its first appearance in Europe in 1979, this species has been implicated in dengue and chikungunya outbreaks and reasonable concern is rising about Zika emergence in Europe in the near future[2,4,10,11]. Thus, mosquito control definitely relieves the biting pressure by aggressive species but, most importantly, it represents the pillar of disease prevention. Therefore research efforts to develop novel effective and sustainable control strategies are strongly encouraged[4,7,12–14].

To face the growing and global challenges in the control of vector-borne diseases, mosquito control must be tackled by Integrated Vector Management (IVM) that is a rational decision-making process consisting of a multi-level approach to optimize the use of different tools and strategies to make it efficient, cost effective, and

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sustainable[12,15,16]. Based on the constant engagement and mobilization of the communities, IVM includes vector surveillance and larval control that can significantly complement adulticiding in the mitigation of disease spread[15,17–20]. In particular, public education and community-based interventions for larval control are crucial in the case of highly anthropophilic and container-inhabiting species (e.g., *Ae. albopictus*) for which larval habitats are ephemeral, unpredictable and ubiquitous within domestic and peridomestic environments[11,12,21,22].

When feasible, the primary intervention for larval source management is the reduction of the availability of larval habitats, e.g., avoiding stagnation of water by everyday observation and elimination of small water containers[4,11,12,19,21,22]. In addition, in Europe, several larvicides are available (a complete list can be found on the European CHemicals Agency website at https://echa.europa.eu/it/information-on-chemicals/biocidal-products) and their adoption is regulated by the legislative act EU 528/2012 on biocide registration and use, that aims to encourage the exploitation of products with low impact on human and animal health and on the environment[11,12]. Essentially, two product categories are available for mosquito larvae control in EU, namely insect growth regulators (IGRs, i.e., chitin synthase inhibitors and hormonal disruptors) and microbial bioinsecticides [i.e., formulates based on *Bacillus thuringiensis* var. *israelensis* (*Bti*) or on the combination *Bti*-*Lysinibacillus sphaericus* (*Ls*)][11,12,21]. Although ascribed as chemicals, IGRs specifically target insect development and thus are relatively safe for non-target organisms with minor effects on aquatic insect fauna[11,23]. IGR-based formulations are important components in IVM since they are effective and long-lasting, especially diflubenzuron-based products. Nevertheless, resistance records have been described and the incidence of resistance should be taken into consideration when using these products extensively, for example planning the rotation of products with different active ingredients[4,12,24].

On the other hand, microbial larvicides based on *Bti* and *Ls* are considered safe for the environment and very specific[25,26]; in particular, they are active by ingestion since the bacteria produce proteinaceous toxins that target the midgut epithelium of mosquito larvae[26–28]. Products based singly on *Bti* or *Ls* present advantages and drawbacks with respect to each other and to other insecticides. *Bti* (i) is scarcely persistent, especially in polluted and organically enriched water, and requires multiple applications[11,29] however (ii) produces a blend of toxins (several Cry and Cyt toxins) and resistance outbreaks have never been registered, although a mild decrease in susceptibility and resistance to single toxins have been described[28,29]. Conversely, *Ls* (i) persists longer and recycle in the environment through infected larvae but (ii) insects are more prone to develop resistance to the binary toxin (Bin) responsible for its acute toxicity[26,30]. To avoid resistance spread no formulates with *Ls* alone are available on the market, whereas bioinsecticides based on both *Bti* and *Ls* have been developed to synergize the toxic effects of both and to partially compensate the lack of persistence of *Bti*. Nevertheless, combining *Bti* and *Ls* or *Bti* and IGRs (e.g., as in VectoPrime®) imposes unnecessarily a selection pressure by *Ls* and IGRs with a real chance of resistance alleles spread in mosquito populations. The use of these combinations would be avoided in the case of persistent *Bti*-based products, by making larval control more targeted and sustainable.

The present work aims to protect the benefits of the use of *Bti*-based bioinsecticides that suffer from lack of persistence but are highly recommended for their environmental sustainability and for their mode of action that prevent resistance development. We have recently developed a suitable delivery method for the oral administration of microorganisms or molecules to mosquito larvae using a chitosan-based hydrogel[31]. Herein the potential of this tool (i.e., "MosChito rafts") for the targeted and long-lasting delivery of a *Bti*-based formulate to *Ae. albopictus* larvae was investigated.

Results

MosChito rafts attractiveness for *Ae. albopictus* larvae. In the present study we intended to validate floating hydrogel rafts consisting of chitosan crosslinked with genipin that have been recently developed[31], for the delivery of a *Bti*-based formulate to control mosquito larvae with bioassays on *Ae. albopictus* larvae (Fig. 1a).

Larvae movement was measured and represented by cumulative heatmaps (Fig. 1b). In the case of control rafts (Fig. 1b, i), light blue-white halos were present in the whole test area except for the borders where yellow and red signals were intense, demonstrating that the larvae were inclined to move intensely and rest for long time at the borders, likely because control rafts were not attractive to them. In contrast, larvae exposed to Y, *Bti*, and *Bti* + Y (Fig. 1b, ii, iii, and iv respectively) rafts showed a tendency of staying around the raft itself, where a red-yellow halo is present, while the rest of the test area remained dark-blue coloured due to fewer movements and/or shorter permanence. In summary, Y, *Bti* and *Bti* + Y rafts attracted the larvae that perceived the presence of yeast, *Bti* or both in the rafts, and tended to stay close to them. These results were confirmed by a higher mean number of crossings of the larvae close to the border of the Petri dish in the case of control rafts compared to the other rafts (Fig. 1c) ($F_{(3, 35)} = 9.548$, $P < 0.001$, with $P < 0.001$ for control vs Y; $P < 0.001$ for control vs *Bti*, and $P < 0.01$ for control vs *Bti* + Y) and higher larvae permanence in the center zone in the case of Y and *Bti* compared to control (Fig. 1d) ($F_{(3, 29)} = 8.495$, $P < 0.001$ with $P < 0.01$ for control vs Y, $P < 0.001$ for control vs *Bti*). In the case of *Bti* + Y rafts no significant difference compared to controls was observed, although a tendency was present ($P = 0.106$) (Fig. 1d). Overall, contrary to previous reports, yeast did not act as a lure[32–35] and attractiveness assays have shown that MosChito rafts attracted larvae per se, thus without the addition of yeast in the hydrogel.

Insecticidal activity of MosChito rafts against *Ae. albopictus* larvae. Bioassays clearly showed that the insecticidal effect of MosChito rafts is (i) dose-dependent, and (ii) slower and lower on 3rd instar larvae compared to 4th instar larvae (Fig. 2). Indeed after 7 h, 3rd instar larvae exposed to the 1× dose were almost all alive, whereas about 50% of 4th instar larvae were dead (Fig. 2a,b). On the contrary, MosChito rafts with the higher dose were highly effective on both larval instars. Survival of 3rd and 4th instar larvae decreased significantly compared to the controls after 4 or 2 h of exposure to the higher dose respectively (for 3rd instar larvae $F_{(2, 21)} = 11.49$, $P < 0.001$; for 4th instar larvae $F_{(2, 33)} = 10.13$, $P < 0.001$) (Fig. 2a,b). After 24 h, MosChito rafts with

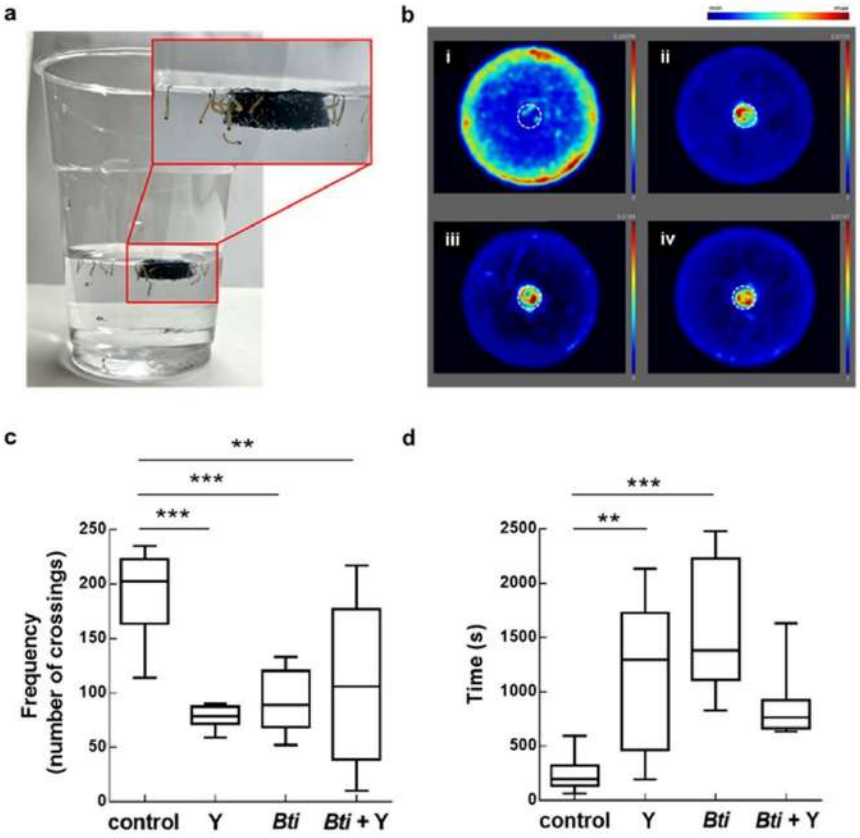

Figure 1. Exposure of *Ae. albopictus* larvae to MosChito rafts (**a**) and results of attractiveness assays (**b–d**). (**b**) Heatmaps of larval movement during the attraction experiment with control (i), Y (ii), *Bti* (iii), and *Bti* + Y (iv) rafts (see Methods for colour interpretation). In (**c,d**), crossing frequency of larvae in the zone at the border of the Petri dish and the cumulative duration of larvae permanence in the center zone are respectively represented. Data are reported as mean ± standard errors (**$P<0.01$, ***$P<0.001$).

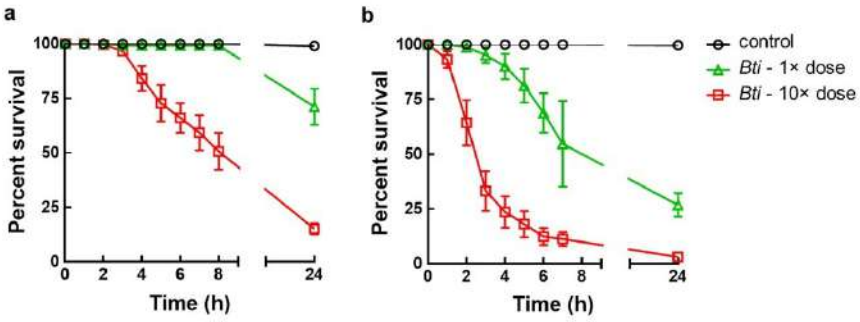

Figure 2. Bioassays with 3rd (**a**) or 4th (**b**) instar *Ae. albopictus* larvae exposed to control and *Bti* rafts. Two different doses of *Bti* were included in MosChito rafts (see Methods for the details) and survival of larvae was recorded at different times during 24 h. The values reported are the mean ± standard errors. Statistical significance of survival decrease in *Bti* exposed larvae compared to controls is reported along with the description of the results obtained.

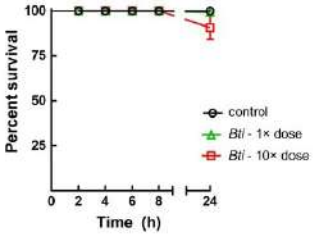

Figure 3. Bioassays with a mix of 3rd and 4th instar *Ae. albopictus* larvae to check whether *Bti* is released by MosChito rafts. Briefly, the rafts were left in 100 ml of tap water for 24 h and then the water was used to perform a 24 h time-course assay of survival with the larvae. The values reported are the mean ± standard error. The only statistically significant difference was observed at 24 h where 10× dose *Bti* induced a statistically significant, albeit small, survival decrease compared to other rafts ($F_{(2, 15)} = 10.76$, $P = 0.0013$, $P < 0.01$).

the 10× dose killed more than 80% of 3rd and almost all 4th instar larvae (for 3rd instar larvae $F_{(2, 27)} = 89.68$, $P < 0.001$; for 4th instar larvae $F_{(2, 33)} = 387.00$, $P < 0.001$).

To unequivocally demonstrate that MosChito rafts are active by ingestion of the hydrogel, a bioassay was performed exposing larvae to the water in which the rafts were left 24 h and then removed (Fig. 3). The data clearly showed that there was no toxicity in the water itself after removing MosChito rafts and thus the toxicity reported in the insecticidal activity test was almost entirely due to the consumption and direct ingestion of the hydrogel with *Bti* by *Ae. albopictus* larvae (Fig. 3).

At the same time, data obtained during a month period demonstrated that MosChito rafts maintained unaltered toxicity against *Ae. albopictus* larvae over time for both tested doses of *Bti* ($P < 0.001$) (Fig. 4). It is worth mentioning that the slightly reduced toxicity in 3rd instar larvae (Figs. 2a, 4a) compared to 4th instar larvae (Figs. 2b, 4b) was likely due to the ingestion of lower quantities of hydrogel containing the *Bti* during 24 h of exposure.

Although MosChito rafts attractiveness assays (Fig. 1) did not show a significant difference in the movement of the larvae between *Bti* and *Bti* + Y rafts, a bioassay was performed to check whether the presence of yeast may have any effect on MosChito rafts toxicity, for instance by phagostimulating the larvae and thus boosting toxicity. This hypothesis was not supported by the results (Fig. 5), indeed the presence of yeast in the rafts with 1× dose *Bti* did not show increased toxicity expected if the presence of yeast would have induced a higher consumption of the rafts ($P = 0.156$). As expected, *Bti* and *Bti* + Y rafts with the higher dose caused similar mortality, closed to 100% ($P = 0.156$).

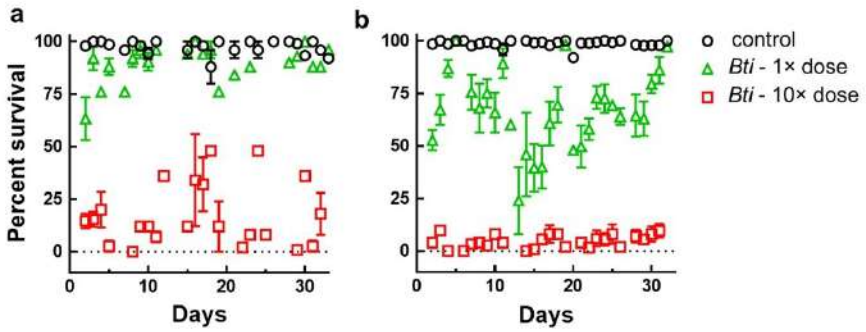

Figure 4. Bioassays with 3rd (**a**) or 4th (**b**) instar *Ae. albopictus* larvae exposed to control and MosChito rafts during long periods. Two different doses of *Bti* were included in the rafts (see Methods for the details) and 24 h survival of the larvae was recorded over a period of at least 30 days with the same rafts. The values reported are the mean ± standard errors. MosChito rafts toxicity was maintained during the test period ($P < 0.001$).

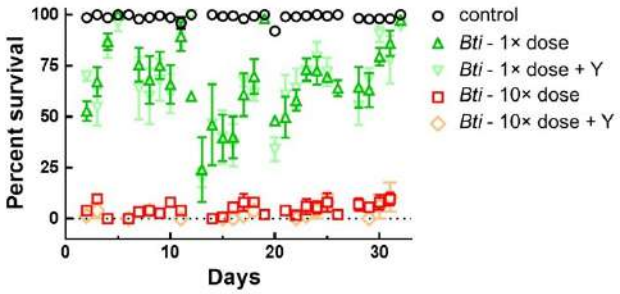

Figure 5. Bioassays with 4th instar *Ae. albopictus* larvae exposed to control, *Bti*, *Bti*+Y rafts (at two different doses, 1× and 10×). 24 h survival of larvae was recorded over a period of at least 30 days. The values reported are the mean ± standard errors. No statistically significant difference due to the presence of yeast between *Bti* and *Bti*+Y rafts was observed ($P=0.156$).

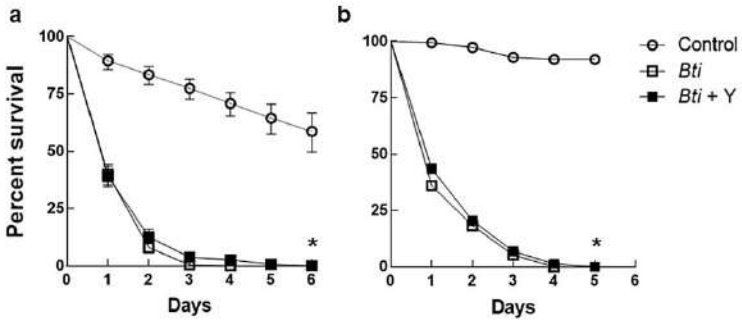

Figure 6. Bioassays in semi-field conditions with laboratory strains of *Ae. albopictus* established about 20 years ago (Rimini strain) or established less than 1 year prior to the experiments (Levate strain). Larvae of Rimini (**a**) or Levate (**b**) strain were exposed to control, *Bti* or *Bti*+Y rafts and survival was recorded every 24 h. The results are represented as mean ± standard error: curves that significantly differ from controls are indicated with an asterisk ($P<0.0001$).

Semi-field bioassays. Efficacy of MosChito rafts in the natural context was then tasted in semi-field bioassays: both strains were highly susceptible to *Bti*-containing rafts since after 1 day of exposure less than 50% survival was observed and after 5 days the mortality increased to almost 100%, compared to the controls (Fig. 6). The results showed that *Ae. albopictus* larvae with a genetic background presumably similar to that of the wild populations were also highly susceptible to *Bti* and did not show any behavioural characteristic that may cause control failure (e.g., lack of attractiveness or erosion activity and ingestion of MosChito rafts in a natural environment), thus validating MosChito rafts as an effective control tool.

Discussion

The control of *Ae. albopictus* mosquitoes is tricky because they can breed in almost any type of water-filled containers and dry-resistant eggs can survive over several months[12,36]. Nevertheless, their attitude to vector arboviruses compels the development of novel and sustainable control strategies. MosChito rafts represent an original, eco-based and user-friendly solution for larval control in aquatic habitats such as saucers and artificial containers in residential or urban environments to prevent the development of the immature stages. Control of floodwater mosquitoes is often performed by predictable, extensive and inundative treatments of wetlands or water bodies by professionals (e.g., by backpack sprayer or even helicopter). On the contrary, larval control of container breeding mosquitoes as *Ae. albopictus* or *Ae. aegypti* requires a localized and targeted treatment of breeding sites which can be better accomplished by hand-application of larvicides to specific containers in private or public urban contexts[11,12,22,36].

MosChito rafts have been conceived with highly tested biomaterials. Chitosan, the major component of the hydrogel, is a renewable resource since it is produced by deacetylation of chitin, the second most widely occurring biopolymer in nature after cellulose[37,38]. Besides availability, chitosan is characterized by non-toxicity, biodegradability, and biocompatibility. It is widely used in food packaging, water and wastewater treatments, cosmetics,

and agriculture to improve crops growth[39–41]. Chitosan also represents a valuable raw material for innovative biomedical applications, as carriers for a variety of drugs, bandages and wound dressing, tissue engineering, and in nerve reparation[38,41]. In addition, genipin was adopted in the hydrogel as chitosan cross-linker, since biobased cross-linkers of plant origin are safe, environmentally sustainable, and renewable[42–44].

The insecticidal activity of MosChito rafts relies on the inclusion of a *Bti*-based formulation to the initial hydrogel. *Bti* is a safe and effective bioinsecticide targeting mosquito larvae and is implemented in current control programs all over the world, including Europe[11,26,36]. The major concern about *Bti* use is the low persistence in the environment, mainly due to UV light exposure and microbic degradation, and thus multiple applications are required[26,28,45]. Available products stuffed with *Bti* as Mosquito Dunks® or Culinex® tabs are designed to be more or less rapidly dissolved in water and thus *Bti* is immediately exposed to water pH and UV light after its release. In addition, the amount of released *Bti* is not adjusted according to larval instar or density but is instead released in very high amounts to guarantee vector control. MosChito rafts' mechanism of delivery is completely different. First, MosChito rafts containing a *Bti* formulate (i.e., VectoBac® 12AS) to be attractive to *Ae. albopictus* larvae even in the absence of a lure, to be highly effective during the 1-month testing period (while the commercial liquid formulate that was used to make MosChito rafts persists fully active only for a few days, as reported in VectoBac® 12AS data sheet), and that toxicity is mediated by the erosion of the soft hydrogel by the larvae mouthparts followed by ingestion. This characteristic is extremely important since it avoids dispersion of the bioinsecticide that is protected by the hydrogel from environmental abiotic and biotic stressors. Importantly, MosChito rafts allow to overcome the need for the addition of other insecticides (as *Ls* or IGRs) in *Bti* formulates to prolong the insecticidal effectiveness, a practice that dangerously imposes a selective pressure on larvae which may evolve in resistance to *Ls* and IGRs.

The European directive No. 528/2012 on biocidal products regulation made *Bti* one of the few larvicides authorized for mosquito control. Notwithstanding the individuation of new bioinsecticides remains a core effort for the improvement of mosquito larvae control strategies, the protection of the benefits of *Bti* use and the optimization of its performances by developing new delivery methods and/or by combining this bioinsecticide with other control strategies are also key issues. Recent works have demonstrated that the effectiveness of a *B. thuringiensis* strain active on lepidopteran pests (*B. thuringiensis* var. *aizawaii*, *Bta*) is enhanced when target insects are immune impaired by RNAi-mediated silencing of genes involved in cellular immune responses[46,47]. In addition, this approach can be exploited in the field by co-administration of the *Bta*-based formulate with transformed bacteria or plants as delivery vectors for immune silencing dsRNAs[48,49]. Similarly, MosChito rafts could be used as vectors for *Bti* in association with dsRNA nanocarriers[50–52] or dsRNA-expressing microorganisms, as mosquito larvae have proved to be susceptible to environmental RNAi vectored by microorganisms, including *S. cerevisiae*[34,53–56]. *S. cerevisiae* in MosChito rafts can therefore be exploited as expression and delivery system for interfering RNAs or for other molecules able to complement or synergize *Bti* formulate activity. Research efforts in this direction are ongoing in our laboratory.

Likewise, the potential of this device has yet to be assessed for other mosquito species. Indeed, the possibility to control mosquito species that often share breeding containers will expand its potential. For instance, in Italy, the overlapping ecological niche and seasonal activities of the populations of *Ae. albopictus* and *Culex pipiens*[57,58], could play to our benefit for a targeted control of both species with a single product. Furthermore, the application of MosChito rafts could be fruitful and promising against the larvae of the species *Cx. pipiens* which normally develop in containers characterized by a larger volume (*Ae. albopictus*: < 5 L; *Cx. pipiens* (s.l.): > 5 L)[59].

In conclusion the present work represents a significant proof of concept that sets the stage for the development of diverse and effective control strategies for mosquito larvae. Indeed, in principle, any bioinsecticide active by ingestion (e.g., formulates that combine both *Bti* and *Ls*) can be included and delivered, and suitably transformed *S. cerevisiae* cells can boost the bioinsecticide activity.

Methods

Mosquitoes, microorganisms and reagents. Bioassays were performed using larvae of the Asian tiger mosquito *Ae. albopictus*. The Rimini strain was established in 2004 from mosquitoes collected in Rimini, Italy[60] and some egg clusters were transferred to the insectary of the Department of Biosciences (University of Milan). The Levate strain was recently established (September 2020) from larvae collected in Levate (Bergamo, Italy). Unless differently indicated, the Rimini strain was used for the experiments. The colonies were maintained in the insectary under standard rearing conditions (27 ± 1 °C, 65%–80% relative humidity, 12:12 h light/dark photoperiod). Both strains were fed with fish food (Tetra-fish, Melle) for all larval instars and with sucrose solution (10% w/v in distilled water) at the adult stage. Females were fed with animal blood to allow egg development. The eggs were stored dry in the insectary and used, by rehydration, no later than 2 months after the laying. For hatching, tap water and different hatching media (broths referred to as HM from now on) were tested, including the medium suggested in literature that include beef meat extracts (0.029 g Lab-Lemco powder, 0.14 g peptone, 0.14 g yeast extract, 0.14 g NaCl in 1 L of distilled water, HM3)[61–63] and 2 media developed in our laboratory (0.14 g Bacto™ tryptone, 0.14 g yeast extract, 0.14 g NaCl in 1 L of distilled water, HM1; 0.14 g Primatone® peptone, 0.14 g yeast extract, 0.14 g NaCl in 1 L of distilled water, HM2). The simplest and cheap medium HM1 was preferred as hatching solution, since no statistical differences were observed in the percentage of hatched larvae after 24 h compared to more complex media (i.e., 70% of egg hatching after 24 h, see Supplementary Fig. S1 online). This method allowed to optimally synchronize the larvae development which is important to perform bioassays with several conditions and replicates at the same time.

S. cerevisiae cells, strain SY2080, included into hydrogels (see "MosChito raft production") were grown in generic yeast extract peptone dextrose (YPD) medium enriched with 2% w/v glucose as nutrient source and with chloramphenicol (1 µg/ml) added as antibiotic. Thirty ml of yeast culture were placed into a 50 ml tube and

pelleted by centrifugation for 10 min at 3500×*g* at room temperature, and the supernatant was discarded. For heat inactivation the pellet was placed in a 70 °C water bath for 2 h (protocol modified from Mysore et al., 2017)[34]. A suspension of 10^7 heat killed cells/ml in water was used for rafts production. The commercial *Bti*-based product used in our experiment is VectoBac® 12AS (Sumitomo Chemicals Italia SRL, Valent Biosciences).

Unless differently indicated, all reagents were provided by Sigma-Aldrich, Italy.

MosChito raft production. A detailed description of the formulation of floating hydrogel baits (rafts) and their properties was reported in Piazzoni et al., 2022[31]. Briefly, "control" rafts were prepared by mixing 1 ml of chitosan solution (10 mg chitosan dissolved in 1 ml of a 1% v/v acetic acid solution and added with 10 µl of 20 mM sodium dodecyl sulphate in water) with 100 µl of 44 mM genipin solution in 10% ethyl alcohol. The other rafts used in the experiments were prepared by adding to the control rafts (i) SY2080 strain *S. cerevisiae* cells (50 µl of an aqueous suspension with 10^7 cells/ml) (i.e., "Y" rafts), or (ii) the *Bti*-based insecticide VectoBac® 12AS (5 µl or 50 µl of the liquid formulate for the rafts used in laboratory tests and 100 µl for semi-field tests) (i.e., "*Bti*" rafts), or (iii) both *S. cerevisiae* cells and VectoBac® 12AS (50 µl with 10^7 yeast cells/ml and 5 µl or 50 µl of the *Bti* formulate) (i.e., "*Bti*+Y" rafts). Then, air bubbles were injected using a syringe pump (KD Scientific, Thermo Fisher Scientific) to allow raft flotation in water; finally, they were placed in aluminum moulds (1.260 ml volume per well) to obtained rafts of discoidal shape of 1.6 cm (diameter) × 0.5 cm (thickness) (i.e., 1.2 ml of volume). The final concentration of VectoBac® 12AS in "MosChito rafts" was thus 4.2 µl/ml (indicated as "1× dose"), 42 µl/ml (indicated as "10× dose"), or 420 µl/ml (in semi-field tests). Moulds were then incubated overnight in a ventilated oven at 37 °C. For mosquito attractiveness assays, the rafts were cut to obtain smaller ones.

MosChito rafts attractiveness assays. To assess whether mosquito larvae were attracted or repelled by control, Y, *Bti*, or *Bti*+Y rafts, attractiveness assays were performed. The dose of VectoBac® 12AS used in these *Bti*, and *Bti*+Y rafts was the 10× dose (i.e., 42 µl/ml). For each assay one raft (0.5 × 0.5 cm) was fixed with a needle in the center of a Petri dish (90 × 15 mm) containing 5 ml of tap water. To record larvae movement, Petri dishes were then placed in the DanioVision™ observation chamber (Noldus Inc., Wageningen, The Netherlands) with a plateholder filled with water to maintain the temperature at 27 °C. One single *Ae. albopictus* 3rd instar larva was tested for each recording. At least 10 larvae for each raft type (control, Y, *Bti* or *Bti*+Y) were tested. During each acquisition, lasting one hour, larvae movements to 3 pre-identified concentric areas in the Petri dish were recorded by automated video tracking (EthoVision XT® software, Noldus Inc.). Starting from the dish center these areas are referred as "central zone", 0.5 cm to 2 cm from the center of the dish, "border zone", corresponding to the most external zone of the dish, near the border, with a 0.7 cm width.

The data acquired were used to generate heatmaps describing larval movements in different zones (the images offer an intuitive and unique view of the data, where the colour represents the relative time spent in a certain area (blue, low; red, high), averaged over all larvae of each experiment) and to establish the frequency of crossings or the duration of the permanence of the larva in a particular zone. Data were then processed with the GraphPad Prism (GraphPad Software Inc. version 8, San Diego, CA, USA).

Laboratory bioassays. Third and 4th instar larvae were exposed separately to the rafts according to the guidelines for laboratory and field testing of mosquito larvicides[64]. Briefly, batches of 25 larvae were transferred by means of plastic Pasteur pipettes to disposable plastic cups containing 100 ml of tap water. Rafts (controls and, depending on the experiment, 1× dose or 10× dose *Bti*, 1× dose *Bti*+Y, or 10× dose *Bti*+Y) were thus gently introduced in the water and experimental cups were put in the insectary (27 ± 1 °C, 65–80% relative humidity, 12:12 h light/dark photoperiod). In the case of the time course analysis of MosChito rafts toxicity (Fig. 2), survival was recorded at different time points within the 24 h period of exposure. In order to check whether the insecticidal activity was exclusively due to the ingestion of the hydrogel containing *Bti* and/or the *Bti* that was released into the water in the cups, the MosChito rafts were left in 100 ml of tap water for 24 h and then the water alone was used to perform a time-course assay of survival with a mix of 3rd and 4th instar larvae (cumulative survival of 3rd and 4th instar larvae was recorded after 2, 4, 6, 8 and 24 h). The experiment was performed in duplicate with 3 cups for each condition (control, 1× dose *Bti* and 10× dose *Bti*) and with 25 larvae for each cup. To measure the insecticidal activity of the rafts during time, after each 24 h bioassay (i.e., larval survival was measured 24 h after the exposure to MosChito rafts), rafts were moved to a new plastic cup, with fresh tap water and 25 larvae, to start a new 24 h bioassay. The insecticidal activity of MosChito rafts was recorded across a 30 days-period. Three batches of 25 larvae were used to measure survival for each experimental condition and experiments were repeated with rafts obtained with at least 2 independent preparations.

Semi-field bioassays. *Ae. albopictus* larvae were tested in bioassays under semi-field conditions to evaluate the larvicidal efficacy of control, Y, *Bti* and *Bti*+Y rafts over time. These experiments were performed in the backyard of the Department of Biosciences of the University of Milan, from June to September 2021. Fifty *Ae. albopictus* larvae (Rimini strain) at different developmental stage were added to plastic containers with 200 ml of rainwater plus pebbles, leaves and sand to mimic the peridomestic environment where these mosquitoes normally breed and larvae develop. Survival was recorded every 24 h, until all larvae died or until all control larvae were pupated. Each bioassay was performed in triplicate and repeated 3 times. The same bioassays were performed following the same protocol using Levate strain of *Ae. albopictus*, a strain that has been established in the laboratory less than one year before the bioassays.

Statistical analysis. Data obtained in attractiveness assays were checked for normality using GraphPad Prism (GraphPad Software Inc. version 8) and statistical significance of differences was assessed with One-way

ANOVA tests followed by Tukey's multiple comparison post-hoc test. Insect survival in laboratory tests was analysed by one-way ANOVA followed by Tukey's post-hoc test (Figs. 2 and 3) or by General Linear Model (GLM, performed with RStudio v2022.2.3.492, RStudio Team 2020)[65] (Figs. 4 and 5). Data from semi-field bioassays were analysed by Log-rank (Mantel-Cox) test and the comparison between groups was adjusted with FDR (false discovery rate). If not differently stated, statistical analysis was performed using GraphPad Prism.

Data availability

All data relevant to the study are included in the article or uploaded as supplementary information. In addition, the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

C.B., S.C., S.E. and S.U. conceived and designed research. S.C., S.L., A.N., G.P., M.P. and S.P. performed experiments. R.Q. performed the experiments with yeasts. S.C., S.E., P.G., V.M. and D.P. analyzed data. S.C. wrote the manuscript. S.E. revised the manuscript. All authors read and approved the manuscript.

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3.3 Third paper: "MosChito rafts as a promising biocontrol tool against larvae of the common house mosquito, *Culex pipiens*"

PLOS ONE

RESEARCH ARTICLE

MosChito rafts as a promising biocontrol tool against larvae of the common house mosquito, *Culex pipiens*

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Abstract

Mosquito control is of paramount importance, in particular, in light of the major environmental alterations associated with human activities, from climate change to the altered distribution of pathogens, including those transmitted by Arthropods. Here, we used the common house mosquito, *Culex pipiens* to test the efficacy of MosChito raft, a novel tool for mosquito larval control. MosChito raft is a floating hydrogel matrix, composed of chitosan, genipin and yeast cells, as bio-attractants, developed for the delivery of a *Bacillus thuringiensis israeliensis* (*Bti*)-based bioinsecticide to mosquito larvae. To this aim, larvae of *Cx. pipiens* were collected in field in Northern Italy and a novel colony of mosquito species (hereafter: Trescore strain) was established. MosChito rafts, containing the *Bti*-based formulation, were tested on *Cx. pipiens* larvae from the Trescore strain to determine the doses to be used in successive experiments. Thus, bioassays with MosChito rafts were carried out under semi-field conditions, both on larvae from the Trescore strain and on pools of larvae collected from the field, at different developmental stages. Our results showed that MosChito raft is effective against *Cx. pipiens*. In particular, the observed mortality was over 50% after two days exposure of the larvae to MosChito rafts, and over 70–80% at days three to four, in both laboratory and wild larvae. In conclusion, our results point to the MosChito raft as a promising tool for the eco-friendly control of a mosquito species that is not only a nuisance insect but is also an important vector of diseases affecting humans and animals.

Introduction

Mosquitoes have coexisted with mankind for thousands of years, and several species have impacted the health and the evolution of humans, in relation to their role as disease vectors. Among them, *Culex pipiens* Linnaeus 1758, is the most common mosquito species worldwide

and is an important vector of several pathogens, both in the tropics and in the temperate zones [1] as including West Nile virus, Japanese encephalitis virus and lymphatic filariae [2, 3]. Its wide distribution in the urban environments in most continents, including Europe, Asia, Africa and the Americas [4], motivates the epithets of "common house mosquito" and "Northern house mosquito".

The term *Cx. pipiens* refers to a polytypic species, or complex, that includes four species *sensu stricto*: *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. australicus* and *Cx. globocoxitus* [5]. Among them, *Cx. pipiens* is further divided into two well-defined forms (or biotypes), one that is typically observed aboveground (*Cx. pipiens pipiens*) and the other-one belowground (*Cx. pipiens molestus*). These two biotypes are indistinguishable at the morphological level, but very different in their ecology. In terms of physiology and behavior, *Cx. pipiens pipiens* is predominantly ornithophilic, feeds and rests outdoors, and requires large spaces to swarm and mate, as well as a blood meal to oviposit the first time; on the contrary, *Cx. pipiens molestus* prefers to feed on mammals, including humans, feeds and rests indoors, and is adapted to confined spaces without the need for a blood meal to complete the first oviposition [6].

In Italy and Europe, mosquito control programs are carried out mostly with targeted, local interventions (e.g., municipal or regional), in air against adults and in water against larvae [7]. The developmental phase that is most easily managed is the larval stage, as larvae live in specific and restricted habitats, such as small water pools and containers that represent the breeding sites [7, 8]. The containment of larval populations reduces the number of adult individuals that can transmit pathogens, outdoor and indoors, and mitigate public and environmental concerns due to the spraying of chemical products such as adulticides, in urban and peri-urban areas [8–10]. Larval control ranges from the reduction/elimination of breeding sites to the use of various insecticides (including bioinsecticides) [8, 11, 12]. Larval management can be implemented under the control of public or private administrations, with medium/wide-scale interventions and citizen involvement. For autonomous, citizen-based applications, the use of safe, eco-compatible and easy-to handle products is strongly recommended [13–15].

Insecticides against immature stages, considering their use in water and their longer permanence into the environment, must have two fundamental characteristics: high eco-compatibility and specificity of action against the target species. At present, only a limited number of compounds targeting mosquito larvae have successfully met the requirements of the European Union biocide legislation: compounds from the class of insect growth regulators (IGRs), like products targeting chitin synthesis (e.g., diflubenzuron), and bioinsecticides, based on use of *Bacillus thuringiensis israeliensis* (*Bti*) or *Bti* in combination with *Lysinibacillus sphaericus* (*Ls*) [7, 10]. These microbial bioinsecticides are generally preferred because they lead to immediate and selective death of the larvae, without consequences in terms of pollution; in addition, resistance in target species has been rarely documented [16–19]. On the contrary, resistance to chitin synthesis inhibitors appears to be more common in insects and has also been reported in natural populations of *Cx. pipiens* in Italy [20, 21]. It is indeed well-known that improper use of insecticides, even for the most efficient compounds, may lead to the overstimulation of larval defenses and to the selection of resistant individuals [22–24].

To counteract the selection of resistance towards bacterial larvicides, delivery systems are required, to protect these bioinsecticides, enhancing their activity and avoiding their use at sublethal doses, due to degradation in the environment (e.g., under sun-light exposure, water pH, and microbial degradation). Several formulations based on *Bti* are commercially available (e.g., VectoBac® 12AS), but some of these still present high biodegradability and, therefore, a low duration of action [25, 26]. Two recent studies [27, 28] proposed the use of a delivery system for a *Bti*-based formulate, obtained by inclusion of this bioinsecticide into a hydrogel matrix composed of chitosan (that we called MosChito raft). MosChito rafts indeed had been

previously tested [28], under laboratory and semi-field conditions, for both its larvicidal activity against *Aedes albopictus* mosquito larvae, through the embedded *Bti* formulate, and for its phagostimulant potential through the embedded *Saccharomyces cerevisiae* yeast. These studies have led to highly satisfactory results for *Ae. albopictus* larval control, in relation to the long duration of the killing action. In the present work, we determined the efficacy of MosChito rafts, both in laboratory and semi-field conditions, on *Cx. pipiens*, a mosquito species that often coexists and competes with *Ae. albopictus* for the same breeding sites [29, 30].

Materials and methods

Mosquito collection, colony establishment, and rearing

Experiments were performed on *Cx. pipiens* larvae from a laboratory strain, and on wild-collected larvae. The laboratory strain of *Cx pipiens* was established for the purpose of this study and was obtained from mosquitoes collected in Trescore Balneario (province of Bergamo, Italy) in 2020. Experiments were performed on larvae after about 20 generations in the insectarium at the Department of Biosciences (University of Milan). Briefly, mosquitoes were maintained in the insectarium in accordance with the habits of the species (24 ± 1˚C, 45%-50% relative humidity, 12:12 hours light/dark photoperiod). Larvae were fed with granular fish food (Tetra-fish, Melle). Oogenesis and oviposition were made possible by feeding adult females on turkey blood. Wild larvae were collected in the field, at the botanical garden "Cascina Rosa" at the University of Milan (45˚28'31.3 "N 9˚14'04.3 "E) between June and September 2022. A subsample of the mosquitoes collected for colony establishment and for the experiments on the wild larvae, were identified by morphological keys and PCR-based gene amplification and sequencing, according to published protocols [31].

MosChito rafts production

Hydrogels rafts were prepared as previously described [27, 28]. MosChito rafts are dishes of 1.6 cm (diameter) × 0.5 cm (thickness), composed of chitosan crosslinked with genipin, containing air bubbles to enable them to float. Two types of rafts were produced: (i) control rafts, composed of chitosan and genipin; and (ii) test rafts containing cells of *Saccharomyces cerevisiae* (strain SY2080; 10^7 cells/raft) and the *Bti*-based bioinsecticide product, VectoBac®12AS (Sumitomo Chemicals Italia SRL, Valent Biosciences). Each MosChito raft contained the commercial product VectoBac®12AS at a final concentration of 420 μl/ml. The bioinsecticide concentration was selected, based on previous data collected on *Ae. albopictus* [28] (also see next paragraph).

Bioassays under laboratory conditions

Since MosChito rafts are expected to control the two co-inhabiting species (i.e., *Cx. pipiens* and *Ae. albopictus*), preliminary bioassays were designed to evaluate whether the VectoBac®12AS concentration, previously used in MosChito rafts that were effective against *Ae. albopictus* [27], may be also suitable for *Cx. pipiens* control. To this purpose, Trescore strain larvae were exposed to the bioinsecticide at a LC_{50} (lethal concentration that causes 50% mortality) determined for *Ae. albopictus* (Levate strain, recently established as Trescore strain, see [28]). Tests were performed in accordance with the World Health Organization guidelines [32]. Briefly, pools of 25 4th instar *Cx. pipiens* (Trescore strain) or *Ae. albopictus* larvae (Levate strain) were transferred into 100 ml of tap water and exposed to 0.370 mg/l of VectoBac®12AS. The bioinsecticide was not added to controls. No food was provided, and alive and dead larvae were counted after 24 h. Bioassays were carried out in the insectarium under

standard rearing conditions and were repeated at least three times, as independent biological replicates, with at least three groups of larvae for *Ae. albopictus* and *Cx. pipiens*, for each condition.

Bioassays under semi-field conditions

Experiments to evaluate the insecticidal activity of MosChito rafts were performed as follows. Semi-field experiments were performed in the backyard of the Department of Biosciences of the University of Milan (45˚28'35.4 "N 9˚14'02.9 "E), in the period between June and August 2022. A breeding environment comparable to that observable in peri-domestic areas was recreated in the insect breeders (Bug Dorm provided by NHBS GmbH) (Fig 1), supplemented with 200 ml of rainwater and environmental enrichments (pebbles, leaves, and sand), in which 50 *Cx. pipiens* larvae at different developmental stages (from 1st to 4th instar larvae) were placed. Each bioassay included: (i) three breeders with MosChito raft *Bti*+Y containing the commercial *Bti*-based product VectoBac® 12AS and *S. cerevisiae* yeast (Y); (ii) three breeders with the control raft (empty). Alive and dead larvae counts were performed every 24 h, until all treated larvae in the breeders died or pupated. Bioassays were performed separately on laboratory Trescore strain larvae and on wild-collected larvae; experiments were repeated three times.

Data analysis

Mortality data obtained from laboratory and semi-field assays were analyzed using GraphPad Prism (GraphPad Software Inc. version 8.0). Larval survival of the different strains in the laboratory assays was statistically analyzed by the Student's *t* test, while the semi-field bioassays were analyzed by the Log-rank (Mantel-Cox) test and between-group comparison, adjusted by FDR (false discovery rate).

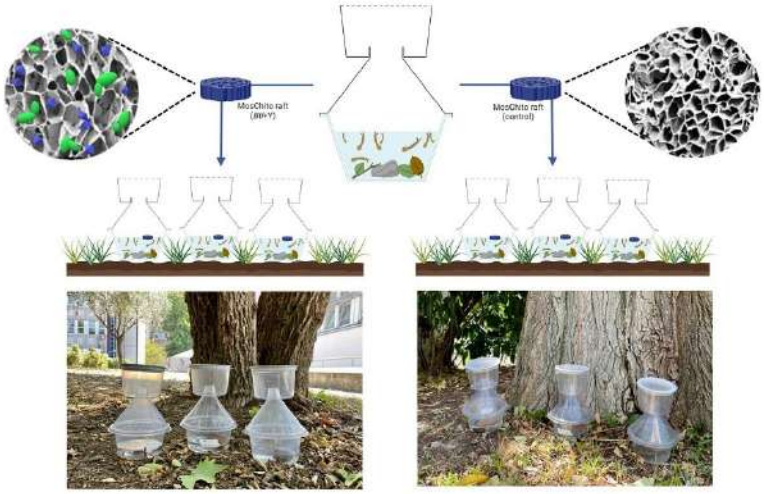

Fig 1. Bioassay in semi-field conditions. Graphical representation and pictures showing the bioassays in semi-field conditions used for *Culex pipiens* larvae exposed to MosChito rafts during summer 2022. Three breeders with *Bti*+Y MosChito rafts and three breeders with control rafts were placed in the backyard of the Department of Biosciences (University of Milan), directly on the ground under the trees and monitored every day, to count alive and dead larvae. Pictures are reprinted from PloSONE under a CC BY license, with permission from BioRender.com, original copyright 2023.

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Results

Mosquito identification by PCR analysis

Mosquitoes from the colony that were reared in the laboratory since 2020 (Trescore strain) and a subsample of the wild-collected larvae were identified as belonging to *Cx. pipiens* through morphological and PCR analyses, according to the published protocols [31, 33]. Thus, PCR products were run on a 1.5% agarose gel, bands of interest were recovered, and the amplified fragments were purified and sequenced. The obtained sequences were compared with public databases and matched with reference *Cx. pipiens* sequences.

Susceptibility of *Culex pipiens* to *Bti* under laboratory conditions

Bioassays under laboratory conditions were performed to determine whether the commercial insecticide *Bti*-based product VectoBac® 12AS had similar efficacy on *Ae. albopictus* larvae (where it has already been tested, also in rafts) and on *Cx. pipiens* larvae, in order to evaluate whether rafts for semi-field experiments could be assembled at the same dosages. Indeed, the LC_{50} dose, effective for *Ae. albopictus* larvae, induced a significantly higher mortality in 4th instar *Cx. pipiens* larvae after 24 h of exposure (Fig 2) (data in S1 Dataset) and thus MosChito rafts composition that resulted effective on *Ae. albopictus* [28] was used in the next experiments.

Susceptibility of *Culex pipiens* to *Bti* in semi-field bioassays

To evaluate the effectiveness of MosChito rafts on *Cx. pipiens*, under conditions that resemble habitats where the tool could potentially be employed for larval control, strains with a different

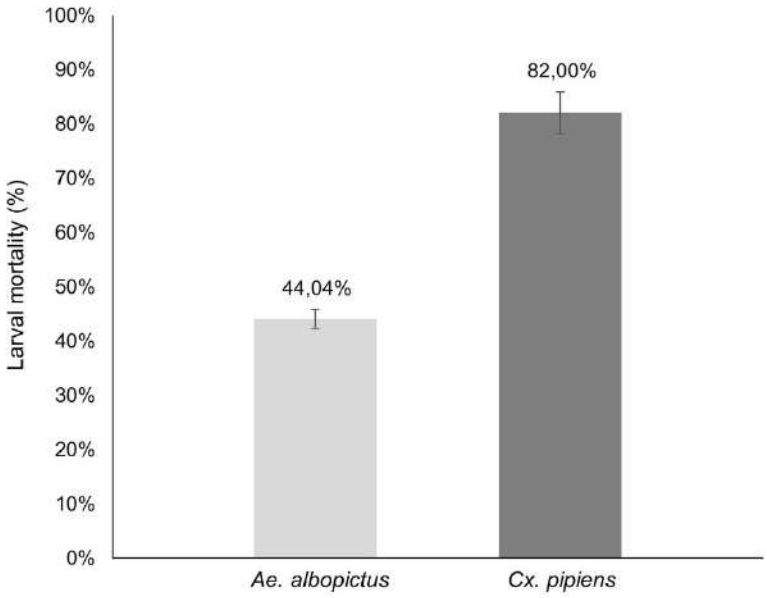

Fig 2. Comparative mortality of *Ae. albopictus* (Levate strain) and *Cx pipiens* (Trescore strain) to *Bti*, under laboratory conditions. Fourth instar larvae of both species were exposed for 24 h with the same dose of VectoBac® 12AS (0.37 mg/L). Data are reported as mean ± standard errors ($t(38) = 7.453$, $P<0.0001$, Student's *t* test). Controls mortality was zero.

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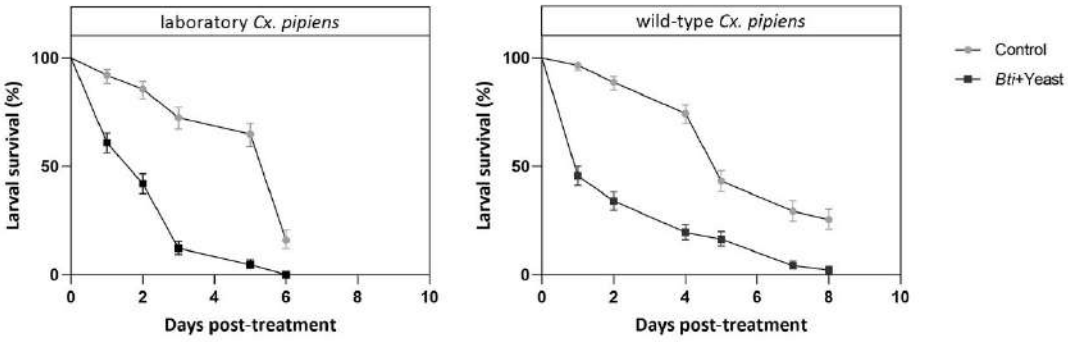

Fig 3. Larval mortality of *Cx. pipiens* in bioassays under semi-field conditions. Results obtained with a laboratory strain of *Cx. pipiens* (Trescore strain, established less than two years prior to the experiments) (a) or with larvae collected in the field (Milan) and immediately used (b). Larvae of both origins were exposed to control or *Bti*+Y rafts and survival was recorded every 24 h. Results are represented as mean ± standard error (N = 3 independent experiments) ($P < 0.0001$).

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origin were used: the Trescore strain and the wild-collected individuals, from Milan. Following the protocol developed in our previous work [27], MosChito rafts were tested on pools of larvae at different developmental stages.

For both *Cx. pipiens* strains, a mortality rate of more than 50% was observed, as early as the second day of treatment, with a more immediate effect for the wild-collected larvae (over 50% already by the first day) (Fig 3) (data in S1 Dataset). 100% mortality was reached on day six or eight for the laboratory and wild strain, respectively. A concomitant decrease in larval survival was observed on the last days, even for control pools, which probably suffered from the lack of food in the semi-field conditions.

Discussion

Human coexistence with *Cx. pipiens* dates to the end of the Neolithic period, and the historic acquaintance with this mosquito is possibly documented by ancient Egyptian papyri and pharaonic sculptures (as long ago as 2000 B.C.) [1, 34]. In recent decades this mosquito has been recognized as a major threat to human health, due to a series of interrelated factors that have modified its habitat, in parallel with changes in its behavior and in the hosts habits [35, 36]. *Cx. pipiens* in origin fed primarily on birds, in warm seasons [37] and in strictly wild and rural areas, but it has now adapted to feed even at lower temperatures and in highly urbanized/anthropized areas on different hosts, including humans [38–40]. Furthermore, urbanization and enhanced commercial trades, combined with gradual climate changes, and rising temperatures, have disrupted the initial balance, reducing distances between the vector, the animal reservoirs of mosquito-borne pathogens and humans, leading to spatial sharing and, consequently, also to pathogen sharing [1, 37]. In particular, in Italy and other European countries, *Cx. pipiens* has been found to occupy a wide variety of natural and artificial water containers in wild, rural, and urban areas, often coexisting with the invasive mosquito *Ae. albopictus* [29, 30, 41, 42].

The sharing of larval habitats of the major mosquito disease vectors (i.e., *Cx. pipiens* and *Ae. albopictus*) provides the opportunity to control both species with a single type of intervention. MosChito rafts was developed as an eco-friendly tool for the delivery of bioinsecticides to mosquito larvae [27] and has recently been tested for its efficacy against the Asian tiger mosquito *Ae. albopictus* [28]. However, the efficacy of larvicides is variable in different mosquito species

[43]. Therefore, with this current study we have determined the efficacy of this tool on *Cx. pipiens* larvae. Our results revealed a high efficacy against this species, paving the way towards the use of MosChito rafts in control programs, aimed at the containment of both *Cx. pipiens* and other species in the areals where they coexist.

Since laboratory experiments showed an even higher susceptibility to *Bti* of *Cx. pipiens* larvae compared to *Ae. albopictus*, MosChito rafts used for the present study contained the *Bti* concentration previously applied to *Ae. albopictus* in the semi-field tests [28].

Semi-field bioassays showed that *Bti*-containing MosChito rafts cause high mortality rates by the third day of treatment, in both mosquito strains (above 50% by the second day). The slower progression to 100% mortality in *Cx. pipiens*, compared to *Ae. albopictus*, possibly derives from the different amounts of bioinsecticide ingested, which in turn is due to the different ecology of the two species. Indeed, mosquito species exhibit different behavioral and feeding habits, at both the larval and adult stages, also in relation to the morphology of head and mouthparts [44]. Species from the *Culex* genus have been categorized as "collector-filterers" that feed in the water column and exhibit immobility in presence of food in the water [45, 46], while the *Aedes* species are generally categorized as "collector-gatherers" and "shredders" on detritus. Therefore, we hypothesize that *Aedes* larvae are more likely to directly shred the rafts and thus ingest micro-fragments of MosChito rafts. This above division of mosquitoes into feeding types should be considered with some caution because mosquito larvae have considerable behavioral flexibility in feeding habits, in response to resource availability and sensory stimuli [42, 46, 47]. In particular, the presence of phagostimulant factors, such as nucleic acids or nucleotides from microorganisms or organic surfaces (such as yeast in our case), attracts them to specific feeding areas [48, 49]. *Culex* larvae have previously been observed moving toward the food source, spending time filtering, and beating their mouthparts near/over debris [42, 48]. During the bioassays, we hypothesized that the embedded yeasts were not released from MosChito rafts, as for *Bti*, as previously demonstrated, in [28]. Thus, the strong attractiveness of yeast [50], trapped within the matrix, is not sensed over long distances by the larvae, however, it could be easily enhanced through the addition of other attractive molecules [51]. Moreover, yeasts could be used in the future, in combination with other insecticides (that present a more repellent effect than VectoBac® 12AS) as bio-factories to produce inhibitory molecules of larval defense systems (i.e., dsRNA, siRNA). The biological control action performed by *Bti* thus would be combined with RNA interference action.

Thus, the use of MosChito rafts is particularly advantageous because the inclusion of *Bti* shields it from sunlight and external agents, that would enhance its degradation. The inclusion of the VectoBac® 12AS product is permanent and avoids release into the environment, preventing phenomena such as deposition in soil or exposition of mosquito larvae to sublethal doses of bioinsecticide (thus preventing resistance phenomena). Unlike current commercial products that act by dispersion/dissolution, MosChito rafts act in a direct and targeted manner on larvae, in the reproduction site where high doses of *Bti* are ingested, while reducing the concentrations used for the single raft. This translates into a reduction in both cost and environmental impact. Although yeasts do not have an attractive action, they can subsequently be engineered to obtain an additional control method. Furthermore, the dark color, buoyancy, biological and erodible composition make MosChito rafts excellent larval bait.

In conclusion, MosChito rafts represent an environmentally friendly tool for mosquito control, which has been shown to be effective on two larval species that are implicated in the transmission of pathogens affecting humans and animals. A single weapon with a safe approach to control two mosquito species that, despite exhibiting different behavioral characteristics, coexist in the same environment and have a remarkable adaptive capacity, may help to reduce the

transmission of different viruses and other pathogens, with a containment of future outbreaks, in Italy and in other countries.

Supporting information

S1 Dataset. Data resulted from multiples bioassay performed in laboratory and semi-field condition and graphical represented in Figs 2 and 3.
(XLSX)

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4. Conclusions and future perspectives

The results obtained in the framework of this doctoral research project represent the proof of concept for the development of a novel biorational product for the biological control of mosquito larval stages based on the entomopathogen *Bacillus thuringiensis* var. *israelensis* (*Bti*). Specifically, MosChito rafts are biobased floating hydrogels that demonstrated to be attractive for the larvae of the Asian tiger mosquito *Ae. albopictus* and those of the common house mosquito *Culex pipiens*. The larvae erode the matrix and ingest the *Bti*-based product included in the raft that cause high mortality within a few hours in laboratory assays and within a few days in semi-field conditions against *Ae. albopictus* and *Cx. pipiens* larvae with a genetic background similar to that of natural populations suggesting their potential for the management of wild mosquitoes. Importantly, effectiveness of larval control is maintained unaltered throughout one month.

The inclusion of the *Bti*-based commercial product Vectobac® 12AS into MosChito rafts solves the problem of short environmental persistence of the formulate, i.e., a few days [Valent BioSciences 2019, Silva-Filha et al. 2021] and thus of constant reapplication of the bioinsecticide to get an effective management of mosquito populations that would translate into higher public costs [Becker et al. 2022]. In addition, the presence of a constant and high dose of Vectobac® 12AS in the matrix allows to prevent resistance phenomena.

Since *Ae. albopictus* and *Cx. pipiens* species are commonly sympatric in urban environments in Italy and in Europe [Carrieri et al. 2003, Marini et al. 2017], the proof of efficacy of MosChito rafts against them would allow their simultaneous control using the same product. In future, the efficacy of these hydrogels may be investigated for the biological control of other invasive mosquito species with vector competence such as *Ae. koreikus* and *Ae. japonicus* that invaded the EU and are now spreading in different Italian regions [Montarsi et al. 2019, Gradoni et al. 2021, Arnoldi et al. 2022].

Although the composition of MosChito rafts includes only environmentally safe components (i.e., *Bti*, *S. cerevisiae* yeast, chitosan, and genipin), it cannot be inferred that the same is true for the final product. Ecotoxicological analyses are necessary to assess these aspects. Preliminary studies conducted on *Danio rerio* and *Daphnia magna* to test motility, vitality, fecundity, and oxidative stress biomarkers showed no statistical differences between treated and control samples [unpublished data]. Further studies on MosChito raft's ecotoxicological effects are needed to determinate if they align with the Organization for Economic Co-operation and Development (OECD) and the EU guidelines for chemical safety assessment [OECD 2023].

Furthermore, additional studies are ongoing to produce MosChito rafts containing a commercial product based on the combination of *Bti* and *Lsph*.

Lastly, yeast was included in MosChito rafts as attractant and phagostimulant, but its presence has not showed to be effective. Nevertheless, yeast cells inclusion could reveal its potential in future. In fact, they could be exploited as a biofactory to produce immune suppressive double-stranded RNA molecules (dsRNAs) to silence target mosquito larva's immune gene in order to weaken larval defences while boosting *Bti* toxicity. A proof of concept for this idea is represented by the increased toxic effect of a *Bt* var. *aizawaii*-based bioinsecticide achieved on *Spodoptera littoralis* larvae previously treated with dsRNAs targeting genes involved in cellular immune responses, i.e., *Sl 102* and *Sl gasmin* [Caccia et al. 2016, Di Lelio et al. 2019; Caccia et al. 2020]. The yeast cells could be exploited for the cost-effective production of dsRNA molecules. Their inclusion into MosChito rafts would guarantee dsRNA protection from environmental factors and their combination with a *Bti*-based bioinsecticide would decrease the selective pressure exerted by the toxins on the genome of target mosquito species reducing, if not eliminating, the risk of resistance onset. The identification of a set of candidate immune genes as well as their characterization would be necessary to proceed with further research. Indeed, differently from adults' immune system that has been widely studied over the last century, little is known about immunity in mosquito larva and further investigations are needed to uncover important aspect for larval management.

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