Bacteria endosymbionts: a source of innovation in biotechnology for the control of vector-borne diseases

Currently available strategies to fight mosquito vector-borne diseases are insufficient, and new approaches need to be developed according to modern economical and ecological sustainability parameters. In this review we discuss the recent advances in the exploitation of endosymbiotic bacteria as biological tool for mosquito vector control, in particular for *Aedes* species, to which the asian tiger mosquito, widely spread also in Italy, belongs.The alfa-proteobacteria *Wolbachia* are among the most common intracellular bacteria and have recently emerged as important drivers of arthropod biology. *Wolbachia* commonly act as reproductive parasites in arthropod inducing cytoplasmic incompatibility (CI) a type of conditional sterility between hosts harbouring incompatible infection types. Two main strategies of using this bacterium for mosquito vector control are presented: (1) the first, treated more extensively, since it directly involves ENEA research activity, exploits cytoplasmic incompatibility as natural source of sterility in place of ionizing radiation for the sterile insect technique (renamed Incompatible Insect Technique), (2) the second, uses the reproductive advantages, conferred by the incompatibility cytoplasmic to infected females, to promote the invasion and ultimately the replacement of natural populations, with individuals carrying advantageous phenotypic traits like low pathogen competence. New scientific evidence regarding the interaction between the symbiotic bacteria and its effects on the hosts, in particular the interference with their ability to transmit pathogens, is further increasing the interest of biologists and entomologists to the study of these symbiotic associations arthropod-bacterium

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Batteri endosimbionti: una fonte di innovazione nelle biotecnologie per il controllo delle malattie trasmesse da zanzare vettori

Le strategie di lotta, attualmente disponibili, per combattere malattie trasmesse da specie di zanzare vettori sono ancora insufficienti e nuove tecnologie devono essere sviluppate nel rispetto dei più moderni parametri di sostenibilità economica ed ecologica. In questa review si analizzano i recenti progressi scientifici compiuti nell'impiego di batteri endosimbionti di artropodi per il controllo biologico delle zanzare vettori di malattie umane, in particolare per le specie del genere *Aedes*, cui appartiene la zanzara tigre ampiamente diffusa nel nostro paese. Gli alfa-proteobatteri del genere *Wolbachia* sono tra i batteri intracellulari più comuni negli artropodi con un importante ruolo di modificatori della biologia riproduttiva dei loro ospiti. *Wolbachia*, normalmente, si comporta come parassita riproduttivo inducendo, tra le altre modifiche riproduttive, una forma di sterilità condizionale (incompatibilità citoplasmatica) tra individui di una stessa specie che presentano un diverso status infettivo (infezione con ceppi diversi, o presenza contemporanea di individui infetti e non infetti). Due principali strategie di impiego del batterio vengono discusse: (1) la prima trattata ampiamente, perché direttamente vede impegnati ricercatori ENEA, che sfrutta l'incompatibilità citoplasmatica come fonte di sterilità in sostituzione delle radiazioni ionizzanti nei programmi di lotta col maschio sterile (SIT); (2) la seconda che sfruttando i vantaggi riproduttivi conferiti dalla incompatibilità citoplasmatica alle popolazioni infette, rispetto a quelle non infette, mira alla in invasione, ed in ultima analisi alla completa sostituzione, di popolazioni naturali, con individui portatori di caratteristiche fenotipiche vantaggiose per l'uomo (ad esempio bassa patogeni trasmissibilità di malattie). Nuove evidenze scientifiche riguardo all'interazione tra il batterio simbionte e i suoi effetti sugli ospiti, in particolare l'interferenza con la loro capacità di trasmettere patogeni, sta incrementando ulteriormente l'interesse dei biologi e degli entomologi verso lo studio di queste associazioni simbiotiche artropode-batterio

Introduction

Mosquito vector-borne diseases such as malaria, leishmaniasis and dengue still represent a significant threat to human health despite considerable national and international efforts [1]. Increased urbanization, migration and poor environmental sanitation are some of the major causes of the emergence and re-emergence of vector-born diseases in developing countries [2]. In addition, due to the effects of global warming [3] re-emerging arboviral pathogens such as dengue and chikungunya (CHIKV) viruses are becoming an increasing threat also in temperate regions [4].

In 2007 an unprecedented outbreak of CHIKV occurred in Italy, supported by the vector mosquito *Aedes (Stegomya) albopictus* (Diptera, Culicidae) [5]. This mosquito species, commonly known as the Asian tiger mosquito, is currently among the most invasive insect species of the world. Following the outbreaks of chikungunya virus in the Indian Ocean islands in 2005-2006 [6] and in Italy in the summer of 2007 (207 cases) [7], the *European Centre for Disease Prevention and Control* (ECDC) closely collaborated with experts in entomology to ensure a comprehensive understanding of the vector-related risk for introduction of the virus in Europe [9].

The concept that vector control remains a key option in the general strategy to reduce the incidence of vector mediated diseases in humans is now consolidated in the scientific community. This should be a strong incentive to focus research efforts towards the development of innovative technologies for vector control.

Scepticism towards the existing vector control strategies

The prevention and control of vector-borne diseases in the early 20th century was effectively achieved through the effective control of vector populations. The best examples are the control of the vector of yellow fever in South America during the early 1900s and the worldwide control of malaria vectors in the 1950s through the 1970s [10]. These successes lead to considerable optimism that these diseases might even be eradicated by using insecticide-based vector control measures and drugs for their prevention and treatment. However, these expectations were short lived because of increasing vector insecticide resistance, harmful effects of certain insecticides on the environment, and lack of resources as well as logistical difficulties associated with implementation of such plans. Clearly, currently available strategies to fight vector-borne diseases are insufficient, and new approaches need to be developed according to modern economic and ecological sustainability parameters.

Aedes albopictus in Italy

A few years since its introduction into Italy in 1990 [8], *Ae. albopictus* has become the most noxious and problematic mosquito species in the country. The colonization process occurred very quickly, mainly through passive transportation, and this species is currently found in all the Italian territory (Figure 1), including the main islands [9].

In Italy, current control methods against *Ae. albopictus* (larval control, source removal, community participation) show unsatisfactory results in terms of sustainable reduction of vector density (data not published). It could be argued that the main reason for the weakness of conventional control practices lies in the eco-ethology of the species that makes it very adaptable to colonize a number of artificial habitats. Larval sites are often located in private properties, thus making control by public institutions extremely difficult because of limited access to private properties and to the high costs of running programs. So far, community participation campaigns to control mosquito breeding sites, even when regularly conducted with professional methods, have never achieved a sufficient level of active participation from citizens for tangible results to be seen (unpublished data).

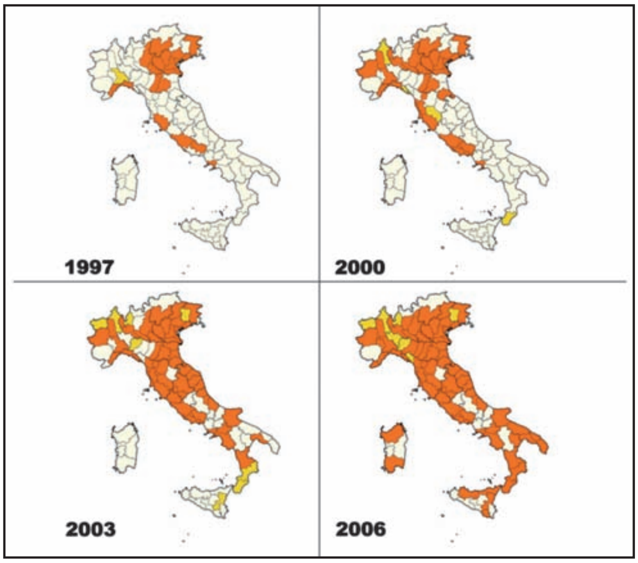

FIGURE 1 Expansion of *A. albopictus* in Italy
Source: Modified from Schaffner et al., 2007

Sterile Insect Technique (SIT) for mosquito control

In the early 2000, the Centro Agricoltura Ambiente "G. Nicoli" (CAA), in cooperation with ENEA (Italian National Agency for New Technologies, Energy and sustainable Economic Development), started developing a sterile insect technique (SIT) to be included in an integrated control program for the Asian Tiger Mosquito in Italy [11].
Nowadays interest in SIT for vector control has resurfaced, driven by the availability of new technologies that have the potential to provide significant cost-effectiveness improvements for SIT, as well as by recognition of the limits of current vector control strategies [13]. SIT are species-specific and environmentally non-polluting methods that rely on the release of large numbers of sterilised insects [14, 16]. Mating of released sterile males with native females leads to a decrease in the female reproductive potential and ultimately, if males are released in sufficient numbers over a sufficient period of time, to the local elimination or suppression of the pest population. Used with an area-wide approach, SIT made it possible to eradicate or suppress pests such as the New World screwworm

Cochliomyia hominovorax Coquel (Diptera: Calliphoridae (from the USA, Mexico and Central America and more recently in Libya)[15]. In Italy this technique was successfully applied in the 1970s to suppress the Mediterranean fruit fly (*ceratitis capitata*) from the isle of Procida by a project carried out by ENEA and coordinated by the IAEA, International Atomic Energy Agency [17].

Some biological and ecological features make *Ae. albopictus* a good candidate for the application of SIT. *Aedes* species are relatively easy to manage under artificial mass rearing conditions, and a pilot model system to rear *Ae. albopictus* has already been set up at the CAA facility in Crevalcore, Italy [12]. The genetic differentiation observed among the Italian populations showed the existence of structured populations at the regional geographic scale with restricted gene exchange among them [18]; the active dispersal of the species is recognized to be limited to a few hundred meters [19, 21]. Moreover, in temperate areas like Northern Italy, the outdoor reproduction is impossible for 5-6 months and winter survival relies on the eggs entering a state of diapauses [19]. The naturally high egg mortality during the winter results in a very low vector density at the beginning of the favourable season [22]. This appears to be the right time to apply a "birth control strategy" against the Asian tiger mosquito in Italy.

Mosquito sterilization: technical issues

Males of *Ae. albopictus* may be easily sterilised through ionizing radiations by exposing mature pupae to a dose of 30-40 Gy γ-rays [23]. Overall, the technique requires laborious handling procedures to prepare pupae for irradiation and transportation, in addition to the need for a radiation source, which is an expensive tool that needs an infrastructure requiring a substantial regulatory framework. This is a minor issue when the SIT is applied following a centrally coordinated area-wide program as in the case of screwworm or fruit-flies eradication programs [24]; in contrast, it can become a constraint when trying to achieve localized suppression of the target pest. In the latter case, the application of SIT requires that most of sterile males releases are concentrated in early summer before the occurring of the first population outbreak. Releases of sterile males should be widely distributed, targeted on those areas predicted to be suitable for mosquito spread like parks, residential gardens and villages and to be supported by community participation for an easier access into private areas.

Developing a technology to produce *ready-made sterile males*, avoiding sterilization with gamma rays, could improve the overall competitiveness of the released insects, allowing to use a *scattered release* approach more suitable against an irregularly distributed species like *Ae. albopictus* in Italy.

Furthermore, the development of *insect transgenesis* technology, is making major progress in this direction and it is about to produce innovative solutions to control insect pests and vectors. The main goals of *insect transgenesis* may be divided into three major categories:

- to make vectors incapable of transmitting the plasmodium protozoa parasite or viruses and use transgenic lines to replace natural vector populations;
- to induce male sterility by way of sterile insect technique, avoiding radiation sterilization and its negative impact on male fitness [25, 26]
- to develop genetic sexing strains to separate sterile males from females [27] for SIT.

Since separation of sexes remains a critical process for SIT approaches, accidental release of infected females should be avoided. Handling a genetic sexing strain would be a strong improvement of SIT. Nevertheless, the effectiveness of the transgenic approach for mosquito (or other pests) population control is under study [25, 26, 28] and the rigorous evaluation of a strain's robustness both in mass-rearing and in field conditions would grant its successful utilization in the framework of SIT programmes. Moreover, issues related to the public acceptance of new technologies that rely on the release of transgenic material have not been resolved yet. In the present state, this aspect likely represents the major limit for the application of this technology due to a very complex regulatory framework for experimental use in open field conditions.

The *endosymbiotic bacterium Wolbachia*: a potent resource for the challenge of mosquito vector control

In the last decades scientists have given more and more theoretical attention to *Wolbachia pipientis*, (Alphaproteobacteria, Rickettsiales) (Figure 2), [29], a widespread intracellular bacterium, carried by an estimated 60% of insect species as well as by some crustaceans, mites and filarial nematodes.

The type species for the *Wolbachia* genus is *Wolbachia* pipientis, which was first discovered in the gonads of the mosquito *Culex pipiens* (Diptera, Culicidae) with no associate pathogenic symptoms [30]. Later the link between Wolbachia infection, the host and the existence of different crossing types was highlighted [31].

The symbiontic relationship between *Wolbachia* and mosquito is the result of a long coevolution whose effects confer reproductive advantages, realized through mechanisms of physiological conditioning. It has been demonstrated that *Wolbachia* infection induces different modifications in the reproduction of its hosts such as feminization of genetic males; parthenogenetic induction, which results in the development of unfertilized eggs; the killing of male progeny from infected females; and sperm–egg incompatibility which is referred to as cytoplasmic incompatibility (CI) [32].

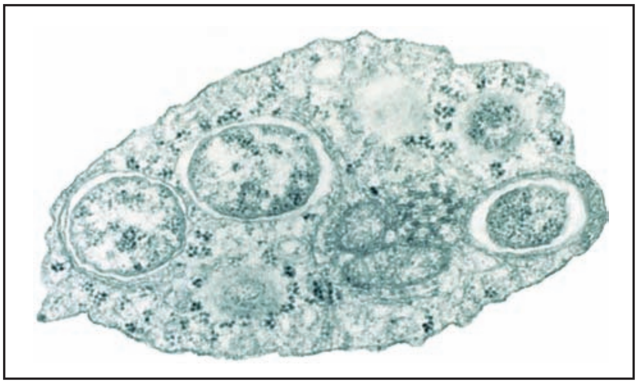

FIGURE 2 The endosymbiotic bacterium *Wolbachia pipientis*
Source: Genome Sequence of the Intracellular Bacterium Wolbachia. PLoS Biol 2/3/2004: e76. http://dx.doi.org/10.1371/journal.pbio.0020076; Author: Scott O'Neill

These attributes are now being studied by many research groups with the aim to develop new technologies and strategies to achieve ground breaking improvements in pest and vector control.

Cytoplasmic incompatibility: a natural mechanism for pest and vector sterility

Cytoplasmic incompatibility (CI) is the most frequently found *Wolbachia*-induced phenotype and has been described in several arachnids, isopods and insect orders [32]. Sperm from *Wolbachia*-infected males is incompatible with eggs from females that do not harbour the same *Wolbachia* type (or types). *Wolbachia*-mediated CI acts through two distinct mechanisms: modification of sperm during spermatogenesis and rescue of this modification in embryos infected with the same strain. When the embryo, originated from a modified sperm, does not contain the appropriate *Wolbachia*, its development is disrupted. The molecular mechanisms that underlie CI remain unknown, despite considerable work on the effect and various proposed mechanisms [33].

CI application instead of gamma radiation for insect sterility

The *Wolbachia*-mediated CI has been proposed as a strategy for insect control as follows: (1) using *Wolbachia*-induced CI as a form of sterility for a mass male release strategy, analogous to a sterile insect technique (renamed Incompatible Insect Technique=IIT); (2) using the reproductive advantage afforded by *Wolbachia*-induced CI as a population replacement strategy to drive wanted phenotypes into natural populations [34] In this review we focus on the first strategy in which *Wolbachia* induced CI is used in alternative to gamma radiosterilization to achieve male sterility and provide *ready-made* sterile males for SIT application.

Application of IIT strategy was originally thought for those species in which incompatible populations coexist (different infection types, or co-presence of infected and uninfected specimens). In fact, the IIT has been used successfully against disease vectors such as *Cx. pipiens* [35], or agriculture pests R*hagoletis cerasi* (Diptera, Tephritidae) [36]. Presently, an operational IIT program is going to be started on multiple islands in French Polynesia to suppress *Aedes (Stegomya) polynesiensis* (Diptera, Culicidae), a particularly efficient Lymphatic filariasis (LF) vector of South Pacific areas [37].

Important mosquito species were cut-off from the application of this strategy by the inability to identify suitable incompatible infection types in the wild populations. This is easy to understand for species like *Aedes aegypti* L. (Diprea, Culicidae) and *Anopheles* spp., that in nature do not harbor *Wolbachia* symbionts. More complex is the case of *Ae. albopictus* which is uniformly superinfected with two *Wolbachia* strains (*w*AlbA and *w*AlbB) throughout its geographical distribution [38] and no evidence of crossing type polymorphism has been reported. Nevertheless, *w*AlbA and *w*AlbB strains are strong CI inducers as shown by fully embryonic mortality found in crosses between superinfected males and females artificially deprived of the bacterial symbiont [39].

***Wolbachia* manipulation and artificial incompatible infection types**

Modern biotechnology techniques allow to artificially transfer this endosymbiont bacterium from a species to the other, offering the possibility to generate new patterns of CI and enlarging the list of target species for *Wolbachia*-based control strategies. The technology used for this purpose, namely transinfection, is based on the microinjection of *Wolbachia* infected ooplasm containing the appropriate *Wolbachia* strain into recipient insect embryos. A complete CI was obtained by microinjecting cytoplasm from *Ae. albopictus* eggs carrying the *w*AlbB *Wolbachia* strain in *Aedes aegypti* [40]. Recently, a stable introduction of a life-shortening *Wolbachia* (*w*MelPop) infection from *Drosophila melanogaster* has also been successfully achieved in the same mosquito species [41].

Regarding *Ae. albopictus*, the production of an incompatible strain may be achieved i) via microinjection of an additional *Wolbachia* strain, resulting in a triple infection, or ii) by removing the natural double-infection and then generating a transinfected line harboring a single new strain of *Wolbachia*. First attempts to modify the infection status of *Ae. albopictus* have been restricted to *Wolbachia* strains harboured by Drosophila species. [42] reported the interspecific transfer of *Wolbachia* (*w*Ri) from *Drosophila simulans* to *Ae. albopictus*. Imperfect CI and vertical transmission failure were observed together with a lower mating competitiveness in transinfected males. Hence, despite the clear potential of the *Wolbachia* based strategy, the control of *Ae. albopictus* population through the IIT approach remains an open challenge.

The transinfected *ARwP* line of *Ae. Albopictus*

In 2009, the Group of Biological Control and Insect Biotechnology of ENEA (now Lab. UTAGRI-ECO), in cooperation with Prof. S.L. Dobson (Department of Entomolgy, University of Kentucky), obtained a transinfected line of *Ae. albopictus* by removing the natural *Wolbachia* double-infection and microinjecting a single new strain of *Wolbachia* (wPip) taken from *Culex pipiens molestus,* a mosquito

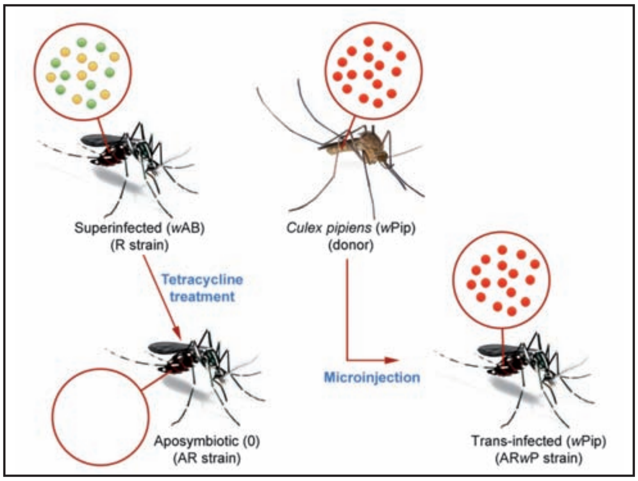

FIGURE 3 Outline of the protocol to generate a new Wolbachia infection in *Ae. Albopictus*.
Isolines of mosquitoes Wolbachia-free are generated via antibiotic treatment of superinfected wild mosquitoes. *Wolbachia* drawn from *Culex pipiens* embryos is then microinjected into embryos of the Wolbachia-free *Ae. albopictus* line.
Source: ENEA

species harbouring a *Wolbachia* strain similar to *w*AlbB, strong inducer of CI (Figure 3).

The new symbiosis has proven to be stable and efficiently transmitted from females to their offspring; males are fully incompatible (induced embryonic mortality close to 100%) over their lifetime when mated to normal double-infected females (Italian patent - application no. RM2009A000678; [43]. The transinfected line so obtained, named ARwP, is bidirectionally incompatible with wild *Ae. albopictus* population. This result is an excellent example in which an exogenous strain of *Wolbachia* imposes the same phenotype alteration (strong CI) on native and new hosts [44].

Benefits of ARwP line use in IIT

Using "ready sterile males" allows for the elimination of radiation technology with its negative effects on sterile male performances. A critical benefit of such approach is that it only requires to set up and maintain a mass-rearing of "incompatible mosquito lines" and to release incompatible males in the field. All this could improve the overall competitiveness of the released insects, with a consequent improvement in program efficiency and a significant decrease in costs. We consider that the results obtained at the ENEA laboratories may open the way to a further development of IIT technology leading to the realization of mass-rearing facilities for the production of incompatible *Ae. albopictus* males. Currently the ARwP strain is going to be evaluated as provider of ready-made sterile males for SIT application on pre-industrial scale within the trans-national project BIOMOS (EurotransBio 2010) funded by the Italian Ministry of Economic Development. It is unlikely that any SIT strain will be a 'cure all' solution to mosquito control, but certainly SIT technology will hold a key role in a wider integrated pest management program.

Risk assessment of using transinfected Aedes mosquitoes for IIT

ARwP line has been generated to support the sterile insect technique against the Asian tiger mosquito by providing sterile males. ARwP mosquitoes are not considered genetically modified organisms [45]. To reduce the risk of fixation in the environment of this artificially transinfected insect, we preferred to focus on a transinfected line harbouring a single incompatible *Wolbachia* strain (*w*Pip) rather than three *Wolbachia* strains (*w*Alb A+*w*Alb B+*w*Pip). In fact, in the first case, the bidirectional CI system guarantees that, whereby transinfected females are accidentally released, they have high probability to be sterilised by wild males. Moreover, transinfected ARwP females suffer of a light fitness cost imposed by the exogenous *Wolbachia* strain. Hence, according to theoretical models [46], there is a minimal probability that the AR*w*P strain will be capable to replace natural mosquito populations (Figure 4).

Although there is no scientific evidence suggesting any negative impact of *Wolbachia* infected mosquitoes on humans, other organisms or environment [47], *Wolbachia* based strategies may be subject to less regulatory issues than genetic control based strategies, providing that *Wolbachia* and infected insects are not genetically modified. Recent releases of transinfected *Aedes aegypti* carried out in Australia for "population replacement purposes" were regulated and approved for use by the Australian Pesticides and Veterinary Medicines Authority. It was concluded that there was negligible risk and the releases would result in no more harm than what is caused by the naturally uninfected populations of *A. aegypti*. The releases in Australia provide an example for other countries to follow; however, regulations will need to be clearly defined by an internationally recognized Institution before large-scale releases and/or the commercialization of *Wolbachia*-based strategies for insect control could take place in practice.

Conclusion and perspectives

As discussed in this review, the prospect of using *Wolbachia* to control insects shows considerable promise. Here we have focused on the use of *Wolbachia* infected males as a mechanism to decrease pest populations by inducing male sterility, similar to the use of sterile male programmes to control insect pest. Other innovative approaches are under

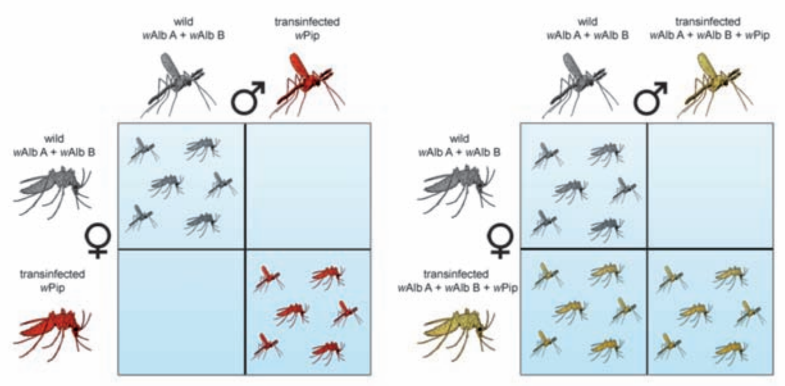

FIGURE 4

Risk assessment of using transinfected *Aedes* mosquitoes for IIT

Relying on a transinfected line harbouring a single incompatible *Wolbachia* strain (wPip) guarantees that, whereby transinfected females are accidentally released, they have high probability to be sterilised by wild males. On the other hand, because all matings of females harbouring three *Wolbachia* strains (wAlb A+wAlb B+wPip) are fertile, we run the risk that the triple infected strain could invade and replace the natural populations.
Source: ENEA

consideration and development, such as using *Wolbachia* to drive desirable traits (for example, resistance to disease) into insect vector populations [32] or to shorten the lives of vectors in which the disease agent requires a long incubation time within the vector, such as Dengue fever in *Aedes* spp mosquitoes [41].

The demonstrated success of artificial horizontal transfer of this symbiont is encouraging for the prospect of *Wolbachia* transfer to major economic pests and disease vectors lacking *Wolbachia* infections in wild populations. One of the future challenges could be the establishment of a *Wolbachia* infection into the gonads of the malaria vector *Anopheles gambiae* or in agricultural pests of worldwide economic importance, like the Mediterranean fruit fly *Ceratitis capitata* (Diptera, Tephritidae) or the olive fruit fly *Bacrtocera oleae* Rossi (Diptera, Tephritidae) [48, 49].

Lastly, the increasing evidence that *Wolbachia* interferes with pathogen transmission [50] highlights the potential of *Wolbachia* as an environmentally-friendly biotechnology to control insect transmitted diseases. It is just in this context that field experiments are ongoing in field to establish an *Ae. aegypti* transinfected with *Wolbachia* *w*Mel from *Drosophila melanogaster*. This mosquito strain has strong anti-dengue properties and limited fitness costs to be used for suppression of dengue transmission in Australia [51, 52]. The above mentioned project provides the first case where wild insect populations have been transformed to reduce their ability to act as vectors of human disease agents. This success with the deliberate release of Wolbachia-infected insects follows early unsuccessful attempts to manipulate insect populations through other genetic control strategies, including chromosomal manipulations and lethal genes [53, 54]. Because *w*Mel and other *Wolbachia* strains inhibit virus transmission in the laboratory [52, 55], there is growing interest around the development of *Wolbachia*-based strategies for mosquito vector diseases suppression. Moreover, exploitation of this bacterium should provide low cost solutions with a relatively simple deployment system suitable for implementation in developing countries.

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